Houseflies (*Musca domestica*) as potential vectors for the spread of ESBL-producing *E. coli* in humans.

Antonio Borg

MSc Student

number

14260818

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Abstract

Antimicrobial resistance (AMR) has emerged as a critical global health challenge, with the spread of Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* being a major concern, particularly in urinary tract infections (UTIs). This research investigates the role of the common housefly (*Musca domestica* L.) as a vector for transmitting ESBL- producing *E. coli* from bovine farms and open markets to human environments. The flies collected were not identified to species level, however the housefly (*Musca domestica*) is considered the most widespread fly species in Malta and is reported to be one of the most abundant and widely distributed in the Maltese Islands. All collected specimens shared similar morphological characteristics and were considered to belong to a single species. Therefore they are referred to in this study as *Musca domestica*. Through a multidisciplinary approach—integrating field sampling, phenotypic and genotypic analysis, and whole-genome sequencing (WGS), the study explores the prevalence, resistance patterns, and genetic similarity of *E. coli* strains isolated from flies and UTI patients.

A total of 177 fly traps and 90 fresh manure samples were collected across Malta and Gozo between 2018 and 2021. Simultaneously, 70 UTI-related ESBL-*E. coli* strains were obtained from clinical samples. Selective culture using TBX agar supplemented with cefotaxime, disc diffusion-based Antimicrobial Susceptibility Testing (AST). The results summarized in Table 4.1, show that a significant number of manure samples harboured CTX-M *E. coli* isolates. Similarly in Table 4.2, houseflies collected from farms and open markets were analysed for the presence of CTX-M type. PCR screening revealed that 42 % of fly samples carried ESBL-producing *E. coli*, with 47 % of these harbouring the bla_{CTX-M} resistance gene. Among the UTI

isolates, 79 % carried the same gene. WGS identified the $bla_{CTX\text{-}M\text{-}15}$ variant as dominant in both environmental and clinical isolates, often carried on IncF-type plasmids and mobilized by the insertion sequence ISEcp1. Crucially, both fly and UTI strains shared virulence genes associated with *ExPEC* and *UPEC* lineages, suggesting a strong potential for zoonotic transmission.

The findings underscore the significance of *M. domestica* as a vector not just for general pathogens but specifically for antimicrobial-resistant, clinically relevant strains of *E. coli*. The overlap in AMR gene profiles and virulence factors between fly-derived and clinical strains strengthens the hypothesis that flies may serve as a critical link in the environmental-human transmission chain of ESBL-producing *E. coli*, although they cannot be considered the sole potential carriers. This highlights an urgent need for improved hygiene practices, integrated surveillance across agricultural and healthcare sectors, and further research into environmental pathways of AMR transmission.

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Declaration by the author

This thesis is composed of my original work and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution of others to jointly authorized works that I have included in my thesis. The content of this thesis is the result of work I carried out since the commencement of my research higher degree candidature, which was interrupted by the pandemic from 2020 to 2022.

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Index of Abbreviations

AI	Artificial Intelligence
AMC	Amoxicillin Clavulanic Acid
AMP	Ampicillin
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Genes
AST	Antibiotic Susceptibility Testing
AT	Aztreonam
Bla	Beta-Lactamase
CAZ	Ceftazidime
CDC	Centre for Disease Control
CFQ	Cefquinone
CFU	Colony Forming Unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CPD	Cefpodoxime
CTX	Cefotaxime
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EXPEC	Extraintestinal *E. coli*
FAO	Food and Agriculture Organization
FOX	Cefoxitin

HGT	Horizontal Gene Transfer
IMP	Imipenem
LB	Luria-Bertoni
Mac	MacConkey Agar
MER	Meropenem
MH	Mueller Hinton
MRD	Maximum Recovery Diluent
NAL	Nalidixic Acid
NDH	New Delhi Metallo β Lactamase
NHS	National Health Service
NIT	Nitrofurantoin
PBS	Phosphate – Buffered Saline
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time PCR
S3	Sulphonamides
SHV	Sulphydryl Variable
SOP	Standard Operating Procedure
TB	Tryptone Bile X-Glucuronide Agar
TET	Tetracycline
UK	United Kingdom
UN	United Nations
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection
WGS	Whole Genome Sequence
WHO	World Health Organisation

WOAH	World Organization For Animal Health
OXA	Oxacillinase

Chapter 1: Introduction and Review of the Study

1.1 Antimicrobial Resistance: The Silent Pandemic

"If we fail to act, we are looking at an almost unthinkable scenario where antibiotics no longer work, and we are cast back into the dark ages of medicine" – David Cameron, former UK Prime Minister, 2014

Professor Sally Davies, England's former Chief Medical Officer and current UK's special envoy on antimicrobial resistance (AMR) warned that the issue of AMR poses a threat is *"more acute* than climate change' and the COVID-19 pandemic will *"look minor*" compared with what humanity faces from the increasing number of resistant bacteria to current drugs making them ineffective and impossible to treat infections.

"If we don't contain AMR, we lose modern medicine", Professor Dame Sally Davies (Davies .2024) Antibiotics have revolutionized modern medicine since their discovery in the 1940s yet bacterial resistance emerged soon after the introduction of penicillin in 1928. Today, AMR is considered a major global health concern , as bacteria continue to evolve to overcome antimicrobial actions (Kohanski, Dwyer, & Collins, 2010 ; Munita & Arias, 2016). In 1914, sanitarian Samuel Miller reported to the Massachusetts Association of Boards of Health and noted that "flies breed disease" and "the fly is a curse" (Miller, 1914) long before it was discovered that flies could transmit antimicrobial-resistant bacteria of clinical importance. Since the discovery of penicillin as an antibiotic often referred to as the "*magic bullet*," bacterial resistance has evolved rapidly confirming Fleming's early warning that misuse of antibiotics could render them ineffective. Nonetheless, the misuse of antibiotics in clinical and veterinary settings has led to the evolution of bacterial resistance to antimicrobial agents, which can then be easily transmitted by houseflies(Adedeji, 2016; Abdus Salam *et al.,* 2023).

"The time may come when anyone in the shops can buy penicillin. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

Fleming during Nobel Lecture December 11, 1945.

Antimicrobial resistance (AMR) has globally become a current problem and one of the greatest threats to modern healthcare and the number of resistant infections has risen rapidly over the last few decades. Resistance refers to the ability of microorganisms, including bacteria, viruses, fungi and parasites, to overcome and resist the action of antimicrobial agents by developing resistance (Salam *et al.*, 2023). Resistant bacteria are frequently isolated from a wide range of sources including environmental, clinical while also depending on the type of bacterial strains, and geographical climate (Bevan *et al.*, 2017;Salam *et al.*, 2023). In 2014, the UK Government commissioned a global estimate report by Jim O'Neill (2014) projected that by 2040, AMR could become the leading cause of death, increasing from 700,000 in 2014 to approximately 10 million by 2050 (O'Neill, 2014). Worldwide, multi- drug-resistant bacteria were responsible for the 1.27 million deaths in 2019, and this figure as already anticipated may rise drastically by 2050 (Lancet, 2022; Tang *et al.*, 2023). World Bank (2017) estimates that drug-resistant infections pose a significant economic threat up to $3.4 trillion annually losses by 2030, with an estimated additional $1 trillion in healthcare costs by 2050 . In Europe alone, the European Centre for Disease Prevention and Control (ECDC), AMR causes 33,000 deaths each year, with healthcare costs of €1.5 billion (ECDC, 2018). Recognizing this risk many countries are implementing surveillance systems, stewardship programs and agricultural reforms in order to minimize antibiotic use and monitor resistance (Ahmed *et al.*, 2024). Van Duin and Paterson (2016) highlight that multidrug-resistant organisms

(MDROs), such as *MRSA*, *CRE*, and ESBL-producing *E. coli* with the CTX-M resistance gene, have made it challenging to treat common infections, ranging from urinary tract infections (UTIs) to pneumonia and sepsis. These antibiotic-resistant bacteria are commonly found in hospitals and healthcare facilities due to the frequent use of antibiotics and patient contact, particularly among individuals with urinary tract infections (UTIs). The antibiotic misuse in both human and veterinary medicine contributes significantly to this crisis.(Salam *et al.,* 2023).

Resistant bacteria are frequently detected in livestock farms where due poor hygienic standards, particularly the presence of fresh manure, attracts houseflies (*Musca domestica*) which can act as a mechanical vector and contributing to the spread of antibiotic resistance bacteria in humans and making it difficult to treat and eradicate mastitis in bovines. Studies indicate an increasing awareness of houseflies in transmitting antimicrobial-resistant bacteria from the environment to humans (Onwugambaa *et al.*, 2018).

Worldwide, multi- drug-resistant bacteria were responsible for the 1.27 million deaths in 2019, and this figure as already anticipated may rise drastically by 2050 (Lancet, 2022; Tang *et al.*, 2023). Studies show that synanthropic flies can serve as environmental reservoir for these resistant bacteria facilitating their transmission to humans and animals. Houseflies are typically found in close association with humans and animals, particularly in environments with decaying organic matter, such as farms a characteristic described as endophilic cosmopolitanism, which means living within human dwellings. Their coprophagous feeding behavior on decaying matter and their mobility enable them to transmit pathogens . Domestic houseflies have been implicated in the transmission of several microorganisms like parasites, viruses, and bacteria. For example, the transmission of the protozoan *Toxoplasma gondii* occurs when house flies pick up the

oocysts of the parasite from a contaminated cat or mouse faeces and transfer them to human food or other surfaces (Hang Yin *et al.*, 2022). Consequently, they are recognized as potential carriers of multi-drug resistant bacteria, including ESBL-producing *E. coli* and showing resistance to the third generation of Cephalosporins and Carbapenems is considered a global threat to the healthcare system (Samreen *et al.*, 2021). Unfortunately, novel research into AMR transmission from environmental sources slowed down during the COVID–19 pandemic, as global attention has shifted on climate change and global terrorism (Al Sulayyim *et al.*, 2022). The transmission of antibiotic-resistant bacteria from farm animals to public health beyond the food chain is usually facilitated by the spread of biological vectors, notably houseflies. The study conducted by Khamesipour *et al.* (2018) provides compelling evidence that the housefly can serve as a vector for pathogens that cause serious and life-threatening diseases in humans and animals. While previous studies have shown the ability of flies to carry pathogenic bacteria, Singh *et al.* (2025) confirmed their role in disseminating antibiotic-resistance genes. A study aimed to evaluate the role of *Musca* spp as a carrier of multidrug-resistant, extended spectrum β − lactamase (ESBL) - producing *E. coli*, to determine the transmission route of antibiotic resistance (AR) within houseflies and patient with infectious disease (Singh *et al.*, 2025). Efforts to control and prevent the transmission of antimicrobial-resistant bacteria are essential in maintaining the effectiveness of antibiotics for treating infections in animals and humans. Antimicrobial- resistant bacteria are frequently isolated from environmental and clinical samples. Microbes are ubiquitous, as are flies, and the identification of their associated resistance genes can help understand the landscape of antimicrobial resistance (AMR) in the local environment, such as in patients with urinary tract infections (UTIs). Houseflies are very common flying insects and are abundant in Malta throughout the year, particularly during the hot summer months that favours mobility and breeding.

Meanwhile UTIs caused by ESBL-producing *E. coli* are surging and expanding in many countries. *E. coli* is by far the most common cause of UTIs such infections must be treated with antibiotics, and treatment is empiric (Kahlmeter *et al.*, 2012; Foxman, 2014; Tandogdu *et al.*, 2016).

1.2 The Situation in Malta: Current and Future Perspectives on Antimicrobial Resistance

It is estimated that worldwide 700,000 people die each year as a result of antimicrobial resistance. In Malta, 53 deaths were attributed to AMR in 2019.Profs. Michael Borg stated that Southern European countries including also Malta, antibiotic consumption remains among the highest in Europe partly due patient expectations and cultural habits and and if we had a Eurovision for Antibiotics, Malta would win every year'. Unfortunately public understanding of antibiotic use remains limited, contributing to lack of knowledge and awareness regarding the management of colds or flu. The Eurobarometer surveys reveal that Malta has the highest antibiotic consumption, and the lack of public knowledge about antibiotics remains below average among European countries.

Malta consumes more antibiotics than, for example, Nordic countries. Malta consumes three times more antibiotics than Sweden. During the COVID- 19 pandemic, however, a 40 % reduction in antibiotic consumption was observed (Saleh *et al.*, 2022). Prof. Borg has long emphasized that excessive antibiotic use increases healthcare costs and resistance rate. Profs Borg's preventive measures might help to slow down this public health concern. Professor M. Borg heads the Department of Infection Prevention and Control at Mater Dei Hospital and chairs the National Antibiotic Committee and holds a professorship at the University of Malta and has advised the European Centre for Disease

Control (ECDC) while recognized by Stanford University among the top scientists worldwide and leader in Antibiotic Resistance.

Minister for Health Hon. Joe E. Abela, speaking on behalf of Malta, stated during a plenary session of a high-level meeting at the UN General Assembly, Malta continues to emphasize the structure of sustainability, efficiency, and effectiveness through the 'One Health' approach. The Minister emphasized the urgent need for the UN to address antimicrobial resistance and achieve Universal Health. This global goal aims to ensure all people have access to the health services they need, thereby protecting more lives in the health sector. Malta continues to recognize the need for rapid and decisive action to address the urgent global threat of AMR. It's crucial to understand that additional financial and technical support is beneficial and necessary to bolster every country's capacity to mobilize global efforts to combat the escalating threat of AMR. This is a battle that we must fight together. However, it's essential to note that significant progress has been made, and action plans for AMRs are being actively implemented. This progress should serve as a beacon of hope, encouraging us to continue our efforts in the fight against AMR. Maltese General practitioners and Pharmacists frequently report pressure from patients or clients to prescribe antibiotics unnecessarily since still believe that antibiotic consumption is considered ‘the holy grail’.. Sometimes, they feel disappointed for not receiving them, and they may also threaten both GPs and pharmacists if they are not prescribed. The same phenomenon occurs in the agricultural sector when treating livestock. There are those who tell you, ‘I came to get antibiotics,’ and when you tell them there’s no need, they keep insisting because their child needs to return to school, and the parent cannot afford to take sick leave, so they must return to work immediately.

Most pharmacists reported that antibiotic prescriptions are no longer over the counter. However, while prescription policies are in place, enforcement can be inconsistent due to

Malta's small community dynamics. Another contributing factor is the inappropriate disposal of leftover antibiotics often through domestic sewage. Pharmacists now encourage the public to return unused or expired antibiotics to designated pharmacy bins. GPs and pharmacists agreed that they play a crucial role in educating patients on the appropriate use of antibiotics, potential drug interactions, side effects, and medication adherence. Addressing antibiotic intake is an initiative aimed at combating antimicrobial resistance (AMR) (Saleh *et al.*, 2022).

1.2.1 What steps can Malta take to reverse this trend and ensure that antibiotics remain effective for future generations?

Serious infections are becoming difficult to cure. Fighting against antimicrobial resistance requires coordinated efforts across human and environmental health sectors, focusing on surveillance, responsible antibiotic use, and infection prevention. In response, the Government, through both the Ministry for Health and the Ministry of Agriculture, Fisheries, and Animal Rights, launched the strategy and action plan for the prevention and containment of antimicrobial resistance in Malta (Strategic Plan 2020-2028) The National Statistics Office conducted the Agriculture Census 2020 in collaboration with the Ministry of Agriculture, (MAFA). Animal husbandry is a section within the agricultural Department concerned with domestic animals and livestock raised for various commodities. Livestock farming, mainly bovine farming, has long been a staple of the local agricultural landscape. However, due to the island's small size, it has faced a scarcity of grazing. Malta's agricultural history dates to the Neolithic period. Livestock farming was also present, with sheep and goats being more common than cattle. Even until the Middle Ages, the cattle population remained small due to the limited space and feed resources. Today, bovine farming remains a small but essential part of the agricultural

sector. The census carried out by MAFA in 2020 shows that the island hosts around 15,000 cattle heads across approximately 248 farms with 100 dedicated to dairy production. with the Holstein-Friesian is the predominant dairy breed. Farmers face challenges including limited grazing land, water scarcity, manure management and environmental impact due to nitrates in groundwater, and compliance oversight by the Veterinary Services Department. Antibiotic use in dairy farms is strictly regulated under EU Regulation (EU) 2019/6 'Antibiotic monitoring on farms and antibiotic growth promoters', Regulation EC No 1831/2003, a continuously monitored by the Veterinary Services Department.

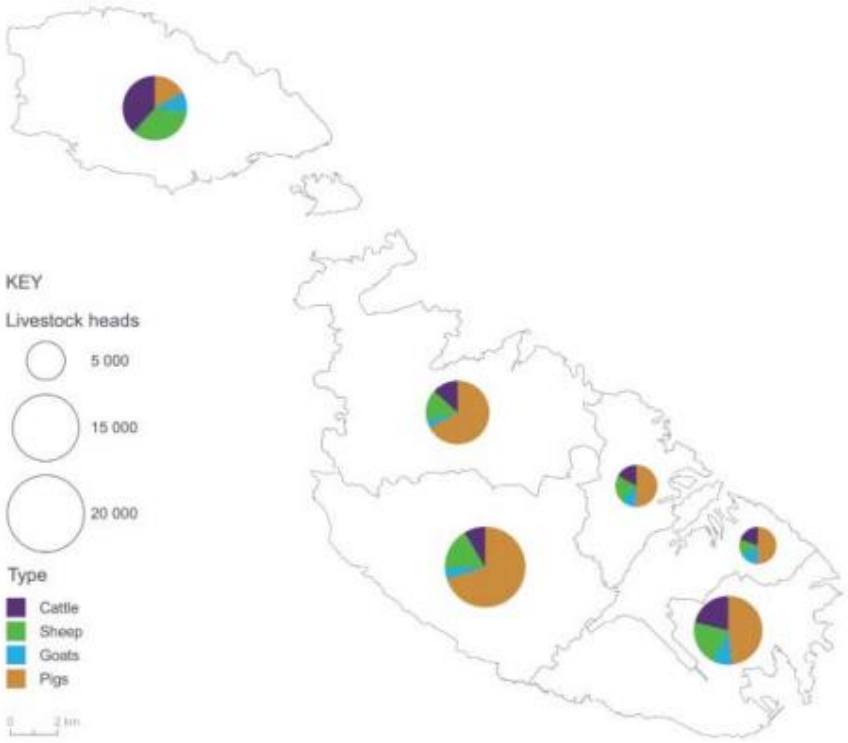


Figure 1.1 Geographic distribution of cattle, sheep, goats and pigs across Malta and Gozo by district. Data from the Agriculture Census 2020, Ministry for Agriculture, Fisheries and Animal Rights (MAFA)

Table 1.1 Distribution of cattle by district in Malta and Gozo

Type of cattle	MALTA	Malta	Southern Harbour	Northern Harbour	South Eastern	Western	Northern	Gozo & Comino
Total cattle	**14,447**	**9,028**	**922**	**1,019**	**3,388**	**1,891**	**1,808**	**5,419**
of which:								
Cattle under 1 year	**4,273**	**2,778**	287	275	1,187	497	532	**1,495**
Cattle between one and two years	**3,288**	**2,030**	196	291	764	433	346	**1,258**
Cattle over two years	**6,886**	**4,220**	439	453	1,437	961	930	**2,666**
of which:								
Dairy cows	**5,996**	**3,662**	353	352	1,275	870	812	**2,334**

Note: Cattle number are recorded according to geographical location of farms, categorized into regional zones as defined in Appendix A (Agriculture Census 2020,MAFA)



Figure 1.2 The Southeastern region of Malta, encompassing locality of Zejtun, hosts a number of cattle farms. Map data © 2025 Google.



Figure 1.3 Typical bovine farm situated on the outskirts of a Maltese village, Zejtun, illustrating the close proximity between rural agricultural areas and residential dwellings. *Image retrieved in 2024 from Google Images*



Figure 1.4 Typical of Maltese bovine farm. *Image retrieved in 2024 from Google Images.*

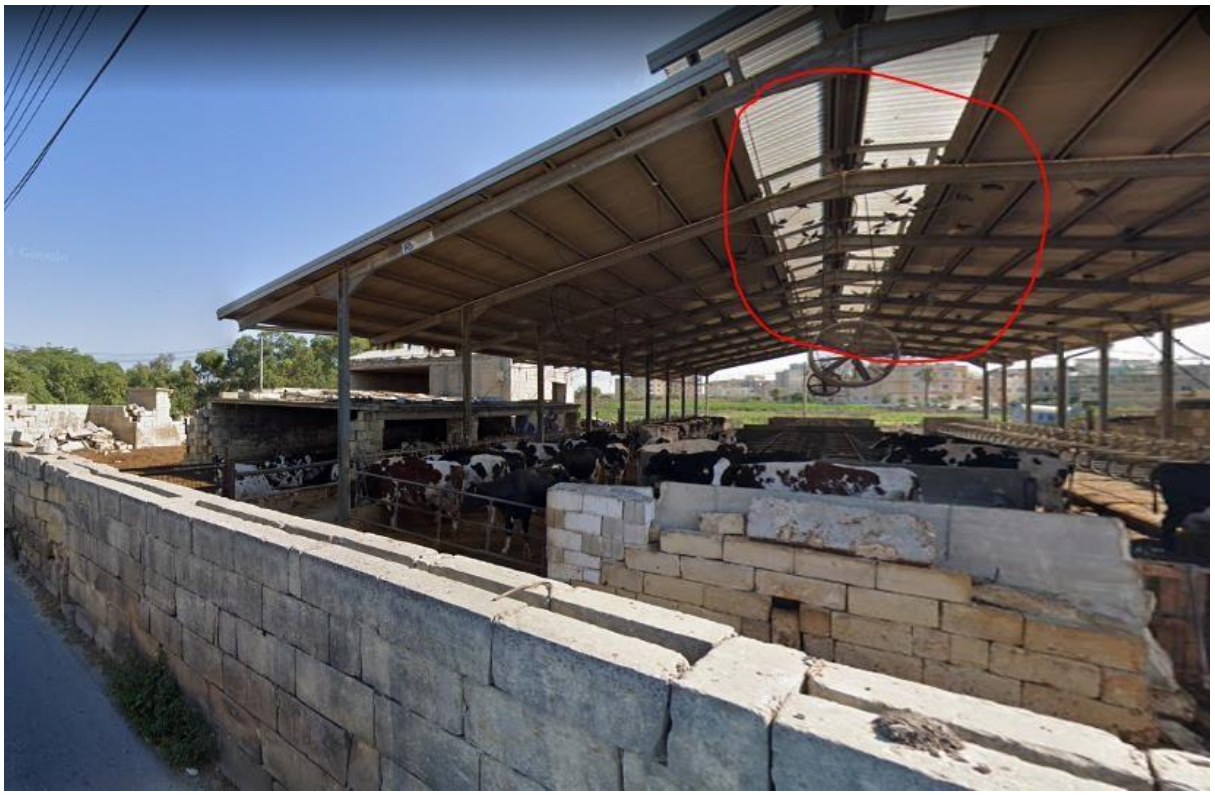


Figure 1.5 Pigeons perching on the roof of a cowshed, illustrating contact and environmental contamination. *Image retrieved in 2024 from Google Images*

Figure 1.3 illustrates a typical bovine farm in a rural area, on the outskirts of a Maltese village, often situated near residential areas. In Figures 1.4 and 1.5, pigeons are perched on the roof of the cowshed. Pigeons pose a risk of transmitting pathogenic bacteria resistant to certain antibiotics. Most bovine farms are located in rural areas of Malta and Gozo, spanning both the northern and southern regions, including the outskirts of Żejtun and Marsa Scala villages, where the majority of samples in this study were collected. The village of Żejtun has a cluster of bovine farms, whereas, in the Western region, smaller clusters of farms exist primarily around areas like the villages of Rabat and Dingli. The southeast region of Malta comprises Żejtun, Marsascala, Marsaxlokk, Birżebbuġa, and Għaxaq.



Figure 1.6 Panoramic view of Marsaxlokk fishing village in southeastern Malta showing traditional luzzu boats in the harbour and weekly open markets.

In most Southeastern villages, street vendors make a significant contribution to the local ambiance. Figures 1.7 and Figure 1.8 show typical open market in Malta, selling local food products



Figure 1.7. Fresh fish and seafood for sale at Marsaxlokk market.

From Madhouse Heaven (2025), *Fresh fish and seafood for sale at Marsaxlokk market The Next Crossing.* https://thenextcrossing.com/marsaxlokk-market-sunday



Figure 1.8 Farmer's market at Malta National Park

From Malta National Park (2023), https://maltanationalpark.mt

Malta's Mediterranean hot climate, especially during dry summers and mild winters, creates an ideal habitat for the growth of fly populations. In brief, climate plays a significant role in the proliferation of the fly population. During the summer, when the temperature reaches 30 °C, the reproductive cycles of flies are enhanced, allowing them to breed quickly and frequently, especially on farms. Livestock farms produce manure from bovine waste, which attracts flies. Bovine farms, poultry farms, and other agricultural operations that generate vegetable waste are all attractive to flies. With climate change, longer, warmer summers will likely enhance fly breeding, resulting in even higher populations over time. Housefly can quickly adapt to these conditions, making them more likely to flourish if summers in Malta become hotter, drier, and longer. During hot summers, flies can be seen roaming around and on fruits and vegetables sold

by street hawkers. Flies are more active during the daytime; even in hot weather, they remain active and land on fruits, especially stone fruits like peaches and plums, as well as other ready–to–eat vegetables like lettuce. Moisture attracts flies, and in summer, most vegetables and fruits produce condensation, as in Malta, most perishable fruits and vegetables are stored in refrigerators before being sold. The changing situation in Malta may contribute to the rise of AMRs in Malta include several. Malta's population has increased drastically recently, with people from developing countries coming to find employment and work here. Unfortunately, compared to regions with different healthcare hygienic standards, the population can contribute to the rise of antibiotic-resistant bacteria in a small country like Malta. Since Malta provides free healthcare to everyone, hospitals may become overwhelmed due to overpopulation as more people seek healthcare services. These conditions contribute to the spread of resistant bacteria. When healthcare services are under strain, an inappropriate intake of antibiotics is often possible. Migrants from countries where antibiotics are not regulated may also bring in resistant strains of bacteria. Since migrants may carry different strains of bacteria common to Malta, they will also introduce these resistant strains into the local population. Crowded housing and poor sanitation contribute to the proliferation of antibiotic-resistant bacteria among the population. Bacteria can spread rapidly in denser populations, and antibiotic-resistant bacteria can disseminate throughout communities. Overpopulation generates more waste, thereby increasing the concentration of bacteria in sewage, which ultimately ends up in seas. The problem of sewage discharge into seawater in Malta can contribute to the rise of antibiotic- resistant bacteria. Untreated wastewater, which may contain pharmaceuticals and leftover antibiotics, enters the marine environment, and finally, the food chain can also be affected. Bacteria in contaminated, nutrient-rich seawater can transfer antibiotic-resistant genes to other bacteria, a process known as horizontal gene

transfer. Sewage discharge into seawater, due to several factors, especially overpopulation, contributes to the rise of antibiotic-resistant bacteria. Overpopulation and migration make Malta more susceptible to antibiotic-resistant bacteria. Migratory birds can also introduce bacteria, including antibiotic-resistant strains, into the local environment. Birds can acquire antibiotic-resistant bacteria through direct contact. Sandegren et al. (2018), Zhao et al. (2020) reported that resistant bacteria can persist for an extended period within the bird's gut, making them an ideal vehicle for transmission from one location to another or among communities. A study has demonstrated that wild bird populations in cities serve as reservoirs of bacteria resistant to many critical human antibiotics. There is an urgent need to understand how human activity influences the spread of zoonotic diseases and antimicrobial-resistant (AMR) pathogens: Professor Samuel Sheppard, INCOS Oxford Institute for Antimicrobial Research and Department of Biology, Oxford University has published a research showing that wild birds like crows living near humans, are likely to harbour and transmit zoonotic diseases, including pathogenic bacteria with antimicrobial resistance (AMR) (Oxford University 2024). Malta, like many other countries around the world, has adopted a national action plan against antimicrobial resistance (AMR), which aims to prevent antibiotic-resistant bacteria and thereby contribute to global efforts.

Some actions which Malta is constantly following, include

- National Action Plan on AMR aligned with the WHO guidelines on AMRs
- Stewardship program on education on how to use antibiotics
- Monitoring and surveillance in collaboration with the European Antimicrobial Resistance Surveillance Network
- Regulating antibiotic use in agriculture

- Investing in research and innovation
- Enhancing infection prevention and control
- Informing the public about risks and training for farmers, veterinarians, and healthcare professions
- Developing new antibiotics, other alternative treatments, and rapid techniques to identify the pathogen
- Improving hygiene and sanitation
- Promoting vaccination to reduce the intake of antibiotic treatments

1.3 Research Gap

There is strong evidence that flies can act as vectors to contaminate food products, animals, and the environment, as they are responsible for carrying antimicrobial-resistant bacteria, such as ESBL-producing *Escherichia coli*. These infections must be treated with antibiotics. Flies almost certainly play a role as vectors and reservoirs of bacteria that are clinically important. However, more research is needed to investigate the transmission chain. The sources of intermediate antibiotic-resistant bacterial contamination from flies require clarification, and practical strategies for preventing the spread of bacterial drug resistance among UTI patients with ESBL-producing *E. coli* should be suggested.

However, limited data are available to determine whether domestic flies are likely to undermine the treatment of ESBL-producing *Escherichia coli* in UTI patients.

1.4 Rationale of the Study

During the initial farm survey in 2018, a notable presence of domestic houseflies (*Musca* spp) was observed flying around manure collection sites and cows. This observation

prompted the research team to conduct a follow-up investigation to access whether these flies could act as mechanical vectors of ARB. Farmers also reported prolonged recovery time in cows treated for mastitis particularly during the Maltese hot summer months, raising concerns about the potential contribution of the farm surrounded environment to the persistence and spread of AMRs.

Similar associations have been reported in previous studies, including Blaak et al., (2014) who detected ESBL-producing *E. coli* on flies at poultry farms. These findings continue to highlight the potential importance of flies as environmental reservoirs and vectors in the spread of resistant bacteria.

1.5 Hypothesis and Objectives

This thesis explains the rise of ESBL-producing *E. coli* with a CTX-M-resistance gene in the community. For patients admitted to hospitals with community-acquired UTIs, the risk factors for acquiring ESBL producing *E. coli* should be considered before initiating treatment. Results from this research will contribute to improved knowledge regarding the potential risks of antibiotic-resistant *E. coli*, which poses a threat to public health due to domestic flies that are continuously exposed to various environmental conditions.

Houseflies (*Musca spp*) are a potential vector for the transmission of bacterial contamination and the spread of resistant bacterial strains that produce CTX-M type enzymes, also known as Extended-Spectrum Beta-Lactamases (ESBL), in *E. coli*, which may threaten or undermine the treatment of ESBL-producing *E. coli* in UTI patients. The aim of this PhD dissertation is to investigate the role of houseflies as potential carriers of ESBL-producing *E. coli* in human environments. Utilizing a multidisciplinary methodology that combines field studies, laboratory analysis, and molecular techniques, this research's objectives are to elucidate the interactions between

houseflies and ESBL-producing *E. coli* in patients with urinary tract infections (UTIs) by, highlighting the role of flies in harbouring resistant bacteria and exploring their association with Urinary Tract Infections in humans.

Chapter 2. Literature Review

2.1 Enterobacteriaceae

2.1.1 Introduction

Depending on the respective cell wall structure and Gram stain properties, bacteria are classified into two main groups: Gram–positive or Gram–negative (Claus, 1992; Berg, 1996). *Enterobacteriaceae* is a large family of Gram-negative, rod-shaped, facultatively anaerobic bacteria, including both commensal and pathogenic species such as *Salmonella spp* and *E.coli* (Berg, 1996). Some strains are harmless and form a commensal part of the typical gastrointestinal microbiome. In contrast, others are pathogenic, such as *E. coli* O157:H7, which can lead to severe gastrointestinal infections and, in some cases, more complicated conditions, including Haemolytic Uremic Syndrome (HUS), resulting in kidney failure (Joseph *et al.*, 2020). Contaminated food and water can be primary agents in transmitting *E. coli*; however, studies indicate that flies can also act as vectors (Lindeberg *et al.*, 2018). Houseflies (*Musca domestica*) are another potential mechanical vector implicated in the transmission of foodborne *E. coli* diseases (Lindeberg *et al.*, 2018).

2.1.2 Escherichia coli is a significant Public Health concern.

E. coli is a Gram-negative, motile bacterium that is a nonsporulating rod, frequently found in abundance in the normal gut microbiome of humans and animals. As a commensal bacterium, it benefits its host by producing vitamin K and preventing colonization with pathogenic bacteria (Berg, 1996). However, *E. coli* can be a pathogen and is among the most frequently found bacteria in community-acquired and hospitalized UTI patients (Khan *et al.*, 2006). This bacterium is one of the most extensively studied prokaryotic

strains. The number of genes in the *E. coli* genome ranges from 4,000 to 5,000, depending on the specific strain, the presence of additional genetic elements such as plasmids, and the methods used to identify the genes (Poirel *et al.*, 2018). Whole-genome sequencing provides a better understanding of the genomic plasticity of *E. coli* (Poirel *et al.*, 2018). In Table 2.1 both *ExPEC* and *UPEC* types originate from the gut but differ in their preferred infection sites and virulence mechanisms.

Strains carrying additional genes, especially plasmid borne ones, may sometimes be associated with diarrhoeal disease, including *Intestinal Pathogenic E. coli (IPEC)*, which are regarded as obligate pathogens in humans, as well as extra-intestinal infections.

Extraintestinal Pathogenic

E. coli (*ExPEC*) strains are facultative pathogens that are part of the normal gut microbiome until they exit the gastrointestinal tract, allowing them to cause infections in other parts of the body (Claus, 1992); (Berg, 1996);(Patwary *et al.*, 2017). Figure 2.1 (Kaper *et al.*, 2004) illustrates a typical *E. coli* showing a variety of colonization factors such as fair- like structures called fimbriae, pili, or fibrillae.



Figure 2.1 Typical *E. coli* showing a variety of colonization factors such as fair- like structures called fimbriae, pili, or fibrillae.

The *E. coli* bacterium is highly adaptable, exhibiting diversity in both commensal and highly virulent strains. In terms of pathogenicity, *E. coli* utilizes virulence factors to invade the host and establish an infection (Kaper *et al.*, 2004).

Table 2.1 *ExPEC* and *UPEC* Types

Feature	***EXPEC (Extraintestinal Pathogenic E. coli)*** (Poolman *et al.*,2015)	***UPEC (Uropathogenic E. coli)***
Definition	Causes infections outside the intestines	Causes Urinary Tract Infections (UTIs)
Common Infections	Bloodstream infections (sepsis), neonatal meningitis, pneumonia, peritonitis	Urinary tract infections (UTIs)
Primary Site of Infection	Blood, lungs, brain, abdominal cavity	Urinary tract (bladder, kidneys, urethra)
Virulence Factors	Capsules, adhesins, iron acquisition systems, toxins	Type 1 and P fimbriae (for adhesion), hemolysins, iron-scavenging siderophores (Melican *et al.*, 2024)
Transmission	Often from the gut to other body sites via bloodstream	Typically from the gut to the urinary tract via the urethra
Common Hosts	Affects both immuno-compromised and healthy individuals	More common in women due to shorter urethra; can also affect men and children (Czajkowski *et al.*,2021)
Treatment	Antibiotics (depending on resistance patterns)	Antibiotics(e.g., trimethoprim-sulfamethoxazole, fluoroquinolones, nitrofurantoin) (Whelan *et al.*, 2023)

Multidrug resistance in *E. coli* has become a significant global threat in both human and veterinary medicine. Originally, *E. coli* was susceptible to most antimicrobial agents; however, it has accumulated resistance through genetic mutations. Over the last decades, more *E. coli* have developed resistance through horizontal gene transfer causing resistance. *E. coli* diseases in livestock animals, especially bovine mastitis, are often

treated with various pharmaceuticals, including antimicrobials such as ampicillin, streptomycin, sulfonamides, or oxytetracycline. Broad-spectrum cephalosporins and fluoroquinolones may also be administered depending on the symptoms (Poiret *et al.*, 2018). *E. coli* is responsible for causing diarrhoea from foodborne outbreaks and hospital infections, such as urinary tract infections (UTIs) and sepsis. The increase in multidrug resistance has made target antibiotics less effective against *E. coli* strains. To date, the global picture indicates that *E. coli* remains responsible for causing bovine mastitis, and the pathogenicity in the bovine udder results from the interaction between its virulence genes and host factors (Zaatout, 2022). ESBL/AmpC genes have been widely recognized in commensal *E. coli* isolated from faecal samples of livestock animals (Poirel *et al.*,2018). There is a debate regarding the transfer of other resistance traits between humans and animals. The role of antimicrobials in treating coliform mastitis is becoming increasingly debated (Poiret *et al.*, 2018). *E. coli*'s key resistance mechanisms include extended-spectrum β-lactamases (ESBLs), which confer resistance to broad-spectrum cephalosporins, and carbapenemases which provide resistance to carbapenems. The spread of carbapenemase genes is primarily attributed to humans and is less commonly found in animals. Carbapenems are reserved to treat severe infections in humans and in veterinary medicine their use is either limited or banned by most European member states(Meletis, 2016). On the other hand, colistin resistance in *E. coli* is widely observed in veterinary medicine due to the extensive use of colistin in livestock as a growth promoter and for infection control (Danaei *et al.*,2023). Plasmid-mediated quinolone resistance (PMQR) genes confer resistance to quinolones and fluoroquinolones, which target bacterial DNA synthesis (Jacoby *et al.*, 2014). However, just one year later, methicillin-resistant isolates were identified. In 1963 development of ampicillin was considered a significant step forward in treating Gram-negative bacterial infections;

however, later that same year, *E. coli* isolates in Greece were shown to produce plasmid-mediated *TEM-1* β-lactamase which provided resistance to ampicillin. When cephalosporins, a new class of antibiotics, were introduced in 1964, they were considered the wonder drugs of their time, providing the medical world with a powerful new ally against β- lactamases (Wilde, 2001). By the 1970s, resistance to cephalosporins had become widespread, due to the production of beta-lactamases, particularly plasmid-mediated and chromosomally encoded enzymes that hydrolyze β-lactam antibiotics (Bush et.al.,2016). Within a year of the introduction of cefuroxime in 1978, strains of *Klebsiella oxytoca* were found to be resistant (Hart *et al.*, 1982). Food is one of the primary vehicles associated with the transmission of pathogenic *E. coli* strains, including Enteropathogenic (EPEC), Enteroaggregative (EAEC), Enteroinvasive (EIEC), and Shiga toxin-producing *E. coli* (STEC). These infections are commonly associated with a variety of clinical settings, ranging from gastroenteritis to severe complications, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Joseph *et al.*, 2020). Food and water contamination can occur at several points, including during animal slaughter, meat processing, transportation, inadequate storage conditions, improper food preparation, and the use of contaminated water for irrigation purposes (Alegbeleye *et al.*,2018). In addition to foodborne *E. coli* infections, which can lead to systemic diseases, the presence of virulence genes and antibiotic resistance genes also contributes to the severity of these infections. The increasing emergence of ESBL-producing *E. coli*, which harbours resistance genes such as $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{OXA}, is a public health concern. Like other pathogens, a study conducted by Lindeberg (2018) demonstrated that *'E. coli can fly'* . In this study, flies served as mechanical vectors for *E. coli*, facilitating cross-contamination of food and other surfaces. flies harbouring pathogenic bacteria can contaminate food, water, or human hands when they land on or touch surfaces. Generally,

flies regurgitate digestive fluids and excrete fecal matter while feeding, introducing *E. coli* to previously uncontaminated food sources. The study describes how cooked rice left exposed to flies had significantly higher *E. coli* contamination than covered rice.

2.2 Antibiotics

2.2.1 Introduction

The first written evidence of using antibacterial substances to cure possible bacterial infections dates to the Bible (Isaiah 38:21)**.** The Bible mentions figs as a treatment for cutaneous anthrax. However, antibiotics with more targeted usage were not discovered until the 20th century (Aarestrup, 2006). Antibiotics are chemical agents that treat bacterial infections by killing bacteria or suppressing their growth (Katzung *et al.*, 2023). Their discovery and development represent one of the most significant medical advancements in history, revolutionizing healthcare and saving millions of lives. However, with the misuse of these antibiotics, especially in animal medicine, bacteria resistant to antibiotics rapidly emerged. Therefore, the spread of antimicrobial resistance bacteria from farm animals to humans became gradually more recognized during the 50s and 60s (Davies & Davies, 2010; World Health Organization [WHO], 2014). Metwaly *et al.* (2021) stated that natural antimicrobial substances were utilized in ancient Egyptian history until the discovery of modern antibiotic therapy in 1928 when Alexander Fleming discovered penicillin (Fleming, 1929). Following the discovery of new drugs like sulphonamides, streptomycin, and tetracyclines which revolutionized antimicrobial therapy significantly improving the treatment of bacterial infections and reducing mortality rates (Brunton, Knollmann, & Hilal-Dandan, 2018). Currently, antibiotics face a growing crisis due to the emergence of bacterial resistance. This is a reminder that each of us has a role to play in preserving the effectiveness of these life-saving drugs while

providing new solutions to combat this life-threatening resistance (Centers for Disease Control and Prevention [CDC], 2019). Excessive misuse of antibiotics in livestock and clinical medicine accelerates the development of antibiotic resistance. The overuse of antibiotics in veterinary medicine is used to treat infections in livestock like bovine mastitis and promote rapid growth in farm animals to increase profit (Galhano *et al.*, 2021). Other routes that facilitate the spread of antimicrobial resistance include inadequate hygienic standards especially in healthcare and community settings. International travel and human migration allow resistant strains to move rapidly across borders. Inadequate food processing can promote the dissemination of resistant strains through the food chain. While high population density increases the contact person – to – person and the risk of bacterial exchange (Aslam et al., 2018). Faecal or oral transmitted diseases, the role of water pollution in both wastewater and fresh water, and especially domestic flies, cannot be ignored in the spread of AMRs. There is interest in supporting the evidence that commensal or environmental bacteria, such as those isolated from domestic flies, are the main factors in the transmission of antibiotic-resistance genes to human pathogenic bacteria through HGT (Davies & Davies, 2010). In the case of flies, bacteria inside the gut can continue to proliferate, serving as plasmid encouragement; mobile genetic elements carrying ARGs can transfer between bacterial species. There is a growing interest in demonstrating that environmental bacteria isolated from houseflies act as an environmental reservoir of antibiotic resistance genes (Davies & Davies, 2010) In the case of flies, the presence of bacteria inside the gut can continue to proliferate, serving as plasmid encouragement; mobile genetic elements carrying ARGs can transfer between bacterial species. Studies of bacteria isolated from closed systems from the pre-antibiotic era have shown that prior to the introduction of antibiotics, few genes coded for antibiotic resistance (Hughes *et al.*,1983). Therefore the rapid expansion of resistant genes is

primarily attributed to the inadequate use of antibiotics in clinical and agricultural sectors which have accelerated the rapid mobilization and horizontal transfer of resistance genes among bacterial populations (Muteeb et al., 2023).

2.2.2 History of Development of Modern Antibiotics

In 1910, Paul Ehrlich discovered *salvarsan* a synthetic prodrug to treat syphilis. Gerhard Domagk discovered sulphonamide, which replaced Salvarsan (Hutchings *et al.*, 2019).

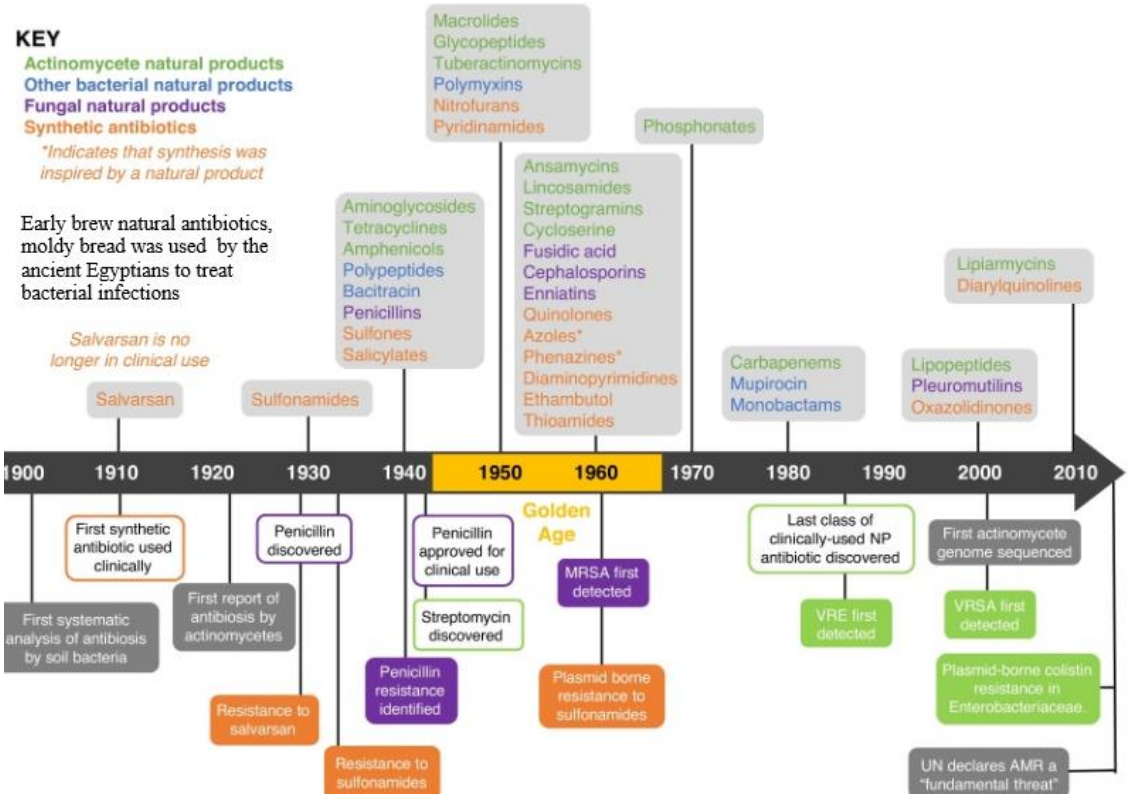


Figure 2.2 The Evolution of Antibiotics: From Discovery to Resistance

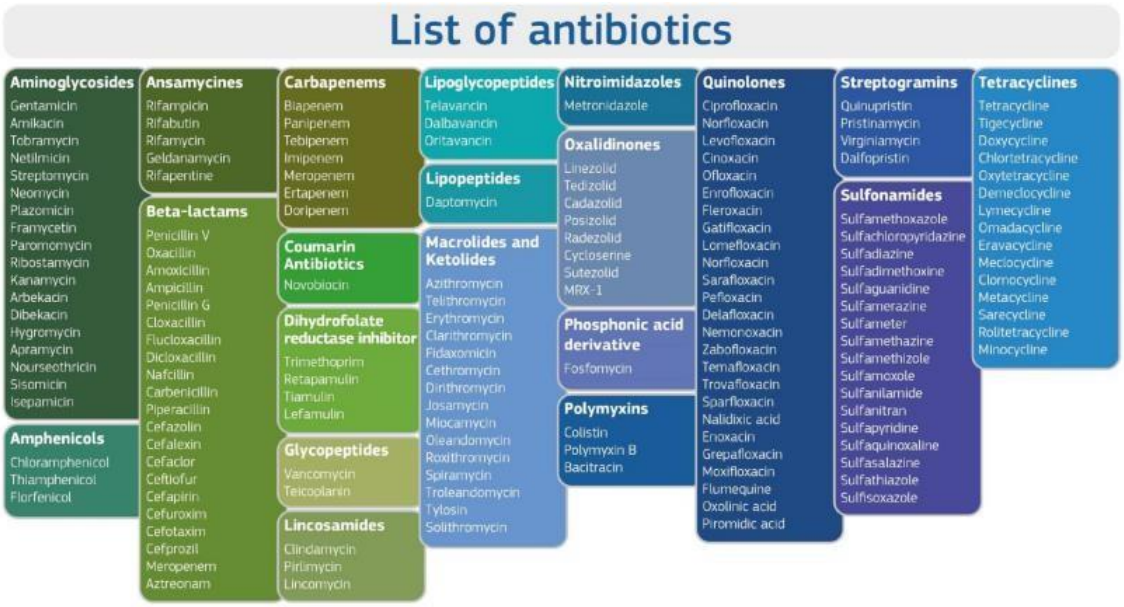
Note: Since 2017, only 13 new antibiotics have been authorized by WHO (Muteeb *et al.*, 2023). (WHO, 2024).

In Figure 2.2 shows an adapted schematic diagram from Hutchings *et al.* (2019) of how antibiotics developed over time. The concept of antimicrobial resistance is as old as the existence of bacteria since resistance mechanisms have developed due to the interaction between microorganisms and the surrounding environment. Bacteria have naturally evolved mechanisms to overcome the antimicrobial action of their co-residents to survive (Kapoor *et al.*, 2017). In 1930, after several discoveries, the American Selman Waksman defined the term antibiotic as '*a compound made by a microbe to destroy other microbes*' (Muteeb *et al.*, 2023) Alexander Fleming accidentally discovered penicillin in 1928. It was later widely distributed, particularly during World War II (Gaynes, 2017). In 1943, following the discovery of penicillin, another important antibiotic, streptomycin, was discovered (Shatz *et al.*, 1994). This antibiotic was the first to treat tuberculosis (Murray *et al.*, 2015).

In 1945, tetracycline was employed to treat infections caused by a ruptured appendix (Nelson & Levy, 2011a; Ramachandran & Schaefer, 2021). Traces of tetracycline–producing fermentation by-products were found in ancient mummies, as documented on an Egyptian papyrus. Additionally, mouldy bread was used to treat infections during this time (Bassett *et al.*, 1980). In 1947, chloramphenicol was discovered (Ehrlich *et al.*, 1947). The discovery and application of antibiotics significantly improved the treatment of previously complex diseases, leading to a worldwide reduction in morbidity and mortality from bacterial infections (Davies, 2006).

Unfortunately, the misuse of antibiotics has caused many bacteria to develop resistance to these drugs. In 1945, Fleming himself predicted the potential for bacterial resistance. During his Nobel Prize lecture, he warned about the dangers of inappropriate prescribing (Nathan & Cars, 2014).

Table 2.2 List of the most common antibiotics grouped into different classes. (Sanseverino *et al.*, 2018).



His prediction materialized in 1947 with the first observed case of penicillin resistance. Since then, bacteria have continued to evolve and develop resistance to various antibiotics. The period between 1940 and 1960 was regarded as the "Golden Age" for antibiotics. The first methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in the UK in 1962. The data indicate that each time a new antibiotic has been discovered, bacteria have subsequently developed resistance to it. Vancomycin, one of the most potent antibiotics, was introduced in 1972 to treat MRSA, but reports of vancomycin resistance began to emerge between 1979 and 1983. Sadly, resistance has been observed in nearly all antibiotics developed. Despite the pharmaceutical industry's ongoing efforts to discover new antibiotics to combat resistance, the rate of new drug development has slowed significantly over the years, with only 13 new antibiotics authorized since 2017 (Muteeb *et al.*, 2023), (WHO 2024).As a result, decades after the first patients were treated with antibiotics, bacterial infections are again becoming a significant threat. In our supposedly modernized world, antibiotic resistance is considered a new plague, rendering

common infections potentially untreatable and even deadly. Antimicrobial resistance (AMR) complicates the treatment of wartime injuries, especially in current conflicts such as those in Gaza, Sudan, and Ukraine. Following the 2018–19 demonstrations in Gaza, a significant increase of 300 % in resistance to specific antibiotics was documented among injured demonstrators. This underscores the sentiment: "Yet another reason why the best medicine is peace." (Spellberg *et al.*, 2014).

AMR is responsible for over a million deaths each year worldwide, with projections indicating an escalating death toll in the coming decades. Addressing AMR remains a formidable challenge since '*antibiotic development is no longer considered to be an economically wise investment for the pharmaceutical industry'* (Ventola *et al.*, 2015).

2.3 Antibiotic Resistance a growing threat to global Public Health

2.3.1 Introduction

"If we do not slow the rise of antimicrobial resistance, we will return to the dark ages of medicine where surgery becomes inherently risky, and currently treatable infections and injuries kill once again.'' This statement about AMRs was made by Dr. Margaret Chan, former Director–General of the WHO (Chan, 2012). Antibiotics have been defined as Godsends to humankind; however, with their increasing misuse, bacteria have evolved and developed antimicrobial resistance. The phenomenon of antimicrobial resistance refers to the potential of microorganisms, including bacteria, viruses, fungi, and parasites, which all thrive to continue to grow and proliferate in the presence of antibiotics designed to inhibit and kill them. Antimicrobial resistance is a complex phenomenon involving a spectrum of antimicrobial agents, bacterial species, resistance genes, and other resistance

mechanisms (Aarestrup, 2006; Salam *et al.*, 2023). Breakdown of the word ‘’antimicrobial’’ from the ancient Greek *anti* means ‘’against ‘’ and *mikros* means ‘’little’’ whereas *bios* mean ‘’life’’. Antimicrobial agents are substances against the life of microorganisms, and according to their definitions, the terms ‘’antimicrobial agents’’ and ‘’antibiotic’’ are not interchangeable. In 1889, the French biologist

P. Vuillemin used the adjective antibiotic for the first time to describe the destruction of one organism by another (Aarestrup, 2006).

The term antibiotic was first used as a noun in 1941. It was introduced by the Nobel laureate microbiologist S.A. Waksman, who defined it as ‘a chemical substance produced by microorganisms that can inhibit growth and destroy other microorganisms’ (Aarestrup, 2006).

2.3.2 Antibiotic Mechanisms

Antibiotics have several mechanisms of action against bacterial cells, including different pathways that can be either bacteriostatic, which prevents growth, or bactericidal, which means they destroy the bacterial cell (Kohanski *et al.*, 2010).

2.3.2.1 Bacteriostatic versus Bactericidal

Static antibiotics, such as chloramphenicol, trimethoprim, and tetracyclines, prevent bacteria from growing by keeping them in the stationary phase. Cidal antibiotics kill and destroy bacteria, such as aminoglycosides, beta-lactams, and quinolones (Kohanski *et al.*, 2010).

There are six main characteristics of action: synthesis targeting the cell wall, inhibition of protein synthesis, inhibition of DNA replication, RNA synthesis inhibitors, folic acid pathway metabolism inhibitors, and disruption of the plasma membrane (Pankey *et al.*, 2004).

2.3.3 Definition of Resistance

Antimicrobial resistance is a relative term. The two most used definitions are based on either in vivo resistance or in vitro resistance. In microbiology, the term 'resistance' refers to the ability of microorganisms to survive and proliferate despite the presence of an antibiotic that would usually destroy them or stop their growth. When bacteria are resistant and not susceptible to a particular drug, it becomes ineffective in treating infections caused by those resistant bacteria (Salam *et al.* 2023). Antibiotic resistance (AMR) is present in every country and can affect anyone regardless of age or gender. The WHO has always considered AMR to be among the top three public health threats. In 2019, an estimated 1.27 million deaths were attributed to AMR infections, while approximately 5 million deaths were recorded and associated with antimicrobial-resistant infections, according to major issues published in January 2022. By the year 2050, it is estimated that the number will go up to 10,000,000 (Antimicrobial Resistance Collaborators, 2022; World Bank, 2027).

Antimicrobial-resistant infection has been ranked 3rd as the leading cause of death after cardiovascular disease. AMRs can impact human health in terms of both therapeutic and preventive consequences (Antimicrobial Resistance Collaborators, 2022).

Healthcare and agricultural practices have been major contributing factors to the spread of AMR (Ghimpeteanu, Pogurschi, & Popa, 2022). Bacteria showing antibiotic resistance can have genes from Intrinsic, Acquired, or Adaptive sources (Lee, 2019)

- Intrinsic Resistance: This is naturally occurring resistance that is not caused by mutations or HGT.
- Acquired Resistance: This resistance is usually transmitted through a plasmid acquired via conjugation
- Adaptive Resistance: Bacteria increase the activity of efflux pumps and proteins

push out antibiotics from the cell (Holmes *et al.*, 2016; Ahmed *et al.*, 2024).

- Intrinsic resistance occurs when a bacterium is inherently resistant to a class of antibiotics, whereas acquired resistance occurs when the bacterium acquires mechanisms that allow it to remain unaffected by the drug. Intrinsic resistance is natural resistance that does not have a site of action for the antibiotic or has an alternative mechanism that prevents the antibiotic from acting (Pankey *et al.*, 2004). Adaptive Resistance: Bacteria increase the activity of efflux pumps and proteins push out antibiotics from the cell (Holmes *et al.*, 2016; Ahmed *et al.*, 2024).

2.3.4 Acquired Resistance

The following steps will help us better understand the actions that enable bacteria to develop resistance to antibiotics and the ability to transfer this resistance to susceptible bacterial strains. Selection pressure results in the increased prevalence and spread of resistance, which are outcomes of natural selection and should be viewed as expected phenomena of the Darwinian Biological principle of 'Survival of the Fittest' (Kahne *et al.*, 2005).

Antibiotic resistance can develop through several mechanisms, and they can be summarized as follows:

- Mutation
- Horizontal gene transfer
- Reduction in uptake
- Efflux pumps
- Modification of the antibiotic target site

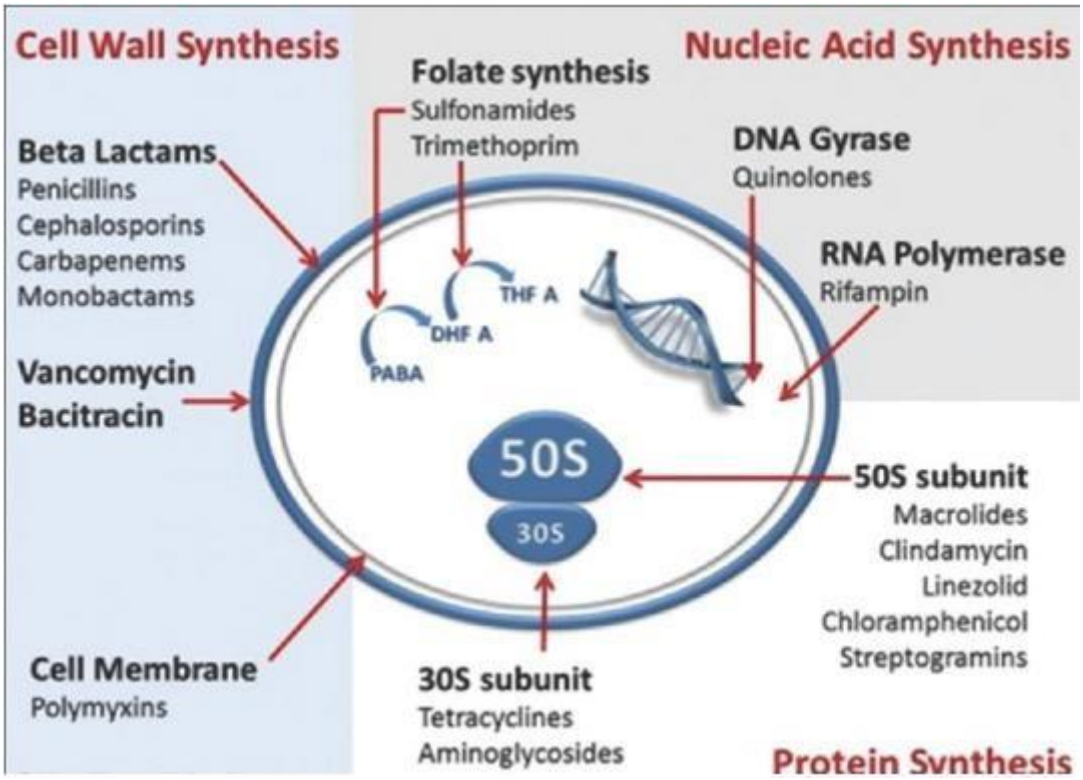


Figure 2.3 Sites of action of antibiotics (Kahne *et al.*, 2005).

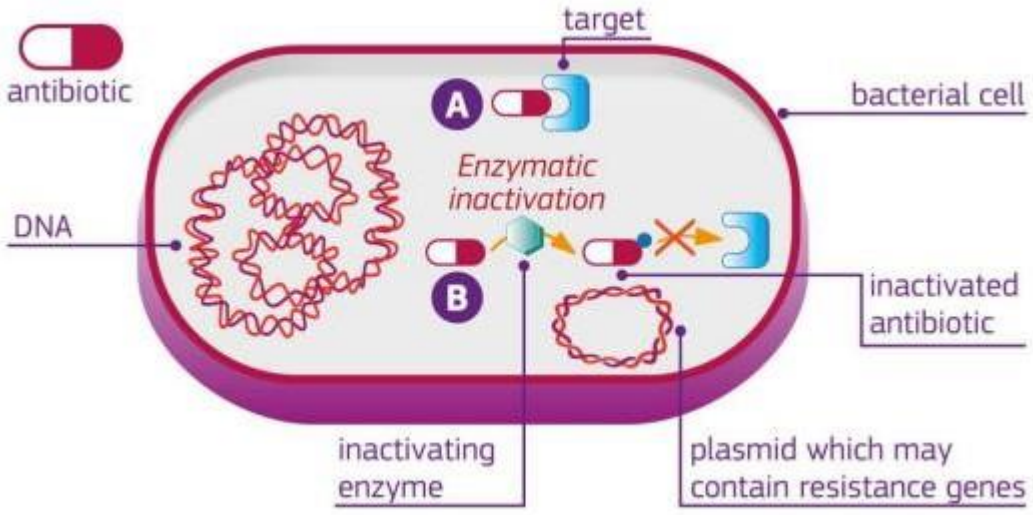


Figure 2.4 The enzymatic inactivation of antibiotics (Sanseverino *et al.*,2018).

Certain bacteria can sometimes become antibiotic-resistant through genetic mutation or efflux mechanism. When the antibiotic enters the bacterium through a porin channel, the efflux pump pumps it out, preventing the antibiotic from killing the bacterium. The bacteria, therefore, develop antibiotic resistance by reducing the antibiotic intracellular concentration (Fernández *et al.*, 2012).

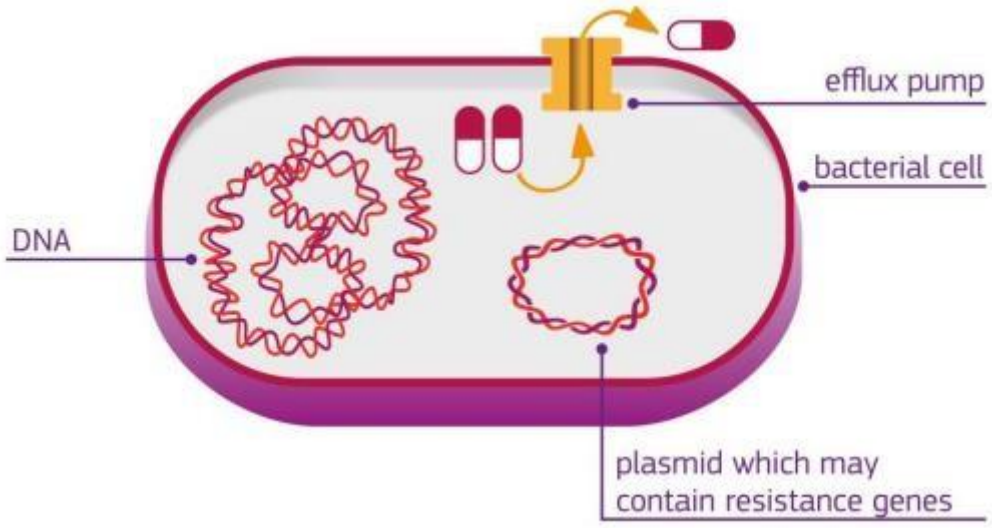


Figure 2.5 Efflux Pumps pumping antibiotics out of the bacterium (Sanseverino *et al.*, 2018).

2.3.4.1 Mechanisms of Antibiotic Resistance Transfer

Antibiotic resistance can be spread through two primary mechanisms Vertical Gene Transfer and Horizontal Gene Transfer

Vertical transfer involves the transfer from the mother to the bacterial daughter's cell during replication. However, the carriage of several resistance genes on the same plasmid results in multi-resistance and mutations in genetic material (Partridge *et al.*, 2015).

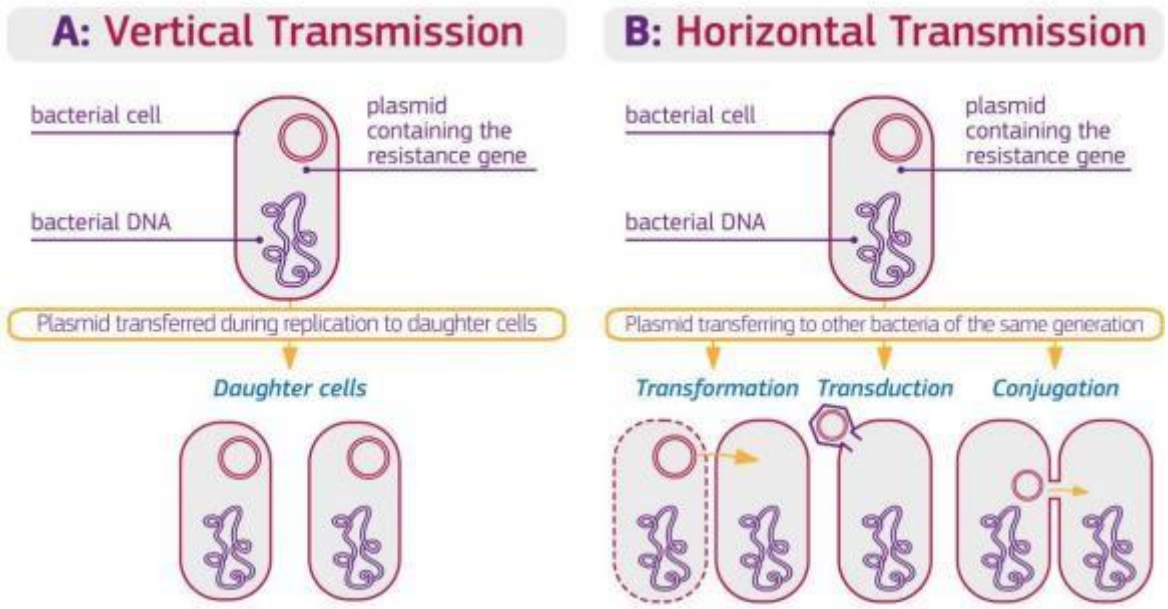


Figure 2.6 Mechanism of the different pathways of vertical and horizontal transmission in bacteria (Sanseverino *et al.*, 2018).

Horizontal Gene Transfer (HGT) Microorganisms use various mechanisms to develop drug resistance, such as recombining other DNA in the bacterial chromosome, HGT, (Klemm et. al., 2006; Burmeister *et al.*, 2015).

Horizontal Gene Transfer (HGT) can take place through 3 main stages:

- Conjugation
- Transformation
- Transduction

Conjugation is where the genetic elements are transferred using a pilus. It is mediated by a particular kind of circular DNA called a plasmid. Plasmids replicate independently of the chromosome. Many plasmids carry antibiotic-resistance genes. When two cells are close to each other a pilus is formed between them. The plasmid replicates and is transferred from one strain to another. With the aid of this process, susceptible bacteria

will acquire resistance to a particular antibiotic agent. Plasmids are extrachromosomal circular rings, like fragments of DNA, that replicate in the host cell. They vary in size in kilobases (kb) and can be horizontally exchanged among bacterial types by conjugation or mobilization (Waters *et al.*, 1999; Francia *et al.*, 2004; Carattoli *et al.* 2005). Plasmids may harbour antibiotic resistance genes and virulence genes. 'Incompatible' plasmids are those plasmids that have the same replication control, whereas plasmids with different replication control are 'compatible.' When two plasmids belong to the same incompatibility group (Inc group), they cannot be propagated in the same cell line. That is, the Inc group shares both identical replication and plasmid maintenance (Datta *et al.*, 1971). Inc groups in the F plasmid family, including IncFIA, IncFIB, and IncFIC, are closely related but not identical and can be subdivided into subgroups FIA, FIB, and FIC (Yang *et al.*, 2015). Some bacterial strains, like *E. coli*, can harbour multiple IncF subgroups in the same cell. Although these subgroups belong to the same F family, they may use different replication initiation proteins. IncFIA plasmids may contain RepA protein and IncFIB plasmids may use RepB for replication. Some *E. coli* strains may have IncFIA and IncFIB plasmids harbouring antibiotic-resistant genes or/and virulence genes. Protein ParA- ParB ensures an even distribution of plasmids during cell division, which permits their long presence from one generation to another (Rozwandowicz *et al.*, 2018). Integrons are genetic elements responsible in assembling, capturing, and expressing gene cassettes. They are found on plasmids or chromosomes and facilitate the spread of multidrug resistance among bacteria. Integrons are not mobile but embedded in mobile genetic elements like transposons or plasmids to facilitate their movement. ThMobile integrons play an important role in gene mobility across bacterial species and contribute to HGT. Class 1 integrons are commonly associated with MDR plasmids, allowing the bacteria to share the resistance genes and promoting multi-drug resistance (Rozwandowicz

et al., 2018). When on a plasmid, transposons facilitate the spread of antibiotic-resistance and virulence genes through HGT. Tn*3*, Tn*5*, and Tn*10* are transposons mediated on a plasmid. Insertion sequences like IS26 allow the plasmid to gain more resistance genes and facilitate transfer between bacterial strains, especially in Gram-negative strains where plasmid mediated exchange is more common (Bennett *et al.*, 2008). Transformation is when the DNA is picked up and incorporated into the bacterial host as 'naked' DNA. Which needs to be inserted either into the chromosome or a plasmid to be stably inherited, or it will be degraded by the cell. When cells die, bacterial DNA can be released into the surroundings and incorporated into other nearby bacteria. This DNA may act as a vector if it contains resistance genes, which may be easily transferred to the reception cell (Bennett *et al.*, 2008). Transduction is where the DNA is transferred by bacteriophage. The host DNA is packaged into the phage head, and specialized transduction results from phage lysogeny.(Norman *et al.*, 2009).

2.3.5 β-lactam Antibiotics

This large class of antibiotics, with low toxicity to humans, contains a cyclic amide called a β-lactam ring as its basic building block. Although they share a standard mode of action, they are the most frequently used antibiotics worldwide (Bush *et al.*, 2016).

The β-lactams include penicillin, cephalosporins, monobactams, and carbapenems, where penicillin was the first to be used. β-lactam antibiotics are usually used in humans and veterinary medicine to treat infections caused by human and animal pathogenic *E. coli* (Patwary *et al.*, 2017). β-lactamase inhibitors, together with beta-lactam antibiotics, prevent the breakdown of the antibiotic by beta–lactamases. Common examples of β-lactam inhibitors include clavulanic acid (Patwary *et al.*, 2017).

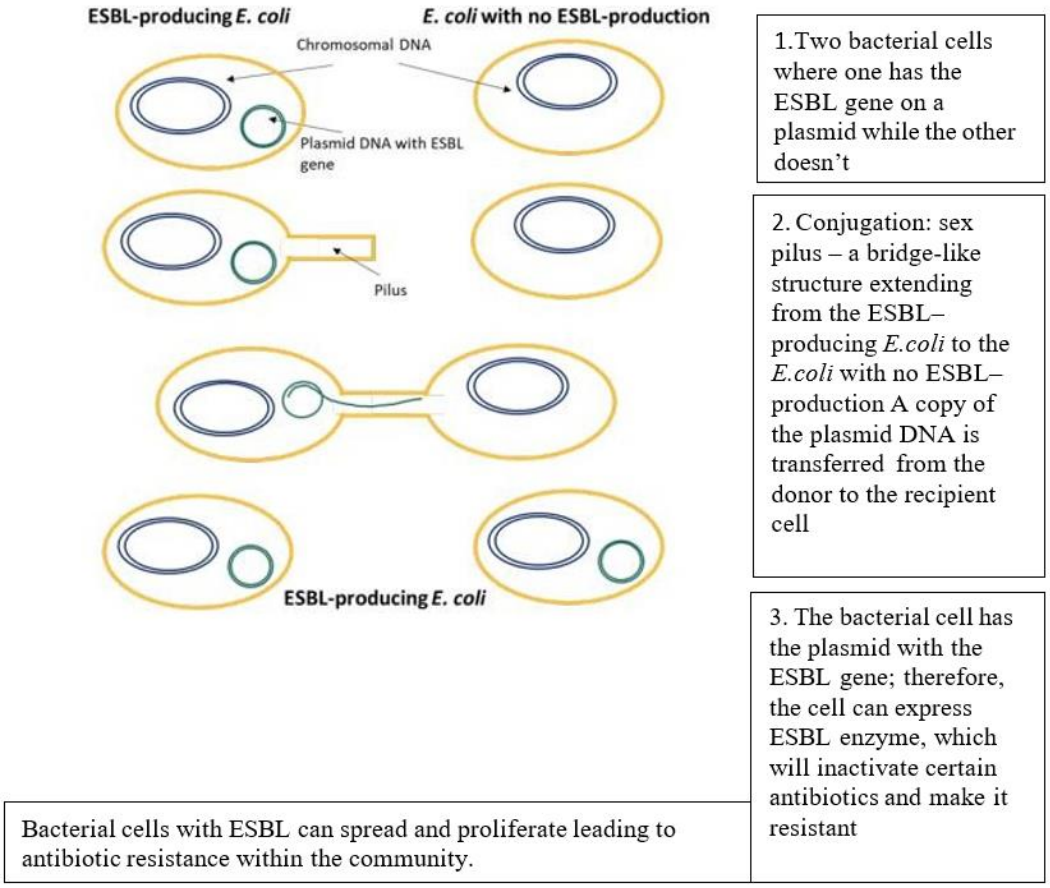


Figure 2.7 The schematic illustration of conjugation (Adapted from Epidemiology of Extended – Spectrum Beta- Lactamase (ESBL) – producing *E. coli* with special reference to outbreak detection.)

β-lactams are the most frequently prescribed group of antibiotics, accounting for approximately 50 % of global antibiotic consumption. This use has inevitably selected for resistance. When penicillin was released in 1941, *Staphylococcus aureus* was sensitive to this agent. However, within one year of its introduction, penicillin-resistant strains of *S. aureus* were found to be no more resistant. The rapid expansion of penicillin resistance among *Staphylococcus* spp isolates worldwide led to a crisis in the 1950s, which appeared to be solved with the introduction of methicillin in 1961 (Lobanovska & Pilla, 2017).

β-lactam antibiotics are divided into distinct classes according to the chemical nature of

the β-lactam ring or the heteroatom included in the ring and are classified into penicillins, cephalosporins, carbapenems and monobactams (Armstrong *et al.*, 1999). The β-lactam ring has a structure like the terminal amino acid residues on the peptide subunits of the peptidoglycan layer.

Therefore, these structural similarities help the β-lactams to bind to the penicillin-binding protein (PBP's). In some bacteria, the production of AmpC enzymes can be 'induced.' Amp C β-lactamase are class C cephalosporins, chromosomally encoded enzymes found in many Enterobacteriaceae (Jacob, 2009). This means that exposure to certain antibiotics like cefoxitin can trigger the bacteria to produce more AmpC, leading to higher levels of resistance *E. coli*. *Amp C* belongs to Class C of the Amber classification and Group 1 according to the Bush, Jacoby, and Mederios classification. Biochemically, they are poorly inhibited by Clavulanic acid and cannot hydrolyse fourth- generation cephalosporin but can hydrolyse cephamycins AmpC β- lactamase is a class of enzymes produced by some bacteria that confer resistance to certain β- lactam antibiotics. These enzymes can hydrolyze and inactivate a broad range of β-lactam antibiotics, including Penicillins, cephamycins (like cefoxitin), and cephalosporins. They typically do not affect the cephalosporin cefepime or the carbapenems. AmpC β--lactamases are commonly chromosomally encoded. They are found on the chromosomes of certain bacteria species. However, certain bacteria can acquire genes encoding AmpC enzymes on mobile genetic elements like plasmids, which can be shared among bacteria (Shayan *et al.*, 2015). In some bacteria, the production of AmpC enzymes can be 'induced.' This means that exposure to certain antibiotics like cefoxitin can trigger the bacteria to produce more AmpC, leading to higher levels of resistance. *E. coli* produces the AmpC enzyme; it is an overexpression that confers resistance (Shayan *et al.*, 2015).

β -lactam antibiotics, including third-generation cephalosporins, are the most frequently used antibiotics worldwide.

2.3.5.1 The β -lactam Ring

These agents work by inhibiting the synthesis of the peptidoglycan layer by enzymes called transpeptidases or penicillin-binding proteins (PBPs) (Bush *et al.*, 2016).

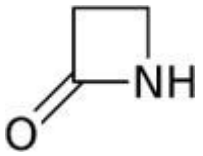


Figure 2.8 The structure of the β-lactam ring

With the increased resistance, protecting the β-lactam antibiotics from degradation was necessary. β-lactam inhibitors are derived from β-lactams, where these β-lactams inhibitors bind to β-lactamases and inactivate them (Bush *et al.*, **2016).**

β-lactams are the most used antimicrobial agents due to their broad-spectrum activity and efficacy. They contain a beta-lactam ring in their molecular structure. This class works by interfering with the synthesis of the bacterial cell wall, leading to its lysis (breakdown). According to the Bush and Jacoby classification, extended-spectrum cephalosporins belong to the group 2 β-lactams.

Cephalosporins are divided into five generations, and the third-generation is the most important due to its extended spectrum against Gram-negative bacteria such as *E. coli* ESBLs are biochemically characterized by their ability to hydrolyze an expanded spectrum of β-lactam antibiotics and their inhibition by β-lactamase inhibitors, specifically clavulanate; they confer resistance to 4th-generation cephalosporins (ESBLs). These enzymes can degrade a wide variety of β-lactam antibiotics.

'β-lactam antibiotics, including the 3rd generation cephalosporins, are essential tools in modern medicines, but their efficacy is threatened by rising resistance.' (Bush, 2010)

2.3.6 Mechanism of Action

β-lactams inhibit the synthesis of peptidoglycan layers of bacterial cell walls. The beta-lactam ring binds to enzymes known as penicillin-binding proteins (PBPs). When this synthesis is interrupted, the bacterial cell wall becomes weak and unstable, causing the bacteria to die. Resistance against β-lactam antibiotics results from the production of β-lactamases. These enzymes break open the β-lactam ring, rendering the antibiotic ineffective (Armstrong et. al., 1999). β-lactamase inhibitors are agents that inactivate Beta-lactamase enzymes produced by some bacteria, preventing the degradation of β-lactam antibiotics. Various β-lactamase inhibitors include: Clavulanic acid, sulbactam and tazobactam. Clavulanic acid inhibits β-lactamases. β-lactamase inhibitors bind to β--lactamase enzymes, preventing them from breaking the antibiotic's β-lactam ring. This allows the antibiotic to remain effective.extended-spectrum β-lactamases (ESBLs).

2.3.6.1 Mode of action

β-lactams act by inhibiting the synthesis of the cell wall, which is achieved by blocking the action of transpeptidases. Specialized acyl serine transferases are involved in the bacterial cell wall assembly. Specifically, they are involved in synthesising the peptide chains which cross link the peptidoglycan chains together to give the walls stability. When these peptide chains are not synthesised, the cell continues to make glycan chains but these are not cross linked and so the walls weaken and eventually the cell wall will rupture. As only growing cells make new walls, static cells are not affected. The structural

properties of β-lactams mimic the D - Ala – D – Ala sequence in that the distance between the carboxylate and the cyclic amide is similar. Thus, these antibiotics are a false substrate for D – alanyl – D- alanyl transpeptidases.

The carboxylate or the sulphonate group of the β-lactams reacts with a serine residue of the transpeptidases (also known as PBPs penicillin-binding proteins) to give an acyl-enzyme, with the formation of a covalent bond. The acylated enzyme is inactive. Such a mechanism is called suicide inhibition or mechanism-based enzyme inactivation. Transpeptidases are in the periplasmic space, directly accessible in Gram-positive bacteria. In Gram-negative bacteria, beta–lactams must cross the outer membrane of the bacteria either through the membrane or via porin channels. The perturbations induced by the β-lactams in the cell wall formation explain the inhibition of the bacteria's growth, but the bactericidal effect results from indirect mechanisms. β–lactams are only active against highly dividing bacteria (Armstrong *et al.*, 1999).

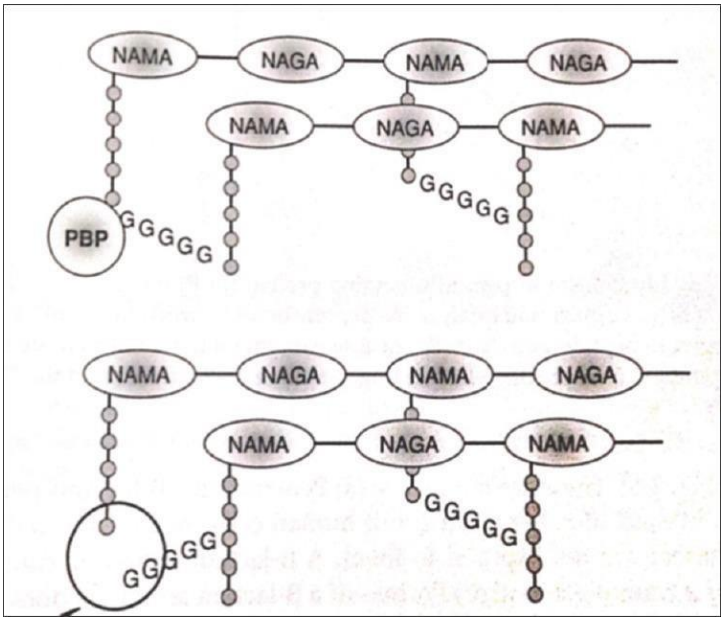


Figure 2.9 Mechanisms of resistance to β-lactams Antibiotics (Džidic *et al*., 2008).

2.3.7 Resistance Mechanism

Bacteria producing AmpC enzymes can resist the action of certain beta-lactam antibiotics by breaking open their Beta-lactam ring, rendering the antibiotic ineffective.

The following is a detailed explanation of the main β-lactams, including their typical features and clinical relevance.

Classes:

Penams

This class includes the penicillins. These are N-acylated derivatives of 6-B-aminopenicillanic acid. The β-lactam ring is fused in these compounds with a saturated five-membered thiazolidine ring containing sulphur. The sulphur heteroatom can be synthetically oxidized to form sulphone, and this will yield such β-lactamase inhibitors as sulbactam and tazobactam (O'Grady *et al.* 1997).

Penicillin has a spectrum of activity against Gram–positive organisms that do not produce β– lactamase and some fastidious Gram–negative organisms. The aminopenicillins, which include ampicillin and amoxicillin, have a slightly broader spectrum than penicillin, with activity against additional Gram-negative organisms, including some members of Enterobacteriaceae. Expanded-spectrum penicillins include carboxypenicillins and ureidopenicillins (mezlocillin and piperacillin). These two groups have an expanded spectrum of activity against *Pseudomonas spp*. and *Burkholderia spp*. Another group is the penicillinase–stable penicillins such as cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin. These are of particular importance in the treatment of penicillinase-producing staphylococci (NCCLS, 2000).

Penems: differ from penams by a double bond between C-2 and C-3. There are so far no known natural penems, but many have been synthesized (O'Grady *et al.*, 1997).

Clavams: the only notable member of this group currently is clavulanic acid, a compound with little antibacterial activity. It owes its therapeutic place to its ability to inhibit bacterial β- lactamases. The structure differs from penams in the substitution of oxygen for sulphur (O'Grady *et al.*, 1997).

Carbapenems and carbapenams: members of this class include thienamycin, imipenem, and meropenem. They differ from penams and penems in the substitution of CH2 for sulphur in the β-lactam ring (O'Grady *et al.*, 1997).

The slight difference in structure to penams renders the carbapenems more resistant to β-lactamase hydrolysis. This property gives them a broader spectrum against both Gram-positive and Gram-negative organisms (NCCLS, 2000).

Cephems: this is the cephalosporin class, and they are N – acylated derivatives of 7 – β aminocephalosporanic acid. These compounds are characterized by a six-membered dihydrothiazine ring containing sulphur and a double bond fused to the β-lactam ring (O'Grady *et al.*, 1997).

The different cephalosporins have different spectra of activity against both Gram-positive and Gram-negative organisms. Depending on the extent of their activity against the more antibiotic- resistant Gram-negative bacteria, these agents are referred to as"first –," "second –," "third –" and "fourth – " generation cephalosporins. Not all representatives of a specific group or generation necessarily have the same spectrum of activity (NCCLS 2000).

Cephamycins: are closely related to cephems, which are substituted at the 7 – position with an alpha–methoxy group. Examples of cephamycins are cefoxitin and cefotetan (O'Grady *et al.*, 1997).

Oxacephems: this class includes the oxygen analogs of cephems with latamoxef being a notable example (O'Grady *et al.*, 1997).

Carbacephems: in this class, which is closely related to cephams, the sulfur atom is replaced by carbon. An example is loracarbef (O'Grady *et al.*, 1997).

Monobactams: these are cyclic amides in a four-membered ring with methylcarboxylate function in the case of nacardicins and sulphonate in the case of aztreonam (O'Grady *et al.*, 1997).

Trinems formerly known as Tribactams: Significant developments have been made in synthesizing and applying tricyclic carbapenems. This class provides a broad–spectrum orally active β– lactam agent, consisting of a fused tricyclic skeleton (O'Grady *et al.*, 1997).

Table 2.3 Different classes of Antibiotics and their actions

Antibiotic Class	**Mechanisms of Action**	**Examples of Antibiotics**	**Bactericidal (BC) or Bacteriostatic (BS)**
β-lactams	Penicillins inhibit bacterial cell wall synthesis by targeting penicillin-binding proteins (PBPs), leading to cell lysis	Penicillins Cephalosporins Carbapenems Monobactams	**(BC)**
Glycopeptides	Inhibit bacterial cell wall synthesis by binding to D-Ala-D-Ala residues.	Vancomycin	**(BC)**
Aminoglycosides	Irreversibly bind to the 30S ribosomal sub-units, inhibiting protein synthesis and bacterial death.	**Gentamicin** **streptomycin**	**(BC)**

Table 2.3 (Continued) Different classes of Antibiotics and their actions

Tetracyclines	Irreversibly bind to the 30S ribosomal sub-units, inhibiting protein synthesis and bacterial death.	Tetracycline	**(BS)**
Macrolides	Inhibit protein synthesis by binding to the 50 S ribosomal subunit.	Azithromycin	**(BS)**
Oxazolidinones	Inhibit protein synthesis by binding to the 50 S ribosomal subunit.	Linezolid	**(BS)**
Quinolones & fluoroquinolones	Inhibit DNA gyrase–blocking bacterial DNA replication.	Ciprofloxacin	**(BC)**
Sulphonamide & Trimethoprim	Inhibition of folate synthesis	Sulfamethoxazole-Trimethoprim	**(BS)**
Chloramphenicol	Binds to the 50 S ribosomal and inhibits bacterial protein synthesis	Chloramphenicol	**(BS)**
Lincosamides	Binds to the 50 S ribosomal and inhibits bacterial protein synthesis	Clindamycin	**(BS)**

Table 2.3 (Continued) Different classes of Antibiotics and their actions

Lipopeptides	Causes ion leakage from the bacterial Cell	Daptomycin	**(BC)**
Polymyxins	Disrupting bacterial membrane integrity by binding to the membrane	Polymyxin B	**(BC)**
Nitrofurans	Damage bacteria DNA	Nitrofurantoin	**(BS)**
Rifamycins	Inhibit bacterial RNA synthesis.	Rifampin/rifampicin	**(BC)**

(Brunton *et al.*, 2018; Katzung *et al.*, 2021)

Antibiotics can be classified by their mechanism of action of either bactericidal or bacteriostatic, broad or narrow spectrum of activity and routes of administration either oral or by injection.

2.4 Extended Spectrum β-lactamases (ESBL)

Cephalosporins which belong to the β-lactam antibiotic family, were discovered in 1945, and used in 1964 to treat penicillin-resistant cases. Since then, several generations of cephalosporins have been used, with the fifth generation still in use (Salam *et al.*, 2023). Cephalosporins were originally useful against Enterobacteriaceae and other gram-negative bacteria. They were never effective against ESBL producing bacteria. It is the bacteria that have developed resistance to the antibiotic, not the antibiotic itself. By the fourth generation, bacteria have acquired significant resistance. ESBLs primarily hydrolyze and therefore confer resistance to extended-spectrum third and fourth-generation cephalosporins and monobactams like aztreonam. They do not typically affect cephamycins like cefoxitin or carbapenems. *AmpC* confers resistance to a broader range of cephalosporins, including cephamycins. However, it typically does not affect the

fourth- generation cephalon. *AmpC* production is the observation of resistance to cefoxitin since *AmpC* enzymes efficiently hydrolyze this antibiotic (Bush et al.,2010**).** According to the Ambler molecular classification system, AmpC β-lactamases are classified as class C β-lactamases, which categorizes β-lactamases based on amino acid sequence homology. The Ambler molecular system divides β-lactamases into four classes: A, B, C, and D. Classes A, C, and D, are serine β-lactamases, utilizing a serine residue at their active site to hydrolyze β-lactam antibiotics, whereas class B comprises metallo-β-lactamases that require zinc ions for activity (Bush etal.,2010**).**

2.4.1 Cephalosporins

There are currently five generations of cephalosporins, each with differing antimicrobial spectrums and activities. First-generation cephalosporins have better activity against Gram- positive bacteria than Gram-negative bacteria, but cephalosporins of subsequent generations have more excellent Gram-negative antimicrobial properties than their previous ones (Okamoto *et al.,* 1994). Cephalosporins are considered the most widely prescribed classes of antibiotics. The first-generation cephalosporins were commonly used to treat community infections, whereas the more recent cephalosporins, with their broad spectrum of activity against Gram-negative bacteria, make them more useful to treat hospital-acquired infections (Klugman *et al.,* 1997). In agriculture, first and second-generation cephalosporins are used to treat mastitis infection in dairy cows, and the third-generation cephalosporin has been approved for respiratory diseases in swine, ruminants, and horses (Hornish *et al.,* 2002) Aminoglycosides are another type of bactericidal antibiotics that bind to the 30S Subunit of the bacterial ribosome and inhibit protein synthesis. These antibiotics are used to treat skin surface infections and infections in the respiratory system (Moellering, 1983).

Glycopeptides are another example of bactericidal antibiotics like β-lactam antibiotics. These inhibit the bacterial cell by inhibiting cell wall peptidoglycan synthesis (Reynolds, 1989).

A known glycopeptide, vancomycin, has been given the most attention in treating MRSA infections. Vancomycin has been identified as a 'last resort' drug used after a failed treatment with other antibiotics (Levine, 1987).

2.4.2 Origin and Transmission

β -lactamase enzymes have been the most studied topic in this field, and they have been studied from different aspects, such as classification, hydrolytic profiles, and kinetic properties.

According to Davies *et al.* (2010)**,** the number of beta-lactamases has increased as described since the 1980s due to increased class A and class D β-lactamases (Bush *et al*., 2010). Among class A beta-lactamases, the extended-spectrum β-lactamases (ESBL) represent a public health issue and are of clinical concern and limiting treatment options for infections (Canton *et al*., 2012). Class A ESBLs mainly include the following resistance enzymes: TEM, SHV, CTX-M, VEB, and GES (Canton *et al.*, 2012). The increase of the enzyme CTX-M all over the world has been described or referred as the 'CTX-Mpandemic'(Canton *et al.*, 2006). ESBLs are generally located on plasmids and are characterized according to their ability to hydrolyze the 3rd and 4th generation cephalosporins and also monobactams but not cefoxitin and carbapenems. (Canton *et al.*, 2012).

2.4.3 Classification of β-lactamases and CTX-M enzymes

Until recently, β-lactamases were classified according to their hydrolytic spectrum,

susceptibility to inhibitors, and whether the chromosome or plasmids encode them. With the rapid emergence of new mutant enzymes, it soon became apparent that this system was inadequate. In 1989, Bush proposed a significant re-organisation. This scheme was adopted in 1995 (Bush *et al.*,1995). The revised Bush scheme classifies β-lactamases by their substrate preference among penicillin, oxacillin, carbenicillin, cephaloridine, expanded–spectrum cephalosporins, and imipenem and by the susceptibility to inhibition by clavulanic acid. A disadvantage of the Bush classification is that point mutations can significantly alter substrate specificity and inhibitor susceptibility, thus changing the group to which an enzyme is assigned. Increasingly, therefore, β-lactamases are classified by a nucleotide sequence, as first proposed by Ambler (1980). This classification is more stable as it is based on fundamental relationships unaffected by mutations. It is also simpler, having only four groups designated A to D (Livermore, 1995). ESBL β-lactamases can be defined and classified by the Ambler classification system and by the Bush–Jacoby–Medeiros classification system as in Figure 2.10 which shows Ambler's A and D classes of ESBL belong to group 2 in the Bush–Jacoby–Medeiros system. Bush and Jacoby later proposed an update to the original Bush–Jacoby–Medeiros functional classification system of β-lactamases for the newly evolved β-lactamases. In both the original version and the updated 2009 version of the classification, ESBL belonged to group 2.

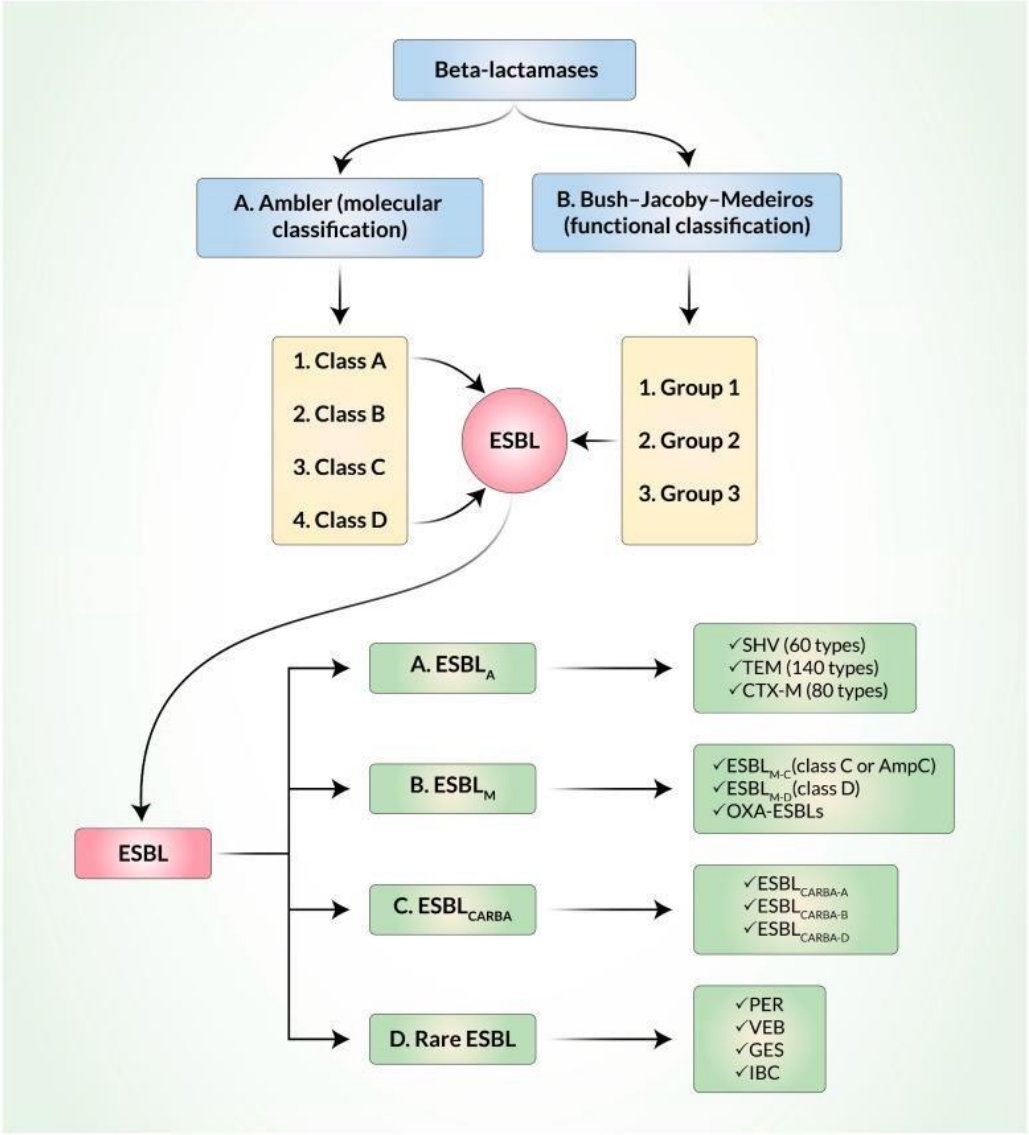


Figure 2.10 Classification of β-lactamases (Husna *et al.*, 2023).

β-lactamases could be grouped into three categories (Patwary *et al.*, 2017)

1. Class A ESBLs ($ESBL_A$) – high prevalence $ESBL_A$ are CTX-M, TEM – ESBLs, SHV- ESBL's; 2. Class M ($ESBL_M$) is further subdivided into two categories into ($ESBL_{M-C}$) and ($ESBL_{M-D}$), ($ESBL_{M-C}$) plasmid–mediated AmpC – Class C, ($ESBL_{M-D}$) – (OXA-ESBLs) – Class D; 3. ESBLs with hydrolytic activity against carbapenems ($ESBL_{CARBA}$). Classification of β-lactamases is based on two central schemes, the Ambler scheme and the Bush-Jacoby and Madeiros group belonging to the molecular class A

beta-lactamases from Ambler's classification (Canton *et al.*, 2012). In this classification, Class A β-lactamases include the TEM, SHV, and CTX-M types showing 'ESBL phenotype' (Canton *et al.*,2012), Class B contains carbapenems but not monobactams. Class C includes AmpC, which is mainly produced by Enterobacteriaceae and *Pseudomonas* (Patwary et al., 2017).

Class D includes the OXA-type enzymes known as the carbapenem-hydrolyzing class D β - lactamases (CHDLs) The Bush-Jacoby and Madeiros groups are based on the substrate and the inhibitor profile of the enzyme: Group 1 includes Class C enzymes; Group 2 includes Classes both A and D; Group 3 includes Class B;Group 4 includes Class A but is specific for penicillins.

2.4.4 Further development of ESBLs

The enzyme CTX-M did not originate from mutations from previous plasmid-mediated enzymes but through mobilization of chromosomal *bla* genes from *Kluyvera spp*. These initially mobilized $bla_{CTX\text{-}M}$ genes hydrolyzing cefotaxime over ceftazidime (Canton *et al*., 2012). CTX-M enzymes can be differentiated depending on their amino acid sequence. Within the CTX-M enzymes, CTX-M 14 and CTX-M-15 are the most important, as CTX-M-15 is the most widespread and found in multidrug-resistant Enterobacteriaceae. Other CTX-M variants include CTX-M-1, 2, 3, 8, 9, 25, 44, and 45 (Shaikh *et al.*, 2015).

FEC-1 (Faecal *E. coli*) related to CTX-M-3 enzyme initially recovered in Poland (Bonnet 2004). The β-lactamase named MEN-1 was the first sequence of a CTX -M β- lactamase (Canton *et al.*, 2012). The type of CTX -M enzyme can vary due to different geographic areas and groups of patients and other factors that might contribute to the current CTX -M scenario (Caratolli, 2008).

2.4.5 CTX M enzymes in Non- Enterobacteriaceae organisms

E. coli is the most important pathogenic strain producing CTX-M enzymes. CTX-M enzymes were initially and commonly associated with the family of Enterobacteriaceae as described in *E. coli, Klebsiella pneumoniae* and *Salmonella spp,* with *E. coli* as the most important pathogenic strain producing CTX-M enzymes (Canton *et al.*, 2006). However, besides Enterobacteriaceae, CTX-M enzymes have also been detected, but are less commonly, in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Canton *et al.*, 2006). *Vibrio spp*. isolates with CTX -M enzymes have also been recognized (Canton *et al.*, 2012). OXA enzymes, also β-lactamases, are characterized by hydrolytic activity against oxacillin and cloxacillin but are hardly inhibited by clavulanic acid. AmpC Beta-lactamases hydrolyze both broad and extended-spectrum cephalosporins as well as cephamycins.

2.4.6 CTX-M β-lactamase Pandemic

The research article by Canton *et al.* (2006) provides a comprehensive and significant picture of the urgent global threat posed by CTX-M-type ESBL variants. These ESBL-resistance enzymes have become the most prevalent resistance genes in hospitals and the community. Due to genetic mobilization, $bla_{CTX\text{-}M}$ genes have spread rapidly among pathogenic bacteria. Those $bla_{CTX\text{-}M}$ genes are commonly carried on plasmids harbouring Class 1 integrons and other associated transposons like Tn*402* derivative Tn*21*-like elements, which promotes their integration into other plasmids (Canton *et al.*, 2006).

Plasmids harbouring $bla_{CTX\text{-}M}$ genes frequently carry other additional resistance genes, such as aminoglycosides, tetracyclines, sulfonamides, and fluoroquinolones. Since the publication by Canton *et al.* (2006), the CTX-M lactamase threat has significantly intensified, becoming a threat to public health. CTX-M-resistance genes have become the

most found ESBL-resistance genes worldwide, with CTX-M-15 being the most predominant among all groups (Castanheira *et al.*, 2021). Another emerging CTX-M variant is CTX-M-151, which is thriving and posing new threats (Mendonça *et al.*, 2022).

2.5 Antibiotic use in Agriculture and its Consequential Resistance in Environmental Sources: A Potential Public Health and Livestock Implications

2.5.1 IntroductionDue to their continuous emissions, Zhi and Cheng (2024) describe antibiotics as 'pseudo- persistent organic contaminants.' After being introduced to human disease treatment, antibiotics were also used to treat farm animals with infectious diseases, and they showed effective results (Ben *et al.*, 2019). By the 1950s, with the rise of modern commercial farming, antibiotics were also used to increase the productivity of livestock animal and herds started to increase in number while infections also surged (Gustafson and Bowen,1997).

While antibiotics helped farmers avoid commercial losses, they contributed to and fuelled the global antibiotic resistance crisis (Maron *et al.*, 2013). Although growth promoters have been banned in European countries, however, they are still practically used in developing countries to produce more herds due to their high-density populations (Maron *et al.*, 2013). Excessive use of antibiotics can stimulate the emergence of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARG) (Pruden *et al.*, 2006).

Antibiotic resistance poses a significant threat to public health because antibiotic-resistant bacteria associated with animals may be pathogenic to humans and can easily be transmitted by zoonosis. These may cause complicated treatments due to prolonged

infections. In humans, this leads to higher healthcare costs and even death. Further, the classes of drugs that are more widely used in agriculture at a global level that and are of public health concern include tetracyclines, aminoglycosides, beta-lactams, lincosamides, macrolides, and sulphonamides. In Europe, most of the use of antibiotics as a growth promoter in animals was banned.

For example the use of antibiotics as growth promoters was banned in Sweden in 1986. In other European countries, avoparcin was banned in 1997 followed by bacitracin, spiramycin, tylosin, and virginiamycin in subsequent years. In 2011, the European Commission published a report stating the importance of introducing the new Animal Health Law (Sanseverino *et al.*, 2018).

Focusing on the proper use of antibiotics by preventing diseases and replacing current animal health provisions based on disease control (Casawell *et al.*, 2023) soil and water have been regarded as important reservoirs and sources of antibiotic resistance, which has greatly impacted Public Health. Antibiotics can enter the agroecosystem mainly through manure fertilizers, wastewater irrigation practices, and the use of biosolids (Fu *et al.*, 2023). Soil contamination with antibiotics has been reported worldwide (Du & Liu, 2012).The usage of antibiotics in livestock animals has increased significantly, raising the concentrations of antibiotic residues in manure, which supports the rise of antibiotic resistance. When it is spread in agricultural fields, antibiotic residues and bacteria carrying antibiotic-resistance genes may be introduced into the environment. The ever-increasing demand for food animal production has been considered the main reason for the intensification of antibiotic usage in farm animals. It has been used not only to treat diseases as therapy but also as metaphylaxis, administering antibiotics to whole herds, even if only very few exhibit clinical symptoms. A mass medication application of antibiotics to eliminate and prevent the outbreak of diseases while preventing economic

losses (Sanseverino *et al.*, 2018). Antibiotics are commonly added to animal feed as a supplement to promote the growth of livestock animals. The ongoing use of antibiotics by the livestock industry and animal consumption, therefore, continuously threatens public health.

The absorption of antibiotics in the animal gut will not be completed, and as a result, substantial amounts of antibiotics are excreted in urine and faeces, which all end up in the animal's manure (Kumar *et al.*, 2005). Another observed factor was the accumulation of antibiotics in field crops under manure fertilization.

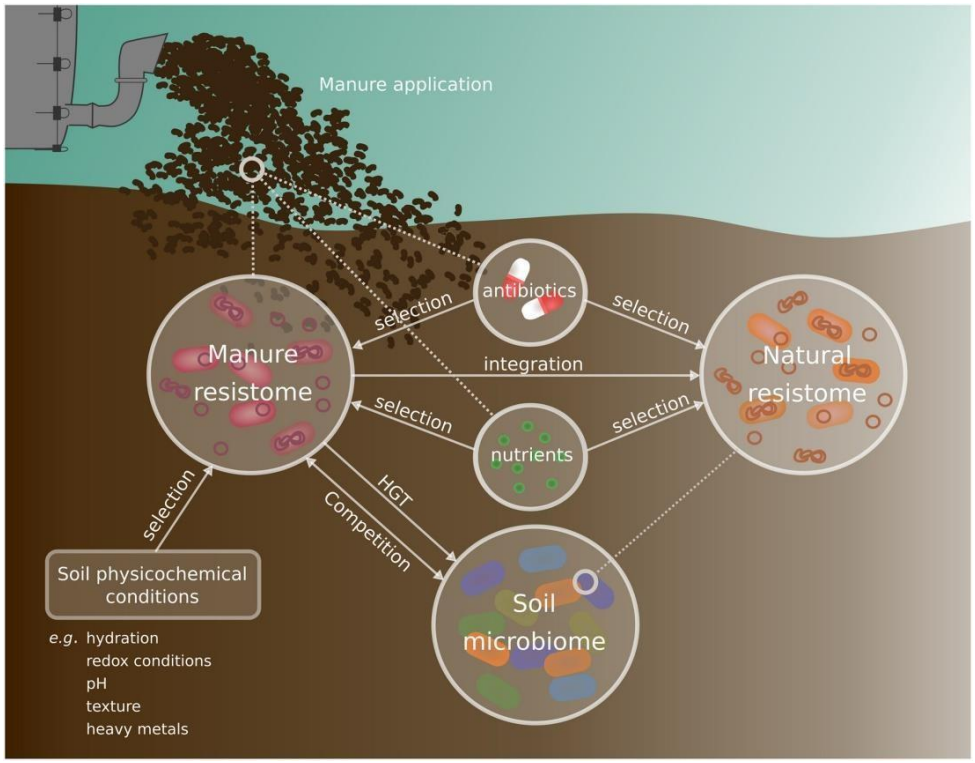


Figure 2.11 Untreated manure in soil

In Figure 2.11 A pathway showing how untreated manure can influence soil microbial flora and help develop and spread antibiotic-resistant bacteria (Jadeja & Worrich, 2022).

It should be noted that when livestock animals are treated with antibiotics, the application of manure as organic fertilizer in agriculture can mediate the contamination of soil and surface waters (Sanseverino *et al.*, 2018). This means that antibiotics may be transferred into the environment through the application of manure, posing an ecological risk from exposure to these contaminants (Lima *et al.*, 2020). Agricultural runoffs are also referred to as a source of antibiotic dissemination. They refer to water leaving farms that run over agricultural lands and then flow in surface water rather than being absorbed into groundwater. Application of manure from antibiotic-treated animals to crops facilitates the spread of anti-resistant determinants into the environment (Manyi-Loh *et al.*, 2018). Studies conducted at the University of Minnesota, US, have shown that vegetables, such as corn, potatoes, lettuce, and others, whether eaten raw or not absorb antibiotics when grown in soil fertilized with livestock manure if enriched with these residues (Kumar *et al.*, 2005; Camitile, 2009). In 2005 researchers in Minnesota conducted an experiment to access the environmental impact of antibiotic use in livestock production. Manure from animals fed with antibiotic supplemented diets was applied to agricultural soils, in which corn, green onions and cabbages were subsequently cultivated. Analytical testing of harvested plant tissues confirmed the uptake of antibiotic residues indicating the potential transfer of veterinary pharmaceuticals in the human food chain. (Kumar *et al.*, 2005; Camitile, 2009). These crops accumulated concentrations of antibiotic residues after they were treated with liquid hog manure enriched with sulfamethazine, an antibiotic commonly found in livestock animals. Other studies show that chlortetracycline concentrations in plants increase with the concentrations of antibiotics present in the manure (Kumar *et al.*, 2005).

Manure is widely used as a natural substitute for fertilizers, providing plants with natural nutrients rather than other chemicals used as fertilizers. The antibiotic accumulation in

vegetables is just another negative consequence of our animal agriculture industry and not surprisingly given to quantify feed to livestock. Some vegetables pose a public health concern since they are eaten raw, like spinach, which is not usually processed but washed with water, allowing antibiotics to remain inside. Tubers, like potatoes, carrots, and radishes, are underground tubers that uptake and store nutrients from the soil. Since such tubers and other crops are found in direct contact with the soil, they may show greater prosperity for antibiotic intake (Camitile, 2009). Previous studies (Mullet *et al.*, 2019) have documented the occurrence of antibiotics in various field vegetables such as lettuce, tomatoes, cauliflower, broad beans, peanuts, and corn. Crop exposure to antibiotics at a relatively high dose may lead to overestimating antibiotic accumulation in plants, posing a risk to livestock animals and human health (Geng *et al.*, 2022). Although manure can enrich soils with natural nutrients and be used for organic farming, some countries impose legislative restrictions on its application. In Malta, the application of manure in fields is restricted. Due to nitrate restrictions under the Nitrate Directive 91/676/EEC, livestock manure is regulated not only because it may act as a reservoir of antibiotic resistance but because it poses a significant risk to ground water in Malta.. (Nitrates Action Programme Malta, 2011).

Antibiotics can enter the agroecosystem mainly through manure fertilizers, wastewater irrigation, biosolid reuse, and act as a reservoir for the spread of antibiotic-resistant bacteria by flies.

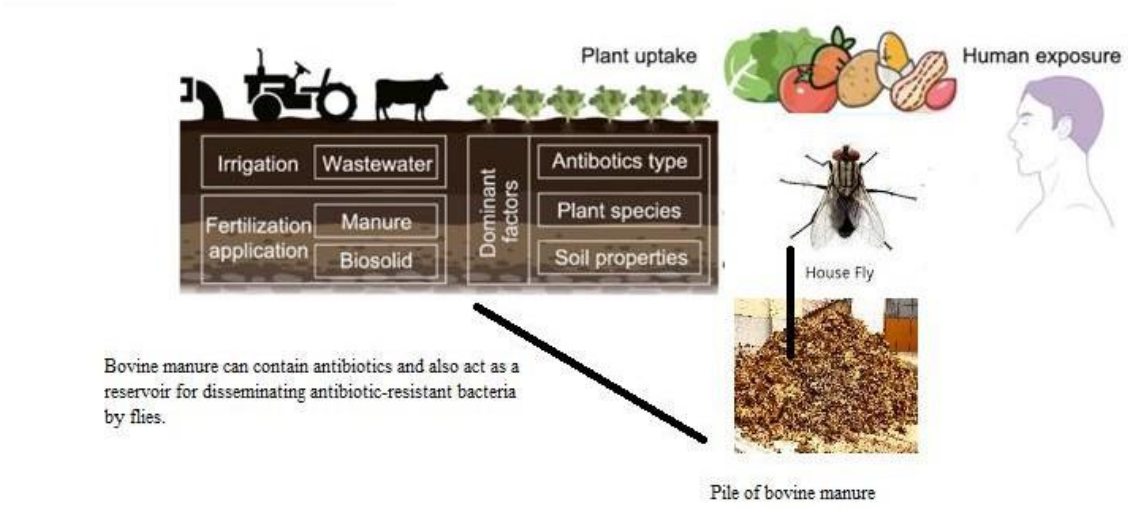


Figure 2.12 Contaminated manure with antibiotics and antimicrobial bacteria Adapted and based from (Sanseverino *et al.*, 2018).

Both antibiotics and antibiotic-resistant bacteria in Figure 2.12 can enter the food chain through contaminated soil and manure (Sanseverino *et al.*, 2018).

Various management practices, including applying animal manure, wastewater, and other wastes containing antibiotic-resistance genes on mobile elements and antibiotic residues, may enhance the abundance and mobility of antibiotic-resistance genes in agricultural soil (Marti *et al.*, 2013). Treatment of manure management can be an important strategy in minimizing the spread of antibiotics and resistant bacteria. Composting manure is used as a treatment to reduce antibiotic residue concentration. While this process can significantly decrease the levels of many antibiotics. Studies have shown that certain compounds such as sulfamethazine are relatively persistent and may not fully degrade during composting. The composting temperatures effectively remove antibiotic residues and antibiotic-resistant genes from those organic fertilizers. Higher temperatures could eliminate pathogenic bacteria and other bacteria-carrying resistant genes. Higher temperatures act as catalysts for the decaying process of harmful substances (Yu *et al.*, 2019). However, it is unclear whether high temperatures destroy antibiotic residues or

whether the Thermophiles growing at high temperatures will exhibit specific roles. A study has reported that tetracycline in animal manure could be effectively degraded after high temperatures. Tetracycline is the most used antibiotic in livestock industries because it can effectively treat diseases and protect animals from infection outbreaks while improving feed efficiency as a growth promoter. The use of antibiotics in food production animals has resulted in certain benefits, such as healthier animals and fewer infectious diseases (Yu *et al.*, 2019).

Despite these benefits, there are considerable public health issues regarding food safety and regulatory perspectives given the application and use of antimicrobials in food animal production (Oliver *et al.*, 2011). Boeckel *et al.* (2015) noted that on a global scale, the average antimicrobial agent consumed yearly per kg of animal produced varied across different breeds, with values of 45 mg/kg, 148 mg/kg, and 12 mg/kg associated with cattle, chicken, and pigs, respectively. Apata (2009) noted that antibiotics were added to water and fed to chickens at subtherapeutic levels for growth promotion and prophylaxis. Even healthy birds were exposed to this treatment. How farmers use antibiotics might influence the level and rate of their utilization in the farming industry. We are a chemical society, and humans are the primary users of pharmaceutical products. We need a better understanding of what happens and how chemicals are applied to food sources, and we must be more vigilant about regulating what we use to grow and what to consume.

In conclusion, the information presented here is from studies obtained by several authors who have previously investigated antibiotic use and antibiotic resistance. The rate of antibiotic usage is alarming and surpasses the red line. Farmers, veterinarians, and local pharmaceutical product vendors must cooperate to prudently use antibiotics in the agricultural sector worldwide.

2.5.2 Bovine mastitis a worldwide impact disease: Causative Factors and Antimicrobial Resistance

2.5.2.1 Introduction

Mastitis is a bovine infection of the udder that causes significant economic losses to milk quality and the production chain of dairy products. It is an inflammation of the bovine mammary gland caused by antibiotic-resistant bacteria and other environmental factors, and it is a significant concern in the long-duration treatment of mastitis on farms.

Common pathogens responsible for this udder infection include both Gram positive and Gram negative bacteria such as *S.aureus*, *E.coli*, and *S.agalactiae*.*E. coli* is the most common Gram–bacterium found to invade and colonize the udder through the teat, proliferate, and initiate an inflammation response in dairy cows.(Smith & Hogan, 1993). Mastitis poses a significant challenge to management through zootechnical and veterinary methods. This is due to various causative factors, such as domestic flies (*Musca domestica),* which may also be vehicles for spreading pathogenic bacteria.



Figure 2.13 The environment is infested with domestic flies. *Image Credit: Srinivasan.Clicks/ Shutterstock*

Mastitis is an inflammation of the udder gland tissue. The infection develops when the organism's defence mechanisms are weakened. The teat canals form the first line of defence against invading pathogens by providing a physical barrier and a source of antimicrobial substances (Stanek *et al.*, 2024). Mastitis can be classified into three classes: clinical mastitis, which causes swelling, rash, pain, and pus formation; subclinical mastitis, which results in impaired milk quality, such as high somatic cell counts and reduced milk production; and chronic mastitis. Bacterial pathogens are causative agents categorised into contagious pathogens from cow to cow and environmental pathogens from the surrounding environment, such as domestic fly infestations and inadequate hygienic standards like bedding and manure. Mastitis caused by *E. coli* exhibits clinical symptoms, including a swollen and red udder and fever. *E. coli* can cause irreversible tissue damage in the mammary gland. *E. coli* persists in the mammary gland, causing recurrent mastitis infections that are difficult to treat, possibly due to its ability to produce biofilm at different levels (Fernandes *et al.*, 2011).

The virulence factors commonly known to enhance the inflammatory response is the endotoxin, which is found on the outer membrane of *E. coli*, known as lipopolysaccharides (LPS). Due to its pathogenicity, *E. coli* is not the only pathogen mediated by single and specific virulence gene factors. A complex of various virulence gene factors, such as toxins, adhesives, invasions, capsule production, ability to resist serum, and iron scavenging, are reported as being necessary to overcome the host's selection pressure, colonize, invade, proliferate, and survive in the udder, and cause inflammatory responses (Kaper *et al.*, 2004. Fernandes *et al.*, 2011). Cow breeding and genetic characteristics influence mastitis. Breeds Holstein – Friesian herds appear more genetically prone and vulnerable to mastitis (Shaheen & Tantary, 2015). Udder structure also affects susceptibility to infection. Cattle with large funnel-shaped teats or pendicular

shapes are at greater risk of subclinical mastitis (Waller *et al.*, 2014).

2.5.2.2 The role of domestic flies (*Musca spp*) as a vector of mastitis

Domestic flies in large quantities on the farm feed on manure and milk.Flies, which breed in manure, regurgitate for defecating bacteria, contaminating the surface of the udder. Flies can transfer bacteria from contaminated surfaces to the teat canal during feeding and resting.

A high fly population on the farm is associated with an increased incidence of environmental mastitis. Flies' role in spreading pathogenic and antibiotic-resistant bacteria complicates the treatment of bovine mastitis due to the spread of multi-drug resistance genes.



Figure 2.14 Flies Colonizing a Cow's Udder. *Image Credit: Srinivasan.Clicks / Shutterstock*

Besides mastitis, flies are also responsible for causing respiratory diseases and transmitting zoonotic diseases to humans (Neupane *et al.*, 2023).

Antibiotics commonly used in mastitis treatment include Penicillin, Ampicillin, Tetracycline, and Gentamycin. An ideal treatment should be long enough to cure subclinical mastitis and short enough to prevent antibiotic resistance once the cow has calved (Cheng & Han, 2020). Dry cow season is the best time of the year to treat mastitis since there is no milk production during this time, and fewer flies will become attracted to the udder.

When a cow is detected with an active mastitis infection, the farmer's first step is to cull the sick cow and the milk completely to eliminate any bacteria or toxins that might be released from the bacteria (Jones *et al.*, 1999). The milk obtained during the antibiotic treatment followed by a waiting period must be discarded since the consumer cannot consume it due to health concerns, including allergies and drug residues that might lead to antibiotic-resistant bacteria (Gomes *et al.*, 2016).

2.5.2.3 Potential Alternative Treatment

Antibiotics remain at the top of the agenda as the primary treatment. On the other hand, to minimise the bacterial resistance genes, other natural treatments might be used to treat this disease. The rise of antibiotic resistance as a public health concern is pushing to limit the usage of antimicrobial drugs. Plants, like thymol, are considered valuable, beneficial natural sources of ingredients to treat mastitis. Research is now gaining interest in treating mastitis using a natural approach(Pasca *et al.*, 2017). Fly control is significant on farms, and this study shows how houseflies carrying multiple resistance genes are considered a significant reservoir and transmit microbial threats to both humans and animals. It

highlights the importance of transmission. Further studies may help identify new strategies or routes to prevent transmission and protect the cows from diseases that cause illness following losses.

2.5.3 Impact of Antibiotic Resistance and Food Safety

Resistance presents one of the most pressing challenges of our time. While antibiotics have long been instrumental in boosting livestock production while controlling infectious diseases among farm animals, the intersection of antibiotic use in agricultural practices, together with the threat of antibiotic-resistant outbreaks and economic losses, their indiscriminate and excessive application has fuelled a silent but devastating crisis. As antibiotics are found in the soil, water, and food systems, these create a reservoir harbouring this resistance that continuously threatens to render essential medicines ineffective (Manyi-Loh *et al.*, 2018). The proliferation of antibiotic-resistant bacteria not only complicates treatment options for humans and animals but creates stress to health care systems while jeopardizes global food security (Oliver *et al.*, 2011). Research has shown that antibiotic residues in manure can persist in agricultural soils and be absorbed by crops, raising concerns over consumer exposure through food consumption (Kumar *et al.*, 2005; Geng *et al.*, 2022).

Abena *et al.*(2024) conducted a study on lettuce and reported the presence of antibiotic resistant bacteria exceeding the WHO safety threshold. The study attributed topoultry manure and irrigation water as a major source of bacterial contamination. Notably molecular analysis confirmed the detection of the bla CTX-M gene in ESBL – producing *E. coli*. This finding possess a serious public health concern, particularly because lettuce is consumed raw.

Sustainability in food is not an option but a necessary and urgent requirement. Food

production should shift away from antibiotic dependency to control diseases and promote growth in livestock farming (Pasca *et al.*, 2017).

2.5.4 Conclusion

Strengthening regulatory frameworks that enforce responsible antibiotic stewardship and foster innovation in disease prevention strategies are considered critical in mitigating the AMR crisis (Van Boeckel *et al.*, 2015). Given Malta's small size and limited buffer zones, wind and other vectors, such as farm equipment and water runoff, can easily spread pathogenic bacteria from farms to nearby fields, contaminating animals, their surrounding environments, and the food chain.

2.6 Urinary Tract Infection and Virulence Factors of Uropathogenic *E. coli* (UPEC)

2.6.1 Introduction

Urinary Tract Infections (UTIs) are a common bacterial infection affecting millions worldwide. The bacterium *E. coli* is the prime causative agent. *E. coli* is a Gram-negative, facultative ubiquitous bacteria in different environmental niches, from the human gut as a commensal organism to pathogenic strains like *E. coli* 0157:H7, responsible for severe infections (Ramos *et al.*, 2020).

The pathogenicity of *E. coli* is determined by virulence factors, which allow the bacterium to colonize, invade, and damage the host cell. These include Adhesives, Toxins, Immune evasion mechanisms, and Resistance proteins (Pakbin *et al.*, 2021).

Uropathogens, such as uropathogenic Enterobacteriaceae like *E. coli*, use pili to enable the bacterium to bind to uroepithelial cells and initiate the invasion process. Uropotheginic *E. coli* (UPEC) is responsible for most Urinary Tract Infections (UTIs) in human cases.

These infectious complications may result in severe renal failure in healthy individuals. There- fore, UTI is considered the most contagious disease in humans (Wiles *et al.*, 2008), (Nowicki *et al.*, 1988).

When colonizing the urinary tract, UPEC may go down towards the bladder to cause cystitis. If left untreated, the bacteria, through the ureter, reaches the kidneys and establishes an acute secondary infection. This causes irreversible kidney damage, leading to kidney failure and death (Bien *et al.*, 2012).

Furthermore, the increasing prevalence of antibiotic-resistant *E. coli* strains, particularly ESBL-producing strains, has made treatment challenging (Martin *et al.*,2013).

The significant rise and resistance to multiple antimicrobial agents in *ESBL*-producing *E. coli* among UTI patients is considered a public health concern. ESBL-resistant genes can hydrolyze most of the β-lactam antibiotics. Carbapenems, such as meropenem or imipenem**,** are often considered the last treatment choice for serious infections caused by ESBL-producing *E. coli*. (Vardakas *et al.*, 2012).

These antibiotics are usually administered intravenously and are reserved for severe cases or when oral options are not viable. However, the overuse of carbapenems can lead to increased resistance and make treatments impossible to treat (Tamma *et al.*, 2022; Husna *et al.*, 2023).

2.6.2 Epidemiology, Mechanisms of Infection and Treatment Options

Urinary Tract Infections (UTIs) are caused by Gram-negative and Gram-positive bacteria and certain fungi. Uropathogenic *E. coli (UPEC)* is the main pathogenic factor for UTIs, and approximately 80% of all UTIs are caused by uropathogenic *E. coli* (UPEC). UTI is considered the most bacterial infectious disease, affecting more than one million people worldwide each year, and is caused by a range of pathogenic bacteria, most commonly *E.*

coli and other Enterobacteriaceae members (Flores-Mireles *et al.*,2015), (Flores-Oropeza *et al.*, 2024).

Urinary Tract Infections (UTIs) are a significant challenge faced by humans worldwide, making the most effective treatment impossible. β-lactamases like CTX-M, TEM-1, SHV and OXA have increased drastically (Paterson *et al.*, 2005).

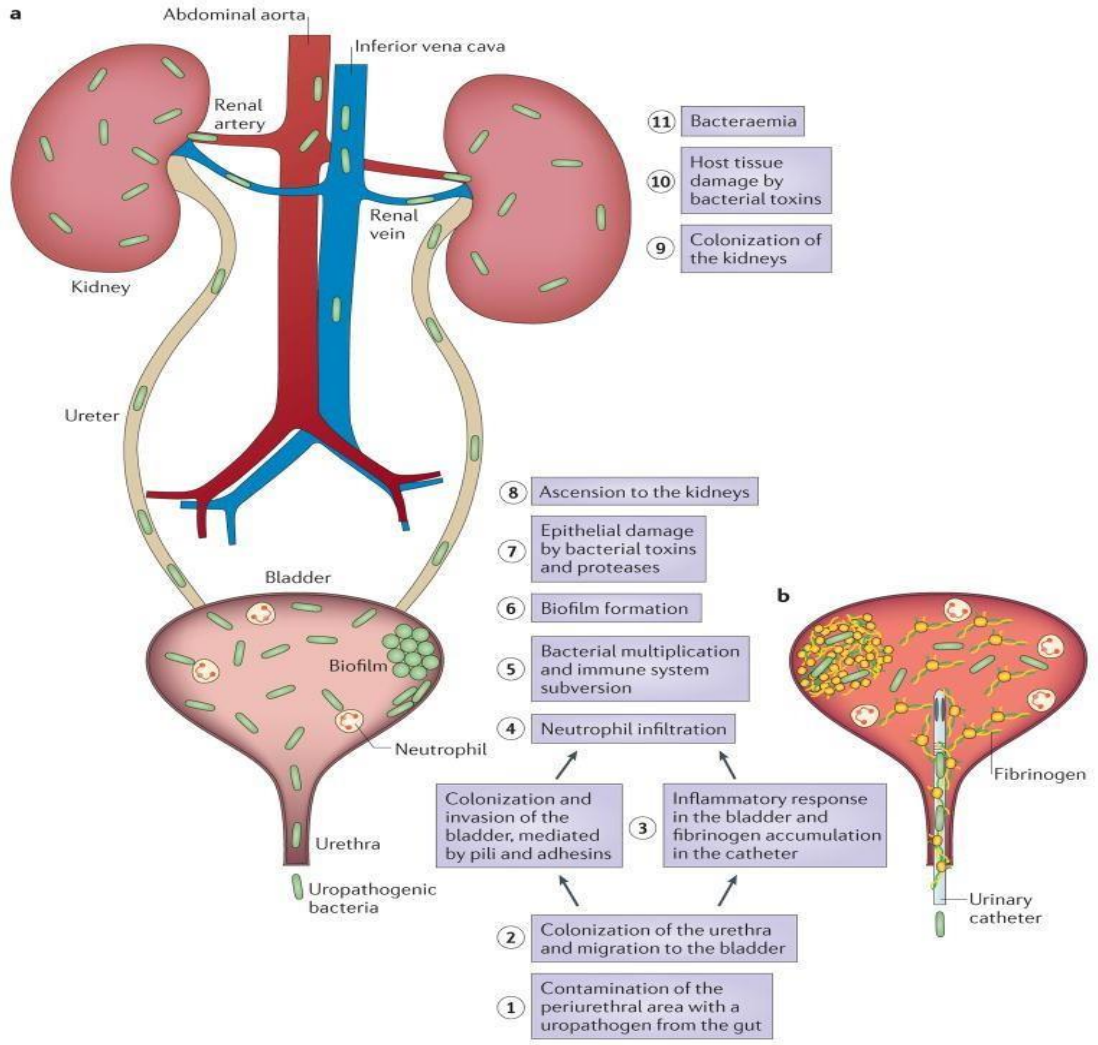


Figure 2.15 Urinary tract infections caused by *Uropathogenic E.coli* (UPEC) (Zhou et al., 2023).

Figure 2.15 illustrates urinary tract infections caused by *Uropathogenic E.coli* (UPEC) (Zhou et al., 2023). Urinary Tract Infection (UTI) is a significant cause of morbidity in infants, boys, older men, and females of all ages. Serious complications include sepsis, renal damage in young children, miscarriages, and other related complications caused by frequent use and misuse of antibiotics (Flores-Mireles et al., 2015).

Upper-tract UTIs involve the kidneys and ureters, whereas lower-tract UTIs involve the bladder and urethra. Commonly used terms for UTIs based on the location of infection are urethritis, an infection of the urethral mucosa; cystitis, an infection limited to the bladder; and pyelonephritis, an infection ascending to the kidneys (Barola *et al.*, 2024). The symptoms are variable, ranging from no symptoms to severely unwell patients suffering from high temperatures, which sometimes may be followed by bacteraemia.

Women have a high risk of a UTI in their lifetime. Recurrent UTI cases can be due to anatomy, sexual activity, menopause, diabetes, hospital catheterization, and immunocompromised patients. In addition, UTIs also affect children within one year of birth. The target areas include bladder, bowel dysfunction (BBD), and vesicourethral reflux (VUR) (Zhou *et al.*, 2023). Clinically, UTIs are categorized as uncomplicated or complicated. Uncomplicated UTIs usually affect otherwise healthy individuals. Complicated UTIs include urinary tract abnormalities, immunocompromised patients, or frequent antibiotic use (Zhou *et al.*, 2023). UTI can be divided into three main categories: acute pyelonephritis associated with kidney failure, acute cystitis, a type of bladder infection, and asymptomatic bacteriuria, which affects the lower urinary tract.

Uropathogenic E. coli (UPEC) is the most common causative agent for uncomplicated and complicated UTIs. The so-called UPEC strains are the most prevalent and are the main pathogenic factor for UTIs (Zhou *et al.*, 2023). Uropathogens such as UPEC survive by invading the bladder epithelium, producing toxins and proteases to release nutrients

from the host cells and break down siderophores to obtain iron (Zhou *et al.*, 2023). UPEC strains carry a variety of virulence factors, including adhesins (fimbriae), toxins (haemolysis, cytotoxic, neutralizing factors, iron acquisition, and immune evasion strategies)

Adhesins recognize receptors on the bladder's epithelial lining, also known as uroepithelium. UPEC are more likely to infect starting from the lower urinary tract.

Such factors enable UPEC to colonize and survive in the urinary tract, resulting in recurrent infections (Zhou *et al.*, 2023). Typically, a UTI starts with periurethral contamination by a uropathogen residing in the gut. This is followed by colonization of the urethra and subsequent migration of the pathogen to the bladder, which requires appendages such as flagella and pili. Bacterial colonization is a crucial step in UTI pathogenesis.

Uncomplicated UTI starts when uropathogenic bacteria in the gut contaminate the periurethral area. When the impact of UPEC on the patient is of health concern, the host suffers from UTI (Zhou *et al.*, 2023).

The physiological events of UPEC in the patient can be divided into six main steps.

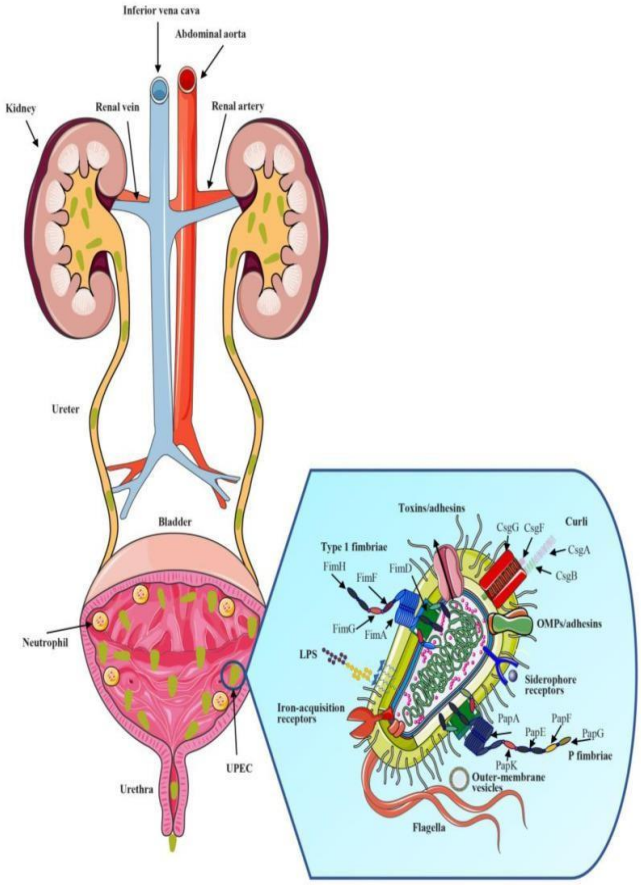


Figure 2.16 illustrates the Pathogenesis of Urinary Tract Infection (Zhou *et al.*, 2023).

Pathogens first colonize the periurethral area and subsequently enter the urinary tract via the urethra, primarily due to poor hygiene and other risks related activities.

UPEC expresses Type 1 fimbriae and P pili which enable adhesion to the urethral line cells, promoting bacterial proliferation and slow down bacterial flow (Zhou *et al.*, 2023). Type 1 pilus adhesin, FimH binds mannosylated uroplakins and integrins that coat the bladder epithelial cells. When FimH binds to these molecules, it helps the bacteria anchor themselves to the bladder epithelial cells, a crucial first step in colonization. FimH facilitates bacterial invasion. This process triggers endocytosis, which allows intracellular bacteria communities to invade and protects UPEC from antibiotic treatments (Zhou *et al.*, 2023). The bacteria proliferate inside the bladder epithelial cells, forming biofilms that continue to protect the pathogenic bacteria from antibiotic treatment.

Biofilm formation also plays an infectious role by contributing to recurrent bacterial infections. It might be hard to treat due to resistance to antibiotic treatment.

Virulence factors produce toxins such as alpha-hemolysin (hIyA) that lyse host cells to release iron. Alpha–haemolysin induces epithelial exfoliation and helps UPEC spread very quickly (Zhou *et al.*, 2023).

2.6.3 Virulence Factors play a dual role in UTI diseases

Virulence gene factors are essential because they encode factors that allow bacteria to orchestrate a complex role and damage the host cell.

However, these actions provide a favourable environment for bacteria to establish and maintain an infection in the urinary tract. Therefore, they create an environment where bacteria are more resistant to antibiotics. For example, biofilms prevent antibiotics from reaching the cell and killing the bacteria (Emödy *et al.*, 2003).

The dual nature of virulence gene factors is that sometimes, they are encoded on mobile genetic elements along with antibiotic-resistant genes so that virulence traits can be linked to resistant characteristics.

Uropathogenic *E. coli* strains that exhibit high levels of virulence genes are also more likely to carry resistant genes, making infections more difficult to treat. There is a pathogenic correlation between virulence factors and antibiotic-resistance genes in UTIs. Many virulence and antibi- otic-resistance genes are found on plasmids, transposons, and integrons. They play a multifac- eted role in UTIs by promoting bacterial adhesion, invasion**,** immune evasion, nutrient acqui- sition, and tissue damage (Emödy *et al.*, 2003; Bien *et al.*, 2012). Figure 2.17 Uropathogenic *E.coli (UPEC)* employ the following virulence genes to facilitate adhesion, evasion, biofilm formation, toxin production, and nutrient requirements (Flores-Mireles *et al.*, 2015).

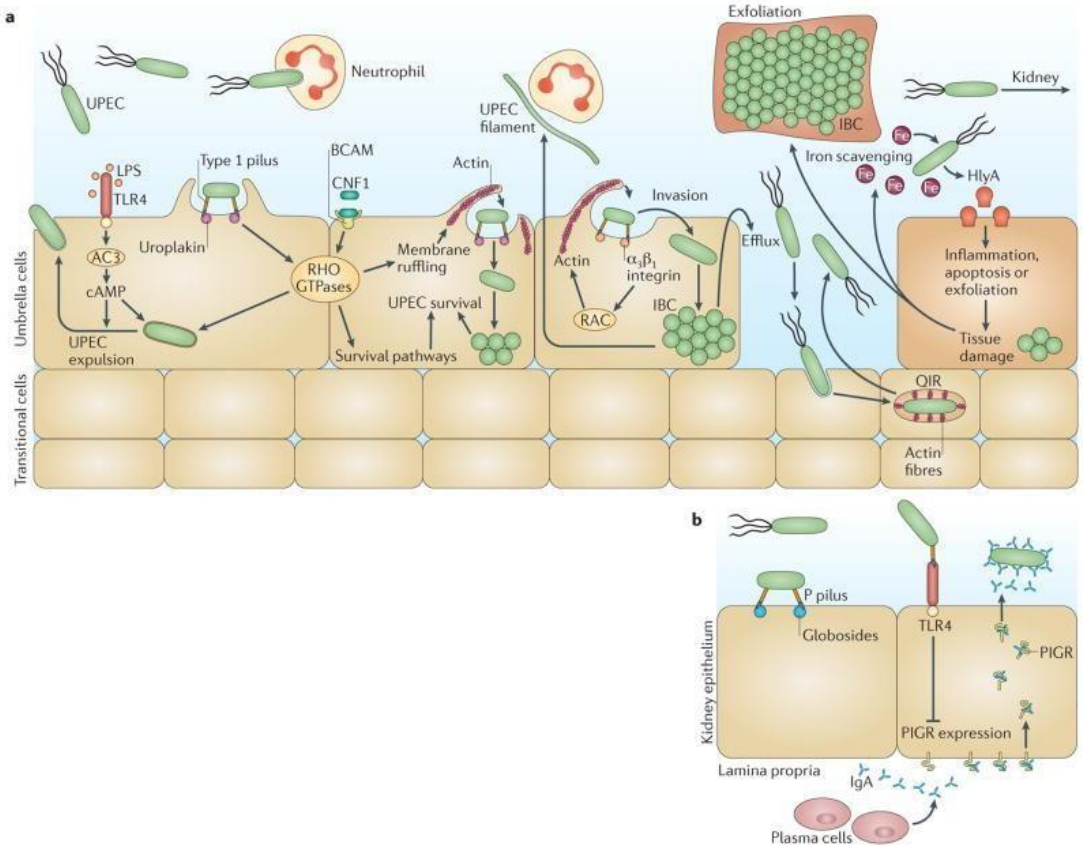


Figure 2.17 Uropathogenic *Escherichia coli* (UPEC) virulence genes and their roles in pathogenesis

Figure 2.17 Uropathogenic *E.coli* (UPEC) employ the following virulence genes to facilitate adhesion, evasion, biofilm formation, toxin production, and nutrient requirements. Virulence factors of *E. coli* have been potentially involved as important genes in the cause of UTI and can be divided into two groups.

Table 2.4 Virulence Genes, factors, and functions associated with Urinary Tract Infection

Virulence Gene	**Associated Virulence Factor**	**Function**
FimH	Type 1 fimbriae	Aids in bacterial adhesion to urothelial cells, promoting colonization
PapG	P fimbriae	Crucial for kidney infection by mediating bacterial Attachment
SfaS	S fimbriae	Involved in adhesion, associated with neonatal meningitis and UTIs
HlyA	Hemolysin	Lyses host cells, causing tissue damage and inflammation
cnf1	Cytotoxic necrotizing factor	Disrupts host cell signaling, leading to increased bacterial survival
IroN	Siderophore system	Facilitates iron acquisition, crucial for bacterial growth
IutA	Aerobactin system	Enhances bacterial iron uptake for survival in iron- limited environments
TraT	Serum resistance protein	Helps bacteria evade host immune responses
OmpT	Outer membrane protease	Protease that degrades host defense proteins, aiding immune evasion
Sat	Autotransporter toxin	Secreted toxin that disrupts host cell function
Vat	Vacuolating autotransporter toxin	Induces vacuolation in host cells, contributing to tissue damage
Usp	Uropathogenic-specific protein	Increases bacterial fitness and survival in the urinary tract

The first group includes virulence factors associated with the bacterial surface, and the second group includes those virulence factors secreted and exposed to the site of action. (Bien *et al.*,2012)

The surface virulence factors include several adhesin organelles (fimbriae) that promote bacterial binding and invasion of the host cell, *fimH, papA, pabC, papG, sfa, foc*.

Virulence Factors *iuc, iroN, irp2* are related to iron acquisition (Bien *et al.*,2012).

The secreted virulence factors include toxin activity against the cells lining the bladder or kidney, which is important for the UTI pathogenicity. The most crucial virulence factor of *UPEC* is *hlyA* α-haemolysin. The role of *hlyA* in UTI pathogenesis is that it is a pore-forming factor that lysis the host cell by causing damage in the uroepithelial cell and promoting bacterial sur- vival and infection. These virulent factors associated with *UPEC* are categorized according to their function in UTI pathogenicity and contribute to *UPEC's* ability to colonize, evade the immune system, and cause urinary tract infections (UTIs), including cystitis and pyelonephritis (Abe *et al.*, 2008; Rahdar *et al.*, 2015). Virulent strains of *E. coli* pose a significant threat in both clinical and agricultural settings.

Søborg *et al.* (2013), demonstrated the presence of virulence genes in agricultural soil and freshwater biofilm, which may be related to soil fertilization with manure and wastewater dis- charge.

Enterotoxigenic *E. coli* (*ETEC*) and enterohemorrhagic *E. coli* (*EHEC*) are significant causes of foodborne illnesses, while multidrug-resistant (MDR) *E. coli* strains complicate treatment options for systemic infections. Acquiring virulence genes through horizontal gene transfer further poses a public health concern and makes treatment difficult. (Malekzadegan *et al.*, 2018; Shah *et al.*, 2019).

This research has the potential to revolutionize the field of microbiology and infectious diseases, offering hope for more effective treatments and prevention methods.

Understanding the virulence factors of *E. coli* is essential for developing new antimi- crobial strategies and preventive measures. Future research should focus on novel therapeutic approaches, including vaccines and targeted antimicrobial agents, to mitigate

the burden of *E. coli*-related diseases.

Studies suggest that infections due to ESBL-producing *E. coli* in non-hospitalized patients are also increasing, but the clinical outcome of patients infected with an ESBL-producing strain of Enterobacteriaceae is still uncertain (Rodriguez *et al.*, 2004).

Naoto *et al.* 2022, in their recent studies, indicate how ESBL-producing *E. coli* transmission in hospitals has become a significant health concern (Okada *et al.*, 2022).

E. coli sequence type (ST) 131 and other sequence types like ST 69, ST10, ST405, and ST38 have been identified as high-risk clones and have been associated with UTI (Kocsis *et al.*, 2022)

Treatment options for uncomplicated UTIs include antibiotics such as nitrofurantoin, trimethoprim, and sulfamethoxazole. Sometimes, fluoroquinolones and β-lactam antibiotics may be prescribed (Jancel *et al.*, 2002 ,Bono *et al.*,2023).

Treatment can sometimes be difficult unless it reduces and controls the overuse and misuse of antibiotics in humans. This can help to slow the spread of *E. coli*, which causes human UTIs. However, adequate and proper hygienic practices may reduce the spread of antibiotic resistance among communities. Alternative non–antibiotic treatments include probiotics and novel natural-based antibiotics. Cranberry extracts and D–mannose are new non–antibiotic approaches (Jancel *et al.*, 2002), (Bono *et al.*, 2023).

2.6.4 Domestic flies are likely to be undermining the treatment of ESBL-producing Escherichia coli in UTI patients?

Studies show that domestic flies can be a continuous vector in spreading pathogenic and antibiotic-resistant bacteria and to define their role in transmission (Yin *et al.*, 2022)

This hypothetical spread shows how domestic flies are likely to undermine the treatment of *ESBL–producing E. coli* in UTI patients. Ongoing research and updated clinical

guidelines are essential to improving the management and outcomes of these infections. Flies are mechanical vectors of pathogenic and other resistant bacteria, including *ESBL-producing E. coli.* The WHO recognizes houseflies' negative impact as a significant vector in the spread of infectious diseases (Rozendaal, 1997).

The study by Shaumburg *et al.*, 2016, described the possible role of domestic flies as vehicles in the spread of antibiotic resistance bacteria. Flies can acquire pathogenic bacteria carrying antibiotic resistant genes from environmental niches which can be easily spread over considerable distances. A hypothetical significant pathway of antibiotic resistance transmission from flies to humans.

2.7 Domestic Housefly (*Musca* spp.)

2.7.1 Introduction

Houseflies (*Musca spp.*) are among Earth's most ubiquitous flying insects. Despite their small size and relatively simple biological features, houseflies play a significant role in public health due to their abilities as mechanical vectors for a wide range of bacterial pathogens. Mechanical vectors carry pathogens on their exoskeleton and in their digestive tract without infecting themselves (Faham *et al.*, 2018). These flying insects have been considered pests of humans and animals since antiquity. In 1914, the sanitarian Samuel Miller reported to the Massachusetts Association of Boards of Health that "Flies breed disease" and "The fly is a curse" (Miller, 1914). In Japan, regarding the transmission of pathogenic bacteria such as *E. coli* from farm animals to children, house flies were identified as the *biotic factor* most closely related to this disease (Hung *et al.*, 2015).

Evidence is that most studies show the detection of pathogens and their antimicrobial resistance characteristics from flies but have no proof for the possible cause of bacterial transmission, contamination, or infectious diseases from flies to humans with UTIs,

making it more difficult to generalize the results (Cook *et al.*, 2025).

The housefly is the most critical vector for spreading infectious diseases among humans and animals. It has contributed to the spread of several pathogenic diseases, such as cholera, dysentery, and tuberculosis, and the transmission of parasitic worms (Khan *et al.*, 2015).

Flies are frequently found in human and animal dwellings, such as household kitchens, animal bedding and manure, dust bins, hospitals, restaurants, and fermented or decaying vegetables or fruits (Nayduch *et al.*, 2023). Flies are ectoparasites and are essential as they are defined as annoyance 'pests' that can easily transmit a mixture of bacterial pathogens (Yin et. al., 2022).

Apart from being a nuisance to humans and especially farm animals, domestic flies act as carriers of pathogenic microorganisms since they carry them in their mouths, on their wings, on their body surface and feet, and can transmit them through regurgitation of gut contents (Bertelloni *et al.*, 2023; Singh *et al.*, 2025).

There is good evidence that flies can act as vehicles to contaminate food products, animals, and even the environment since they are responsible for carrying antimicrobial-resistant bacteria such as ESBL-producing *Escherichia coli.* These infections must be treated with antibiotics.

House flies are common pests of significant concern for transmitting foodborne pathogens such as *E. coli* and *Salmonella spp*. They can feed, especially on animal waste, such as cattle manure, from farms. These flying pests are closely associated with animal waste from which human pathogenic bacteria, viruses, and even parasites can be acquired (Barreiro *et al.*, 2013). *M. domestica* is considered a driver of transmission of various pathogenic bacterial species and is an excellent candidate for the distribution of susceptible and resistant bacterial strains (Nayduch *et al.*, 2023).

2.7.2 Typical Housefly (*Musca domestica*)

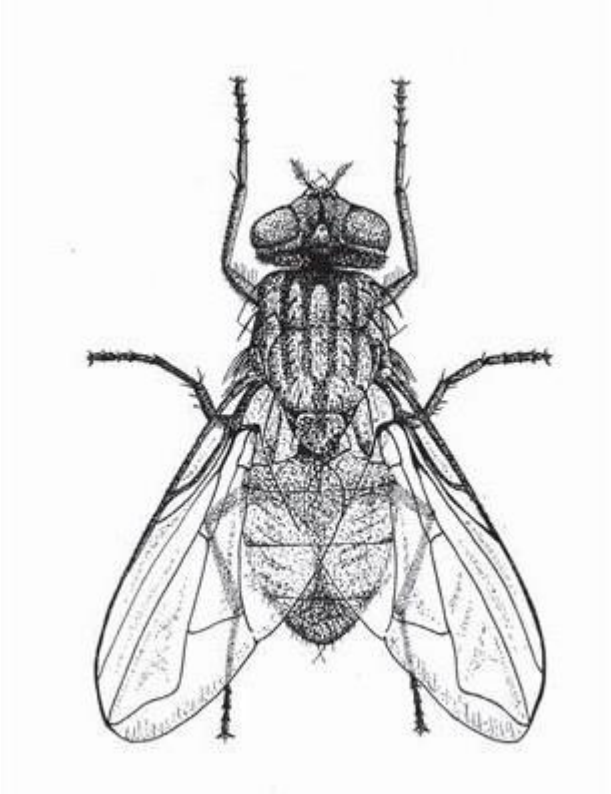


Figure 2.18 *Musca domestica* .L (Stafford, 2008)

Adult flies may have a single pair of wings Figure 2.18 They have six legs, each ending with a ‘tarsus' segment. On the tarsus, they have a small claw and a little sticky pad that permits the fly to hang upside down and cling to smooth surfaces (Stafford, 2008)

‘I’d rather have ten snakes in the house than one fly’

Mark Twain, letter to Albert B.Paine, March 1910 **(**Stafford, 2008) ‘Filth flies’ are defined as flies that use excrement and decaying matter for nutrition and oviposition. They are also coprophagic, which means they feed on animal manure and other faeces. The

common houseflies mentioned in this study are muscoid flies, filth flies, or synanthropic flies since they coexist with both humans and animals. They are commonly found on food and decaying matter in habitats created by agricultural practices and other human activities. Houseflies are closely associated with people, livestock animals, and poultry. They are associated with poultry farms, cattle and dairy sheds, horse stables, and pig farms. Houseflies and other flies can also breed in decaying organic matter in garbage, animal bedding, and human excrement (Onwugamba *et al.*, 2018).

The scientific name of flies, like other organisms, is given in two parts: Genus and species. These flying insects are classified as follows in Table 2.4

Table 2.5 Classification of Housefly

Class	Order	Family	Genus	Species	Describer
Insecta	Diptera	Muscidae	*Musca*	*domestica*	Linneus

Entomologists define a fly as having a holometabolous development, where the immature stage is strikingly different from the adult stage as shown in Figure 2.18

Houseflies (*Musca domestica*) are ubiquitous cosmopolite pests that inhabit a variety of environmental niches worldwide. The housefly (*Musca domestica L.* (Diptera Muscidae) is the world's most common and widespread fly species. It is said to have originated from the savannahs of central Asia and spread across the globe. It can be found in both rural and urban areas of tropical and temperate climates (Hemmatinezhad *et al.*, 2015; Khamesipour *et al.*, 2018). It has been in existence since the origin of human life and is well adapted to live in human habitations which belong to a group of flies often referred to as ‘filth flies’, whereas the other members belong to the families Calliphoridae and

Fannirdae (WHO, 2005; Cottone *et al.*, 2014; Sarwar *et al.*, 2014).

Musca sp is a synanthropic endophilic species, i.e., it lies closely in association with humans and is viable to complete its entire life cycle within the habitations of humans and domestic animals (Smallegange *et al.*, 2007).

Houseflies are often abundant in areas of human activity such as hospitals, food markets, slaughterhouses, and poultry and livestock animals. They also act as a potential vector of diseases (Awache *et al.*, 2016).

2.7.3 Life Cycle

Female house flies can lay over 100 eggs in a single batch, with over 500 in a lifetime. Under optimal temperature, larval development can be completed within a week.

Female flies directly consume microbe–rich substances such as manure, while adult male flies prefer CHO-rich food sources. Females are attracted to protein-rich options. This preference is likely due to their anautogenous reproductive physiology, which requires protein for egg development (vitellogenesis). Female flies in agricultural settings often feed on proteinaceous secretions and excretions of animal eye exudate, mucus, weeping wounds, blood, and manure. Female flies have a greater abundance of bacteria than male flies, possibly due to their prosperity in microbe-rich environments (Nayduch *et al.*, 2023). Domestic flies have four life stages: eggs, larvae (maggots), pupae, and adults.

They lay their eggs in compost, trash, and moist, microbial-rich decaying organic matter near people's houses and farms (Khamesipour *et al.*, 2018)

During their lifetime female fly can oviposit four up to six times and produces between 100- 150 eggs (Smallegange *et al.*, 2007; Khamesipour *et al.*, 2018) and if the environment conditions are favourable, moist and at a temperature of 25 °C to 30 °C, therefore the eggs hatch within 8 to 12 h (Khamesipour *et al.*, 2018) The first-instar larvae

feed on bacteria in nutrient- rich environments and pass through a further two instars before becoming larvae (maggots) that migrate into a dark, dry, and cool place where they pupate (Smallegange *et al.*, 2007; Khamesipour *et al.*, 2018). Adult flies emerge 2–4 days later when ambient temperatures range from 32 °C to 37 °C, meaning the entire life cycle of houseflies is very rapid, spanning 10–21 days.

Thus, all life stages can be exposed to various pathogens in unsanitary environments, which can be mechanically transmitted to people (Chaiwong *et al.*, 2012).

Adult flies can move over distances of up to 20 miles in their lifetime, which means they are ubiquitous in the environment and capable of spreading pathogens from unsanitary areas into people's homes and places of work (Murvosh *et al*.,1966)

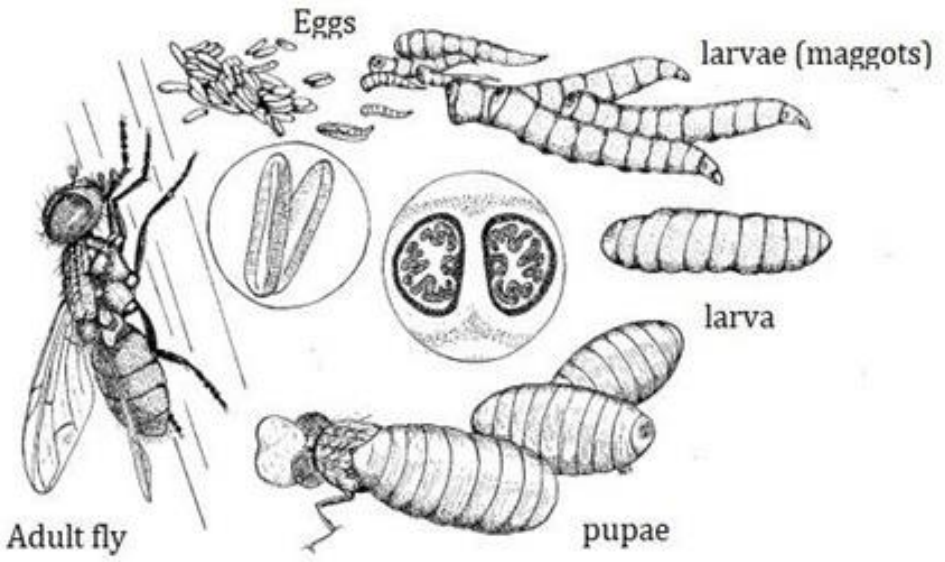


Figure 2.19 Typical life cycle of a house fly showing a muscoid type development **(**Stafford, 2008).

2.7.4 The Housefly and Transmission of Diseases

Houseflies are often abundant in areas of human activity, such as food markets, slaughterhouses, livestock farms, and poultry. Moreover, they act as potential vectors of diseases. They are highly mobile and can fly several kilometres in search of food (Förster *et al.*, 2008). Their flying ability to travel between food sources and breeding sites, which are often located in different places.

- Flies are frequently found in a wide range of habitats
- They move freely due to their strong ability to fly, sometimes covering distances as much as 10 km
- They can fly at a speed of up to 7 km/h (about 4.3 mph)
- House flies are attracted to a variety of food sources, particularly those that are decaying or fermenting
- When not flying, houseflies rest on walls, ceilings, and floors.
- They are often found near food sources or potential breeding sites.

  Most flies stay within a half mile or mile from their breeding place. House flies are not naturally migratory and generally do not make long flights. They move around to explore their environment but tend to remain within a radius of 100 – 500 meters from their breeding site as long as they find suitable food, breeding sites, and shelter (Stafford, 2008).

A general housefly dispersal distances at 0.8 km ,1.6 km , and 3.2 km from a release point in Figure 2.20. Flies move upwind with steady 3.2 – 11.3 km/h wind towards attractive sites. Otherwise, movement is non-directional ie ‘random’; another study was 16.1 km/h for directed movement **(**Stafford, 2008). Morris and Hansen (Graham *et al.*, 2009) did not find clear evidence for the influence of the wind. Released flies were recovered at all farms in a 1.6 km zone and at the maximum trap distance of 8.25 miles.

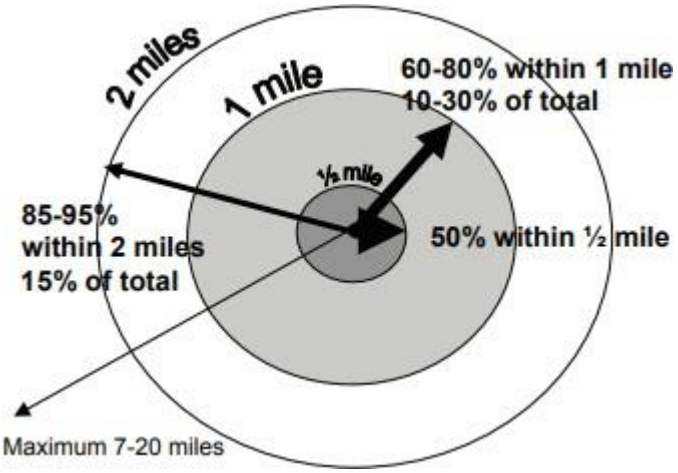


Figure 2.20 General Dispersal Distances of Houseflies from a Release Point **(**Stafford, 2008)

Most adult house flies are oriented on wind-borne odours from farms (Ranjbar *et al.*, 2016). Most of the flies were recovered upwind. In summary, flies tend to remain close to the breeding source, and the maximum dispersal distance for most of the fly population is about 3.2 km (Graham *et al.*, 2009). Houseflies can pick up and transfer various pathogens (Gioia *et al.*, 2022).

Their movements between contaminated and clean areas facilitate the spread of diseases. Beyond their role in disease transmission, houseflies are a common nuisance due to their persistent buzzing and presence around food.

The more significant threat posed by house flies is their ability to acquire and move pathogens from low-risk areas, such as manure, to high-risk areas, such as animal and human feed (Nayduch *et al.*, 2023)

Being both zoophilic and synanthropic, house flies bridge the gap between filth and animals within and across the environmental niches (Yin *et al.*,2022)

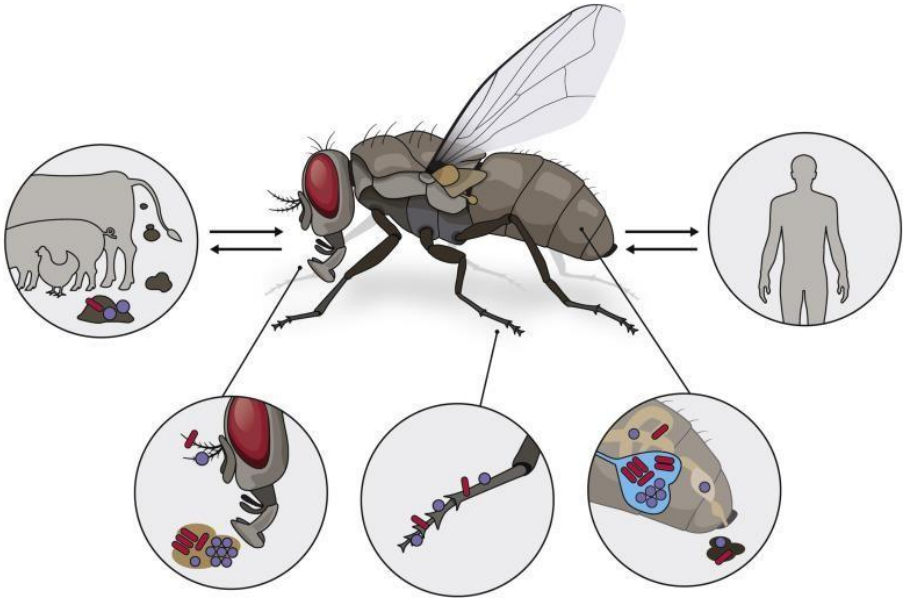


Figure 2.21 The role of 'filth flies' in the spread of antimicrobial resistance (Onwugamba *et al.*, 2018).

Flies can obtain many kinds of bacterial species from various sources by eating or direct contact which can transfer pathogenic bacteria critical for public health to their body surfaces or colonize their alimentary canals by ingestion (Awache *et al.*, 2016; Shing *et al.*, 2025).

Non-biting flies have sponging-sucking mouthparts that they use to consume liquid foods. They can ingest these foods by sucking them up or by moistening the food surface with regurgitated liquid. This liquid contains saliva with enzymes that help liquefy the material for easier ingestion. The antennae of the flies are responsible for detecting airborne odours, while their feet are used for tasting. In female flies, the compound eyes are widely separated, in contrast to male flies, where the eyes are more narrowly spaced (Onwugamba *et al.*, 2018).

Houseflies might be seen as a nuisance, but these are more than an annoyance for public health and may sometimes play an essential role in the transmission of a range of pathogens, including in the transmission of ESBL-producing *E. coli* and are involved in the

horizontal and vertical transmission of antimicrobial resistance genes (Yin *et al*., 2022). Flies can move from farms where they were bred and have fed on animal waste and may move to ready-to-eat foods like fruits and vegetables. They are attracted by the sugary content or fermented odours of seasonal fruits like figs, peaches, plums, and melons or by other volatile compounds present in the food (Murillo *et al.*, 2018).

Even though flies generally stay close to their breeding source, like, for example, on farms, they may also move over considerable distances. Adult flies are ubiquitous in the environment and can move approximately 3.2 km in their lifetime. Therefore, they can act as vehicles and disseminate pathogens from unsanitary areas into people's homes, dwellings, or hospitals. Bacteria can quickly become attached to their sticky leg pads, hairs, and electrostatically charged exoskeleton. In Figure 2.21 contaminated ingested material containing pathogenic bacteria is initially stored in the crop, from where it passes down the digestive tract (Khan *et al.*, 2015; Yin *et al.*, 2022) Bio-enhanced transmission occurs when the bacteria inside the flies can propagate in their mouthparts, intestines, and regurgitation spots, and translocation occurs from the exoskeleton and defecation. Factors like weather, fly population, geography, hygiene, food chain, and proximity of livestock farms may influence the transmission route. Since domestic flies share the same environment as both animals and humans, transmission of antimicrobial resistance might be possible. Some investigators have investigated bacteria carried internally within the digestive tract (Nayduch *et al.*, 2001; Joyner *et al.*,2013). Collectively, these studies highlight the role of the domestic fly as a potential vector of pathogenic bacteria within their digestive system.

Ingested material containing bacteria is initially stored in the crop, passing down the digestive tract through the proventriculus midgut, hindgut, and rectum (Figure 2.22).

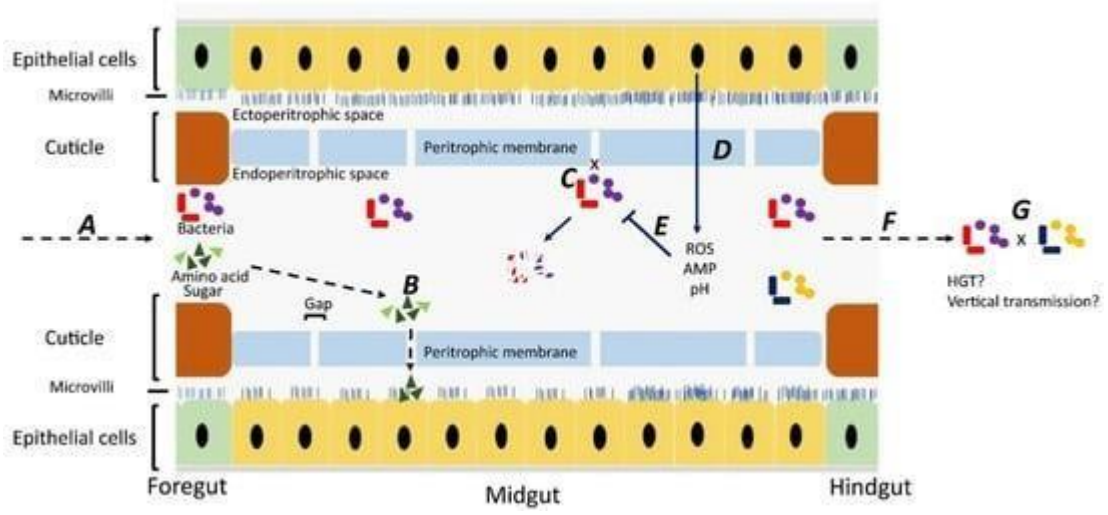


Figure 2.22 The fate of bacteria in flies' digestive tracts

Figure 2.22 illustrates the fate of bacteria in flies' digestive tracts (midgut). A: Ingested food with bacteria is predigested with saliva in the crop. The epithelial cells in the foregut are covered by a cuticle, which prevents bacterial invasion. B: In the midgut, digestion products can pass through gaps in the peritrophic membrane and enter the ectoperitrophic space to be absorbed by epithelial cells. C: Bacteria cannot pass through the gaps on the peritrophic membrane and remain in the endoperitrophic space. D: Bacteria are trapped in the endoperitrophic space, triggering an innate immune response in epithelial cells to produce reactive oxygen species (ROS) and antimicrobial peptides (AMP). E: Trapped bacteria are killed by ROS, AMP, pH changes, and digestive enzymes. F: Some bacteria survive in the hostile environment, pass through to the hindgut, and are shed. G: There might be horizontal gene transfer between bacteria surviving in the digestive tract, and bacteria may be transmitted vertically to offspring (Adapted from Yin, Kelly & Wang, 2022).

The fly has been demonstrated to carry enough pathogens on its body surface to cause an infection (Neupane *et al.*, 2020). The quantity of pathogens present in the gut is usually higher than the quantity present on the body surfaces, suggesting that faeces and vomitus

may also serve as a significant route of transmission of pathogens (Nayduch *et al.*, 2001; Khamesipour *et al.*, 2018)

Shing *et al.* (2025), in their recent study, showed that *E. coli* was frequently isolated from the flies' internal organs rather than from their external surfaces. This could be because houseflies feed mainly on faeces and other animal waste, a rich Enterobactericeae source (Khamesipour *et al.*, 2018). Also, in lab experiments, house flies were reported to be capable of carrying several viruses. Most of these viruses were of veterinary importance, including the Seneca virus A, whose natural hosts are pigs and cows. Other studies demonstrate the ability of the housefly to carry the Ebola virus in lab experiments. However, its role in the transmission of the virus is still to be confirmed (Khamesipour *et al.*, 2018).

Nevertheless, there is enough evidence to show that house flies can carry pathogens capable of causing severe diseases in humans and domestic animals and should, therefore, be controlled. Control of the house fly can be achieved through physical means, such as composting the manure. The most common method today, chemical pesticides, is quickly losing ground due to the growing resistance of houseflies and other pests to them. Other targets include biological controls. Furthermore, the sequencing of the house fly's genome presents new opportunities for identifying novel targets for controlling the house fly, understanding the mechanisms of resistance to insecticides, and determining the house fly's genetic adaptation to high pathogen loads (Khamesipour *et al.*, 2018).

2.7.5 The role of domestic flies in the spread of AMRs

Domestic houseflies (*Musca sp*) have great potential to contribute to the spread of antimicrobial resistance, and studies show an increase in awareness of their role in transmitting antimicrobial-resistant bacteria from the environment to humans

(Onwugamba *et al.*, 2018). Investigations must be conducted to identify whether the bacteria and resistance genes are effectively transmitted from flies to animals and people. Flies might pose threats to the public like UTIs.

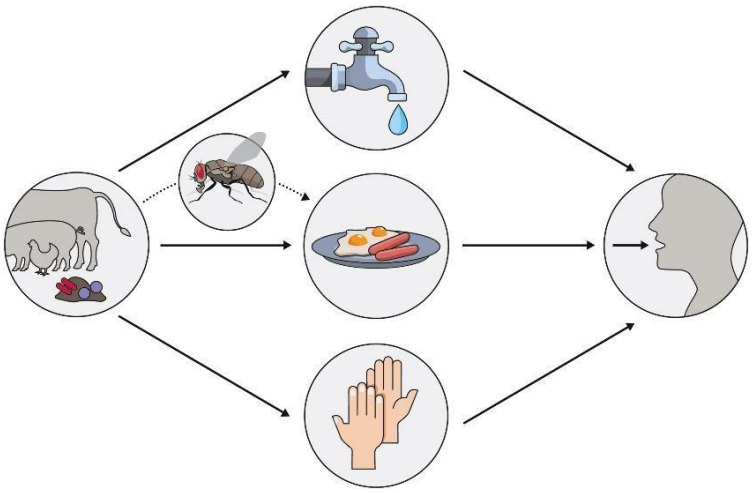


Figure 2.23 Flies act as a vector to enhance the cross-contamination of food, as no direct contact with food and faeces is necessary (Onwugamba *et al.*, 2018)

Houseflies can easily pick up antibiotic-resistance genes from livestock animals and spread them to and between communities and other animals. Cosmopolitan, invertebrate synanthropic flying insects, are considered potential vectors in the spread of pathogens through mechanical transmission (Singh *et al.*, 2025).

Flies can harbour and carry a wide range of microorganisms. A systematic review revealed that more than one hundred microorganisms have been identified in houseflies including bacteria, viruses, fungi, and even parasites (Cottone *et al.*, 2014). The predominant bacterial pathogens include *Klebsiella spp., Salmonella spp, Pseudomonas aeruginosa, Campylobacter jejuni, Clostridium spp.*, and *Yersinia enterocolitica.* Studies have shown that houseflies can acquire SARS-COV-2 and spread its genomic RNA into the

environment for up to 24 h following exposure (Balaram *et al.* 2021; Laguipo, 2021)

Marshall *et al.* (1990) were the first to discuss and show through studies that the domestic fly can represent a route of transmission for AMRs among farm animals. The study sought to understand the natural conditions that contributed to the bacterial spread and movement of plasmids in the environment. Rybarikova *et al.* (2010) found the spread of resistant strains in flies and manure, suggesting that flies spread resistant pathogenic strains from manure.

Usui *et al.* (2015) suggested that flies can spread AMR genes within individual farms as well between distant farms. Similarly, Sola-Gine *et al.* (2015) found the exact ESBL-producing *E. coli* clone isolated from flies within two broiler farms, and it was concluded that it may happen because flies can travel long distances (dos Santos Alves *et al.*, 2018). The continuous use of antimicrobials in livestock favours the presence of AMR pathogens in commensal microorganisms due to selective pressure (Oliver *et al.* 2011).

Therefore, there is a high risk that domestic flies will spread antimicrobial-resistant bacteria from livestock to humans (Schaumburg *et al.*, 2016). In 2016, Schaumburg *et al.* investigated antimicrobial resistance in bacteria from rural and urban areas of Munster City in Germany and compared them to human isolates. Because of the high similarity of the isolates, it was suggested that there could be a common source of transmission of bacteria from flies to humans (dos Santos Alves *et al.*, 2018). It is now well known that pathogens carrying antibiotic- resistant genes can be transmitted mechanically on domestic flies' surfaces, from their faeces, or through direct contact with other surfaces, especially food (Shing *et al.*, 2025) and there is growing evidence that flies' digestive tracts might serve as a perfect location for Horizontal Gene Transfer (HGT) (Shing *et al.*,2025) In addition, an in vivo study showed that third- generation cephalosporin resistance genes, including beta–lactamases, $bla_{CTX\text{-}M}$ and $bla_{CMY\text{-}2}$, were successfully transferred from *E. coli* to

Achromobacter spp and *Pseudomonas fluorescens* within the intestines of houseflies (Phan *et al.*, 2015). Vertical transmission of pathogens can also occur in houseflies. When flies are fed different concentrations of *Salmonella enterica*, *Cronobacter sakazaki*, and *Listeria monocytogenes*, the bacteria can be detected in the next generation's eggs. *E. coli,* containing plasmids with antimicrobial resistance genes, fed to houseflies were found in the subsequent immature state and adults' life cycles. Furthermore, in poultry that had been given immature fly stages with *E. coli* containing plasmids with AMR genes, the resistant *E. coli* persisted in the caecal contents for at least 16 days (Pava-Ripoll *et al.*, 2015). The fly has been demonstrated to be a potential vector for disseminating a sufficient quantity of pathogens on its body surface to cause an infection. The quantity of pathogens that might be present in the gut is usually higher than the quantity on the external surface of the fly, suggesting that faeces and the regurgitation process by vomiting may also serve as a significant route of transmission of pathogens (Onwugamba *et al.*, 2018).

Bacteria can proliferate in colonies inside the gut, first in the digestive tract, which is transferred to the gut or by regurgitation, leading to the concept of '*Bioenhance'*. This could be because houseflies feed mainly on contaminated faeces and other waste contaminated with enteric bacteria (Kobayashi *et al.*, 1999).

As noted by Onwugamba *et al.* (2018) *' There is thus growing evidence that houseflies are not only mechanical but also biological vectors and as such, they might facilitate the maintenance of antimicrobial resistance genes.'*

2.8 One Health Approach

2.8.1 Introduction

The One Health Approach integrates human, livestock, and environmental health which must be addressed to combat antimicrobial resistance effectively (AMRs) and ensure a

well-coordinated response to their spread. The misuse of antibiotics in health care services and the agricultural sector accelerates resistance, threatening the efficacy of treatments and public health. This multidisciplinary framework integrates both clinical and veterinary medicine as well agriculture and environmental management to enhance collaborative solutions and to mitigate the spread of bacterial resistance. The WHO Global Action Plan and the EU on the One Health Action Plan emphasize the development that follows other countries implementing their own National Strategy Plans on AMR.(Cella et al. 2023) Antimicrobial resistance (AMR) is one of the most significant global health threats of the 21st century and according to a study 'Lancet Global Burden of Disease'(2021), AMR directly caused 1.27 million deaths and contributed to nearly 5 million deaths worldwide during 2019. Such a death toll has surpassed AIDS and malaria cases. The O'Neil report (2014) highlighted that if left unchecked, AMR cases could reach 10million deaths annually by 2050 highlighting a global economic loss exceeding USD 100 trillion. Based on these alarming figures, the need for a unified strategy such as the One Health Approach is highlighted. Globally the FAO (2020) reports that approximately 70% of all antibiotics are administered to livestock animals rather than to humans. High antibiotic consumption in livestock animals significantly contributes to the development of resistant bacterial strains that can be transmitted to humans through the food chain, water, or via mechanical vectors such as domestic houseflies. Furthermore the WHO (2023) estimates that 60% of human pathogens originate from animals, highlighting the zoonotic potential of resistance transmission. Zoonotic infections are responsible for the spread of AMRs. This is because the same antibiotics such as while fluoroquinolones are used to treat UTIs in humans are also used to treat infections in domestic pets. Therefore, this helps in the transmission of resistant strains from animals to humans. Overuse and misuse of fluoroquinolones in veterinary medicine leads to

quinolone resistant *Salmonella* species that infect the human gut.

The core objective to implement the One Health Approach framework is to ensure coordinated surveillance, stewardship, education, awareness and other policy initiatives that prevent and control resistant infections across sectors and social dimensions influencing antibiotic use and misuse.

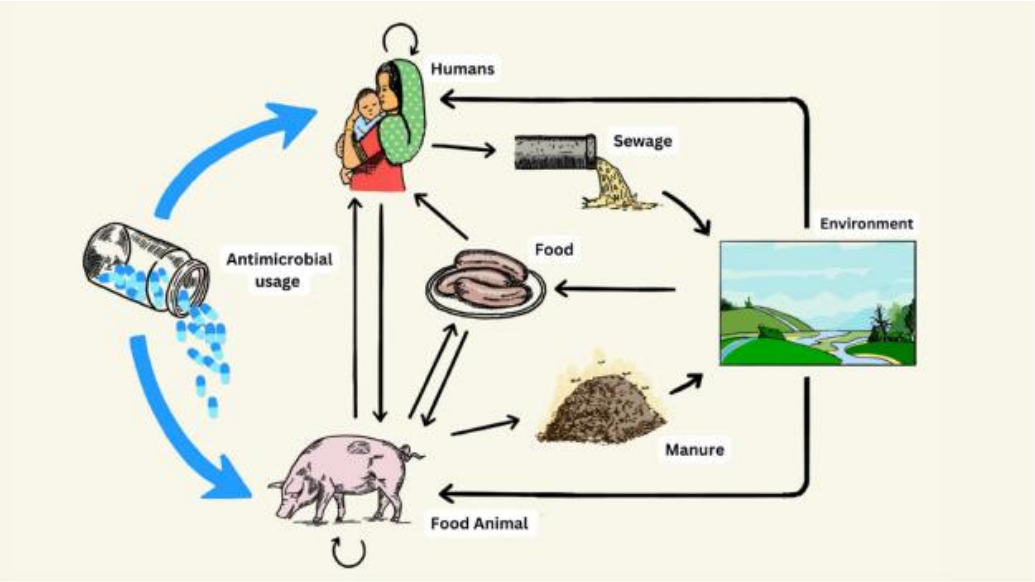
2.8.2 An Interdisciplinary and Intersectoral issue to tackle Antimicrobial Resistance

Antimicrobial resistance is one of the most recognized public health challenges . This one health framework has been set up to be multisectoral, transdisciplinary, and collaborative to tackle not only global health security in humans but also animals and the environment, addressing significant issues such as AMR, zoonotic disease, food safety and security and other emergent diseases. The Global Action Plans on AMR (2015) by the WHO, which has developed five pillars to help combat antimicrobial resistance and providing a framework for countries to develop National Plans, tailored to their specific healthcare systems and agricultural practices. The WHO, FAO and WOAH jointly promote the One Health strategy to ensure and implement a unified and sustainable response. This plan targets a broad spectrum and aims to collaborate as a network to combat resistance. It seeks to implement this model by minimizing cross- contamination between humans, animals and environmental sectors as well as reducing antibiotic use, promoting infection prevention and enhancing waste management. Under the One Health Approach countries are encouraged to establish national systems to monitor antimicrobial use and resistance patterns in humans, livestock and the surrounding environment. Education and awareness programs aimed at healthcare professionals, veterinarians, farmers and the general public are essential for reducing the inappropriate use and misuse of antibiotics while strengthening infection control practices.

Malta has aligned its National Action Plan on Antimicrobial Resistance jointly with the WHO's Global Action Plan and also European One Health Action. This strategy and Action Plan for the Prevention and Containment of Antimicrobial Resistance (AMR) (2020-2028) represents a joint initiative between the Ministry for Health and Ministry for Agriculture, Fisheries, Food and Animal Rights. Addressing on reducing antibiotic improper intake and misuse in both human and veterinary medicine through implanted program of continuous surveillance, stewardship, awareness, public education and international research collaboration on innovative technologies.

The plan against AMR is composed of seven strategic objectives to control AMR while preserving the effectiveness of antibiotics including: Legislation and policy development such as implementing regulatory framework; Antimicrobial Stewardship programs antibiotic usage through national guidelines; Surveillance and data collection to track antimicrobial resistance and intake; Infection Prevention and Continuous Control to limit the AMR spread across all sectors including health settings, veterinary practices and food production sectors. Educational and Awareness Campaigns among professionals, farmers, and the public; Encouraging Research and innovation, including the study of innovative rapid technology for the rapid laboratory investigation of AMR presence, utilizing AI tools; strengthening international collaboration through cross-border policies The UK 5 – Year AMR Strategy (2024) provides a strong example of effective One Health implementation. Following a partnership between the NHS, public health agencies and agricultural stakeholders. Key components include Scope and Prioritization where Malta has developed regulatory frameworks and surveillance systems where the UK-S-5-Year Strategy focuses more on how to reduce antibiotics such prescriptions in health care settings; Surveillance and monitoring where the UK has an advanced Real-Time National AMR Surveillance System whereas Malta is establishing a national AMR Surveillance

database; Research and Development as regards the UK which is investing in the development of new alternatives of antibiotics, Malta is focusing on local AMR drivers; Infection Prevention and Public Awareness. Furthermore, both the UK and Malta demonstrate that the success of One Health depends on sustained political and community commitments and intersectoral cooperation. . The Role of Media in raising AMR awareness through the One Health Approach plays a vital part among communities since media platforms can play a crucial role in educating the public about resistance. Sharing life stories of individuals affected by AMR, for example, ECDPC has launched a series of patient stories, giving people a voice to share their experiences and the impact that these resistant infections have had on patients who have experienced prolonged hospital stays, followed by uncertain recoveries and complex treatments. The European Antibiotic Awareness Day provides an annual platform on or around 18 November, supporting national campaigns on the responsible use of antibiotics in humans, healthcare settings, and veterinary medicine. The emergence of ESBL-producing *E. coli* isolates is a significant public health concern, especially in community-acquired infections. Although this recent study by Woolhouse. (2024) adopts a One Health approach to antimicrobial resistance (AMR); where in the study does not explicitly mention flies as potential vectors. Given their established role in bacterial transmission as shown hereunder in ‘Figure 1 produced exactly as presented in Woolhouse. (2024) retaining its original numbering on page 145. Copyright 2024 by Springer Nature.



*Legend: This is not included in the list of Figures in this thesis (Woolhouse, 2024).

Future studies should investigate the role of flies in the dissemination of AMR to determine whether they are significant contributors. Integrating such findings into AMR models would provide a general understanding of resistance transmission within a One Health Framework

2.8.3 Conclusion

The One Health Approach offers a holistic response to the current global threat of antimicrobial resistance (AMR) by bridging human, livestock and environmental health while promoting interdisciplinary collaboration and providing a sustainable framework for continuous prevention and control.

Malta's small geographic area and high population present a unique challenge. The proximity between residential zones, livestock farms, wastewater and other environmental sources facilitates bacterial resistance transmission via other mechanical vectors like houseflies. Therefore through the One Health Approach, Malta aims to bridge these gaps by linking human, veterinary and environmental surveillance.

Ongoing campaigns promote hygiene, responsible prescribing, and safe disposal of antibiotics to reduce environmental contamination and limit the transmission of resistance genes. Finally, combatting AMR's through One Health Approach ensures the control of antibiotic effectiveness and the protection of global health especially food safety and security.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter describes the experimental investigation and methodologies used to explore the role of domestic housefly (*Musca* sp) as potential mechanical vectors in the spread of ESBL- producing *E. coli*. This Chapter sought to investigate the phenotypic and genotypic characteristics of ESBL-producing *E. coli* strains isolated from environmental fly samples and clinical Urinary Tract Infection (UTI) samples across Malta, focusing on the distribution of the CTX-M type-resistant gene. To achieve this, the study of housefly (*Musca domestica*) and UTI samples was processed for the isolation and confirmation of ESBL-producing *E. coli*. Using standardized disc diffusion methods for antimicrobial susceptibility profile and molecular techniques for the detection of resistance genes and mobile genetic elements to determine the mobility through Horizontal Gene Transfer (HGT)

The phenotypic methodology was adapted from Blaak et al., (2014) who investigated the presence of ESBL-producing ***E. coli*** in flies in poultry farms. Starting with the preparation of culture solid and liquid growth media and other reagents used throughout the methodology, followed by detailed descriptions of sampling procedures for bovine fresh manure, fly traps and clinical UTI isolates. Subsequent sections present both microbiological and biochemical methods used to isolate and detect ***E. coli*** strains, as well as the AST performed according the CLSI guidelines to measure and determine the resistance profiles. All lab procedures were conducted following established SOP's, quality control (QC) of prepared cultured media and the use of reference control strains of ESBL and non – ESBL ***E. coli*** to ensure accuracy and reproducibility. Throughout the laboratory work aseptic measures and techniques were continuously applied to prevent

cross – contamination. Molecular techniques, including DNA extraction, Real -Time (RT) PCR for the detection of the resistant gene CTX-M, and the Whole Genome Sequence (WGS) are further described and to characterise resistance mechanisms and genetic relationships among ***E. coli*** isolates.

3.1.1 Methodology Workflow

Table 3.1 Schematic flow chart illustrating the overall methodology workflow from sample collection to comparative genetic analysis

Table 3.1 Schematic flow chart of the overall methodology workflow

A. **Sample Collection (2018–2021)**	• Bovine manure samples (Malta and Gozo farms) • Traps – houseflies from farms and markets ESBL-producing *E. coli* UTI isolates (Mater Dei Hospital)
B. **Sample Processing and Culture**	• Manure: 1 g in 9 ml MRD • Houseflies: homogenisation in PBS • Inoculation on TBX + 1 µg/ml cefotaxime (CTX) Subculturing of UTI isolates on LB agar
C. **Microbiological Interpretation**	• Enumeration and detection of ESBL-producing *E. coli*
D. **Antimicrobial Susceptibility Testing (AST)**	• Disc diffusion on Mueller Hinton agar Interpretation following CLSI guidelines
E. **Molecular Detection of CTX-M Resistant Gene**	• DNA extraction (QIAamp DNA Mini Kit) Real-time PCR for blaCTX-M screening
F. **Whole Genome Sequencing (WGS)**	• Selection of representative isolates • Preparation following MicroBesNG protocol (Appendix) Hybrid assemblies and long–short read sequencing
G. **Bioinformatic Analytical Online Tools**	• Detection of blaCTX-M variants • Virulence factors profiling • Plasmid type determination Genetic mobile elements characterization
H. **Comparative Analysis and Interpretation**	

3.2 Preparation of Culture Growth Media

3.2.1 Maximum Recovery Diluent (MRD)

Following the manufacturer's instructions the liquid medium was employed in the study to prepare serial dilutions of manure samples. The liquid medium was sterilised and autoclaved at 121°C for 15 mins.

3.2.2 Mac Conkey Agar

Dehydrated media was prepared according to the manufacturer's instructions, following the ISO Standard for Media Preparation and the ISO Standard for Quality Control and Guidelines for Media Preparation. The media was prepared under aseptic conditions. The dehydrated agar was dissolved in distilled water by gentle heating on a hot plate stirrer until boiling. The pH was checked and found to be within the range. Once the dehydrated media was dissolved entirely, it was sterilized by autoclaving at 121°C for 15 mins. After autoclaving, the sterile medium was cooled to 45-50 °C in a water bath to safely add the CTX supplement without degrading it.

Cefotaxime supplement CTX was used as a marker for ESBL-producing *E. coli* strains. To avoid contamination, a sterile stock solution of cefotaxime at 1mg/mL was sterilized aseptically through a 0.22 µm membrane filter.

A 2 mL stock solution was prepared and added to the 1 litre 45-50 °C agar, gently mixed to prevent the introduction of air bubbles, and poured into sterile Petri dishes.

3.2.3 Tryptone Bile X- Glucuronide (TBX) Agar

TBX agar was prepared following the ISO 16649-2 Standard for detecting and enumerating *E. coli,* especially β-glucuronidase-positive strains. Following the manufacturer's directions, the dehydrated medium was dissolved in 500 mL distilled water and sterilized by autoclaving at 121°C for 15 mins. After autoclaving, the liquid

medium was left to cool down between 45- 50 °C. Since 500 mL of TBX was prepared, the volume of the cefotaxime supplement was adjusted accordingly to maintain the desired final concentration of 2 µg / mL, and 1 mL of the 1 mg/mL cefotaxime stock solution was added to the cooled medium after sterilization.

TBX + 1µg/mL Cefotaxime (CTX) was poured into sterile, clean petri dishes.

3.2.4 Luria-Bertani (LB) Agar

Following the manufacturer's directions, the dehydrated ingredients were added to 1 L of distilled water and sterilized by autoclaving at 121 °C for 15 mins. After autoclaving, the medium was tempered between 45-50 °C and was aseptically poured into clean, sterile Petri dishes. The molten medium of the LB agar was poured aseptically under the laminar flow cabinet in 90mm petri dishes and left to solidify at room temperature with the lid slightly opened to avoid the formation of condensation inside the plate. The ready-prepared LB Agar plates were labelled and stored at 4 °C.

3.2.5 Tryptone Water

Following the manufacturer's instructions, tryptone water was dissolved in distilled water and brought to a boil. Once dissolved the broth was dispensed in 5 – 10 ml clean test tubes with screw taps and sterilized by autoclaving at 121 °C for 15 mins. After autoclaving, the tubes were left at ambient temperature and stored at 2 – 8 °C.

3.2.6 Mueller Hinton Agar and Broth

3.2.7.1 Mueller Hinton Agar

For antibiotic susceptibility testing, this medium was prepared following the manufacturer's instructions at the School of Biosciences laboratory, Nottingham University. The dehydrated ingredient was dissolved in 1 l of distilled water and sterilized

by autoclaving at 121 °C for 15 mins. After autoclaving, the liquid medium was tempered between 45-50 °C and aseptically poured into 120 mm x 120 mm square Petri dishes. These were left to solidify with the lid slightly opened to avoid condensation inside the plate. The ready-prepared Mueller–Hinton agar plates were labeled and stored at 4 °C.

3.2.7.2 Mueller Hinton Broth

Following the manufacturer's instructions, this medium was used for antibiotic susceptibility testing and prepared at the School of Biosciences laboratory, Nottingham University. The dehydrated medium was dissolved in 500 ml distilled water and sterilized by autoclaving at 121 °C for 15 minutes. The liquid medium was dispensed in 8 mL volume in sterile, clean

15 ml glass tubes were later used for AST procedures to prepare standard 0.5 McFarland.

3.2.8 Scheme of work of Good Quality Media Preparation

Quality Control of Solid and Liquid Prepared Media

Without proper quality control (QC) or improperly prepared media, the cultures may fail to support the desired microbial growth, leading to false-positive or false-negative results. QC was performed in association with sterility testing plates of the prepared cultured medium and the use of control strains in order to ensure and provide compliance with laboratory standards and regulatory guidelines following ISO/IEC 17025:2017

Sterility testing for prepared plates was performed on already prepared plates, with reference strains *E. coli* ATCC 25922 used to detect growth morphology and *E. coli* reference strain ATCC 35218 as a negative control (Appendix A.2). Suspected positive ESBL-producing *E. coli* colonies were green on TBX agar + CTX-M 1mg/mL and pink-

red colonies on MacConkey agar + CTX-M 1mg/mL. Following the typical growth observations, typical ESBL-producing *E. coli* strains were tested within a series of biochemical tests, including the indole and oxidase strip tests in Table 3.1

Table 3.2 Growth and Biochemical Characteristics of ESBL and Non-ESBL *E. coli* Strains on Selective Media

Medium	ESBL strain	Non – ESBL strain
TBX+CTX-M 1mg/mL	Blue/ green colonies	No Growth
Mac Conkey+ 1mg/mL	Pink reddish colonies	No Growth
Biochemical Tests		
Indole	+	
Oxidase*	-	

*Biolife, Italy

3.3 Buffers, Reagents & Solutions

3.3.1 Phosphate-Buffered Saline

A sterile buffer solution to maintain pH stability and osmotic balance while maintaining the viability and morphology of strains

3.3.2 QIAamp DNA mini kit available buffers ATL; AL; AW1; AW2; AE

(Appendix A.1)

3.3.3 Cefotaxime (CTX)

Cefotaxime sodium salt was purchased from Sigma, Germany (C7912-1G). It is a third–generation cephalosporin antibiotic commonly used in research for its broad–spectrum

antibacterial activity, particularly against Gram-negative bacteria and to inhibit the growth of non–ESBL *E. coli* on TBX supplemented with 1 μg/ml

3.3.4 Kovac's Reagent

Kovacs´ reagent was purchased from Scharlab, Spain (RE0007G100). Kovac's reagent is a commonly used in microbiology to detect indole production. Detection is performed by adding Kovac's drops to the inoculated tryptone water. *E. coli* is indole positive.

3.4 Study Design - Collection of Bovine Manure: A Pilot Project 2018

A pilot project was carried out by the research team in 2018 across all dairy farms farms in Malta and Gozo. During this preliminary investigation fresh bovine manure was collected from cow sheds to analyse the presence of ESBL- producing *E. coli*

3.4.1 Microbiological investigation for the isolation of ESBL-*E. coli* strains in bovine manure

Bovine manure was collected fresh directly from underneath the cows, which was still soft but not warm. In the laboratory, the collected manure samples from the same farm were pooled as onesample representing different zones or geographical areas of the island.

1g of the fresh manure sample was diluted in 9 ml MRD (10^{-1} dilution) and vortexed to homogenize the solution well. Following homogenization, 100 µl from both 10^{-1} and 10^{-2} dilutions were streaked on a chromogenic medium TBX (Tryptone Bile X-glucuronide agar) plate and another plate supplemented with TBX + 1µg/ml cefotaxime (CTX).

All cultures were incubated for 3 to 4 h at 36 ± 2 °C followed by 18 to 20 h at 44 ±

0.5 °C (Ibrahim *et al.*, 2022) to increase selectivity for *E.coli**,** and* typical colonies were identified and confirmed further by biochemical tests, including indole and oxidase tests.

3.5 Musca sp. sampling and microbiological analysis

3.5.1 Collection of *Musca spp* samples 2018-2019, 2021 from Bovine Farms and Open Markets

Flies were collected independently from multiple bovine farms and open markets using insect traps over two years, 2018 - 2021, precisely during April and October. This process was disrupted for more than one year during the pandemic.

Sampling was conducted during meteorologically stable conditions to ensure that environmental factors such as rainfall and winds did not interfere with the collection process. The collection occurred on days with temperatures between 25 °C and 35 °C, which promotes high fly activity. Bright yellow sticky sheets, commercially available from agricultural nursery shops as pest control, were utilized to capture flies.

These sheets are non-toxic, waterproof, and coated with strong adhesive, making them highly effective in trapping flying insects upon contact. The yellow coloration is a powerful visual attractant for houseflies as they instinctively draw to bright surfaces. The sticky sheets, approximately 20 cm by 30 cm, were strategically placed at multiple locations in the farm, both inside the cowsheds and in the external areas surrounding the farm, ensuring that flies were collected from diverse areas within the same farm. In the case of open markets, the sticky sheets were suspended inside the vegetable trucks parked in open markets and positioned in areas on the truck accessible to flies but discretely placed out of direct public view. Once the flies adhered to the sticky sheets, they were carefully removed from their locations and immediately placed in temperature-controlled coolers to maintain sample integrity. The traps were then transported to the Institute of

Earth System, Division of Rural Sciences and Food Systems laboratory at the University of Malta, ensuring they reached the microbiology laboratory within 4 h of collection. The samples were either processed on the day or stored at -20 °C for future analysis. At this stage, morphological identification of the flies was not conducted but was based on collection methodology and known fly populations in bovine farms and open markets. They were presumed to be *Musca spp*. Although the yellow sticky sheets provided an effective passive and non-invasive method of fly collection, they had one notable disadvantage. Due to the strong adhesive coating, parts of the flies' bodies sometimes remained stuck to the sheet without entire bodies when picked up with sterile forceps. However, to mitigate these limitations and ensure adequate sample weight, fly specimens were pooled together from multiple sticky sheets collected from the same farm and vegetable trucks, combining 8-12 whole flies per sample.

This pooling approach ensured sufficient bacterial load was available for microbiological analysis, particularly for isolating and identifying extended-spectrum beta-lactamase ESBL- producing *E. coli*.

3.5.1.2 Microbiological investigation for isolating *E. coli* strains in flies on TBX + 1µg/ml Cefotaxime (CTX)

3.5.1.2.1 Sample Preparation, Homogenisation, and Inoculation of Growth Media TBX

Four sticky insect trap sheets containing the captured flies were pooled for each identified farm to ensure a representative sample. Each pooled sample consisted of 8-12 whole flies collected from a specific farm within a defined geographical zone (Zones A-G). These zones represent (Appendix A) distinct districts across Malta and Gozo where dairy farms are located. Flies were collected from representative farms within each zone In the laboratory, flies were carefully removed from the sticky traps and transferred into 100 ml

sterile homogenized bags containing 25 ml Phosphate-Buffered Saline (PBS) supplemented with 0.5 % Tween 20. The flies were manually crushed in the homogenization bag using both thumb and forefinger and were then further homogenized using a Stomacher 400 (Seward, UK)

(A)



(B)



Figure 3.1 Flies were captured on insect sticky traps (A) then homogenized in Phosphate Buffered Saline (B)

From the homogenates, 100 µl was streaked on two chromogenic medium TBX (Tryptone Bile X-glucuronide agar) plates and another plate supplemented with TBX + 1µg/ml Cefotaxime (CTX). All cultures were incubated for 3 to 4 hours at 36 ± 2 °C followed by 18 to 20 hours at 44 ± 0.5°C (Ibrahim *et al.*, 2022) to increase selectivity for *Escherichia coli* (Blaak *et al.*, 2014).

3.5.2 Sub-culture of UTI ESBL – Producing *E. coli* Isolates from National Hospital

ESBL-producing *E. coli* isolates were obtained from urinary tract infection samples collected at the National Hospital, Mater Dei Malta. The UTI samples originated from patients diagnosed with ESBL-producing *E. coli* as outpatients or during hospitalization. The bacterial strains were preserved in the Microbiology lab of the Pathology Department at -80 °C using cryobeads. For further analysis, the isolates were sub-cultured on Luria-Bertani (LB Agar) and incubated at 37 °C for phenotypic and genotypic analyses.

3.5.2.1 Isolation, Identification, and Confirmation of Suspected ESBL-*E. coli* isolates

TBX agar is formulated to enumerate *E. coli* in water, food, and environmental samples. On TBX agar, ESBL-producing *E.coli* appear as blue/green round colonies whereas Non–ESBL–producing *E.coli* also appear blue/green. Presumptive *E. coli* colonies on TBX supplemented with cefotaxime (CTX) were identified and confirmed by biochemical tests, including indole and oxidase tests. Observing green colonies were considered suspect ESBL isolates (Ibrahim *et al.*, 2022). Suspected colonies were sub-cultured on Luria-Bertoni Agar (LB Agar) and incubated for 24 h at 36 ± 2°C.

After incubation, the typical colonies were observed for their typical blue-green morphology.

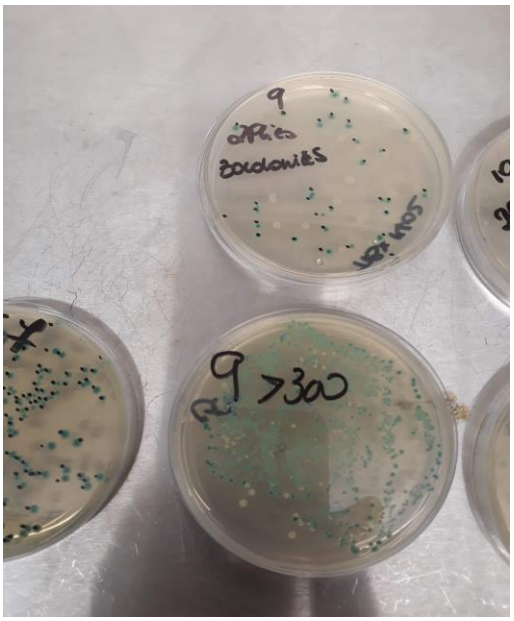


Figure 3.2 TBX + 1µg/ml Cefotaxime (CTX). agar showing typical ESBL-producing colonies.

Typical colonies in Figure 3.2 were collected, and tryptone water was inoculated with a single pure colony and incubated overnight at 36 ± 2 °C. After incubation, a few drops of Kovac's reagent were added to the tryptone water, and if a red circle formed at the top of the tube, it indicated an Indole-positive sample.

Detection of *Enterobacteriaceae* species like *E. coli* was performed according to International Standards ISO 16649-2 (Enumeration of *E.coli* in Food samples)

3.6 Antimicrobial Susceptibility Testing as per CLSI Guidelines

3.6.1 Phenotypic Detection of ESBL-producing *E. coli* - The Disc Diffusion Technique

The most used technique for identifying antimicrobial susceptibility is determined by the Disk Diffusion Method.

This technique consists of spreading the test bacterium onto agar and adding a paper

disk impregnated with different concentrations of antimicrobials. Interpreting the Mueller Hinton Agar involves inhibiting microbial growth, which will occur around the disk. The zone formed around the colony indicates whether the strain is sensitive, and if no zone surrounds the culture, the strain is resistant to that antibiotic. The zone is usually used as an indication.

Phenotypically confirmed ESBL-producing *E. coli* isolates were tested for antibiotic susceptibility against 16 antibiotics using the disk diffusion technique cultured on Mueller Hinton Agar. The zone of inhibition caused by the antibiotic was measured the next day after 16 – 18 h of incubation.

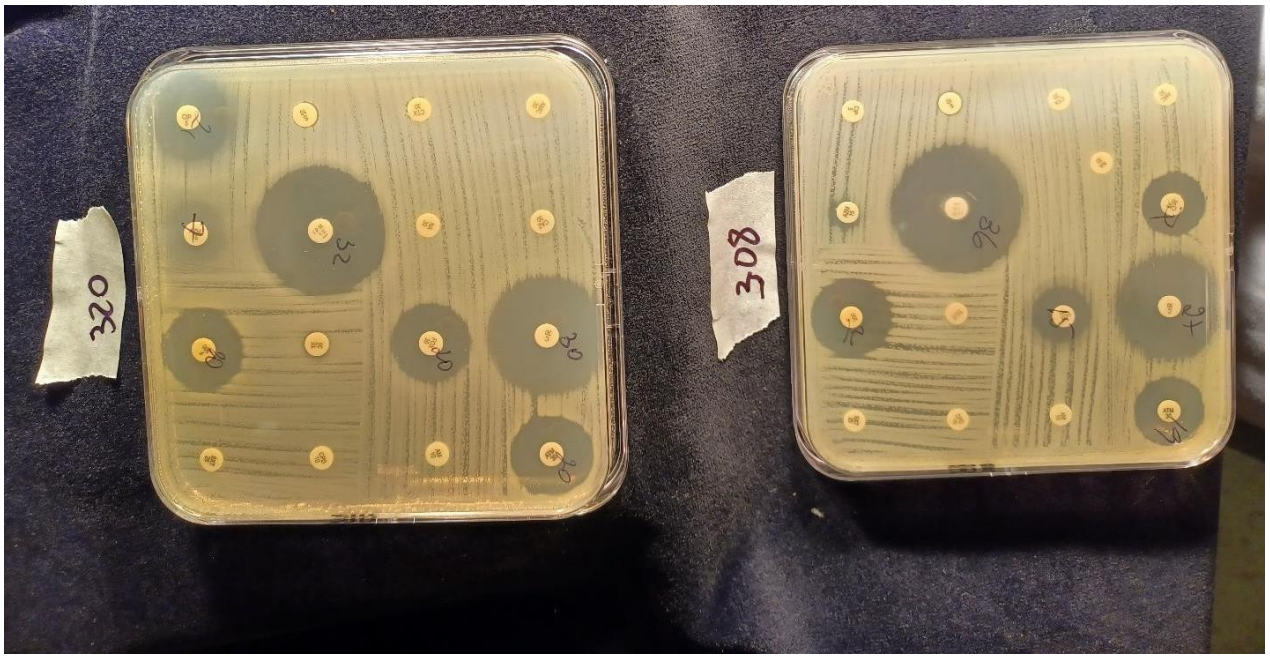


Figure 3.3 Zones of inhibition were measured on Mueller-Hinton agar indicating bacterial sensitivity or resistance to the corresponding antibiotic disc

Antimicrobial resistance against the tested antibiotic disks is being interpreted using the Clinical and Laboratory Standards Institute breakpoints (CLSI).

Susceptibility to antimicrobials was performed by an agar diffusion test using

antimicrobial discs consisting of impregnated paper discs as described by the Clinical Laboratory Standards Institute (CLSI) guidelines. Antimicrobial resistance against the tested antibiotic disks in Figure 3.3 is being interpreted using the Clinical and Laboratory Standards Institute breakpoints (CLSI).

Phenotypically confirmed ESBL-producing *E.coli* isolates were tested for Antibiotic susceptibility against 17 different antibiotics using the disk diffusion technique cultured on Mueller Hinton Agar. The zone of inhibition caused by the antibiotic is measured the next day after 16 h of incubation.

Antimicrobial resistance against the tested antibiotic disks was interpreted using the Clinical and Laboratory Standards Institute breakpoints (CLSI).

3.7 Scheme of work

Antibiotic sensitivity testing was conducted using the disc diffusion method set out by the Clinical and Laboratory Standards Institute (CLSI). Antibiotic SIRscan, discs France and their concentrations used were ampicillin (AMP; 10 µg), amoxicillin-clavulanic acid (AMC; 20 µg/10 µg), cefoxitin (FOX; 30 µg), cefotaxime (CTX; 30 µg), cefpodoxime (CPD; 10 µg), cefquinome (CFQ; 30 µg) or ceftazidime (CAZ; 30 µg), aztreonam (ATM; 30 µg), imipenem (IMP; 10 µg), streptomycin (S10; 10 µg), oxytetracycline (OT; 30 µg), ciprofloxacin (CIP; 5 µg), nalidixic acid (NA; 30 µg), sulfamethoxazole-trimethoprim (SXT; 23.75 µg/1.25 µg), chloramphenicol (C; 30 µg), nitrofurantoin (F; 300 µg) and azithromycin (AZM; 15 µg). Quality control strains used were *E. coli* ATCC 25922.

Before commencing the AST testing, the dispenser and disc were taken out of the fridge for approximately 2 h to warm up at room temperature. When up to temperature, the discs SIRscan discs were uploaded into the dispenser as per the layout of the sheet. This technique ensures that the zones on the agar do not overlap should the isolate be sensitive

to antibiotics.

Isolates were sub-cultured on Luria-Bertoni agar and incubated overnight at 36°C ± 1°C for 20 h incubation. After incubation, 2 to 3 typical pure single colonies were selected and aseptically transferred in an 8 mL Mueller-Hinton broth with a sterile cotton swab. The swab was first aseptically dipped in sterile Mueller-Hinton Broth, and then with the same swab, the selected colonies from the LB agar were picked up and again aseptically transferred in the same Mueller-Hinton broth. McFarland standard was used as a reference, and the spectrophotometer reader was used to adjust the turbidity of the bacterial suspension to fall within a specific range. The colony was suspended in 8 ml sterile 0.85% NaCl solution to a .05 McFarland standard into a falcon tube. The sample readings were between 0.8 and 1, measured on a spectrophotometer in a fresh, clean cuvette—OD 650. Before starting, the spectrophotometer was uploaded with a blank saline solution.

The number of bacteria within a given range recommended by the CLSI guidelines: to use bacteria with 0.5 McFarland turbidity for antimicrobial testing.

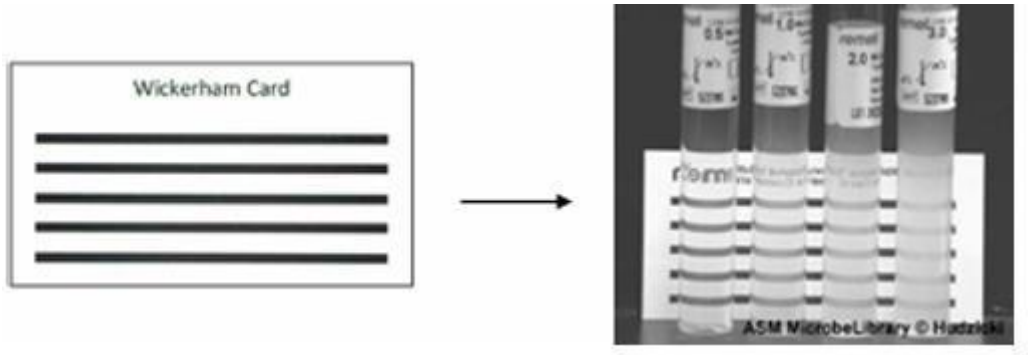


Figure 3.4 illustrates how the turbidity of bacterial suspension in Mueller-Hinton broth is visually compared against McFarland standard using a Wickerham card before antibiotic susceptibility testing (AST)

Aseptically, another new fresh swab was dipped in the Mueller-Hinton broth with the

bacterial suspension. It was evenly spread over the entire 120 mm x 120 mm Mueller-Hinton Agar plate in a rotating motion. The swab was reloaded, and the plate was rotated approximately 120° and repeated 3 times. Lastly, the swab was dipped again and run around the edge of the plate to provide a confluent lawn, followed by a side-to-side swipe of the petri dish, and the plate was left to dry for 5 mins. This ensures a thin but uniform growth of bacteria on the dish. Static electricity was removed from the prepared plate using a static gun. The 16-disc dispenser was aligned over the open plate and firmly pressed down, and the discs were dispensed onto the agar.

All the antibiotic discs were dispensed carefully on the plate by the 16-disc dispenser, plus another disc, Cefquinome (CFQ), was added onto another agar plate. Agar was inverted and incubated at 37 ℃ for 16-18 h. The loaded agar was put away and incubated within 15 min of the application of the discs.

After 16-18 h of incubation, inhibition zones surrounding the discs were observed. The diameter of the inhibition zone was measured using a ruler to the nearest millimeter, and it was interpreted as susceptible, resistant, or intermediate. The readings were recorded and uploaded on a supplied AST spreadsheet. The conditional formatting turned the provided red cells red if they were resistant, yellow if intermediate, and green if susceptible.

The readings were based on the CLSI standard for AST on Enterobacterales.

3.8 Molecular Methods: Detection of CTX-M resistant gene in *E. coli* Isolates

3.8.1 DNA extraction from *E. coli* Isolates

DNA was extracted from suspected ESBL–producing *E. coli* using an automated QIA cube connect. The QIA cube is designed to fully automate the purification of nucleic

acids from a freshly prepared 24-h ESBL-producing *E. coli* strain.



Figure 3.5 illustrates the QIAcube connector used during the automated DNA extraction process as part of the sample processing workflow

3.8.2 Detection of CTX-M resistant gene in *E. coli* Isolates by Real-Time PCR

All suspected ESBL-producing *E. coli* were screened for the presence of the resistance gene CTX–M. Bacterial drug resistance kits are intended to detect genetic elements

responsible for bacterial resistance to various antibiotic drugs.

These kits are qualitative tests that allow detection by Real-Time PCR based on the amplification of the genome-specific region using specific primers. In Real-Time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescent intensities during the reaction allows the detection of accumulating products without re-opening the reaction tubes after the PCR run.

The analysis of the DNA specimens using Bacterial Drug Resistance Kits by **Sacace** (Appendix A.2.2)

Biotechnologies includes the following stages:

- Preparing the Real-Time PCR: **Rotor-Gene 6000/Q**
- Real-Time PCR analysis
- Data analysis with the software of Real-Time PCR instrument
- Results analysis and conclusions



Figure 3.6 Real-Time PCR instrument (Rotor-Gene 6000/Q, Qiagen) used for amplification and detection of resistant gene CTX-M.

The necessary number of ready–to–use PCR tubes (extracted samples + positive control + negative control) were removed from the fridge, left at room temperature, and prepared. Extracted frozen DNA stored at -20 °C was removed and left at room temperature.

The provided reagents, including the Taq polymerase mix, positive control, and negative control vials, were vortexed for 3-5 s, and 7µl was aseptically added to each PCR tube. The strip tubes provided by the kit were placed into the loading block, and 7µl of extracted DNA from the sample was added to the corresponding PCR tubes.

(A) 

(B) 

Figure 3.7 ROTOR-GENE Q setup illustrating molecular analysis workflow, how samples were prepared and loaded and positioned (A) pipetting of samples into PCR tubes and (B) loading of tubes into the ROTO ring rotor prior to RT-PCR amplification.

The strip tubes were inserted into the 36-well rotor, ensuring each tube was in the correct position. Tubes were vortexed for 3-5 s to collect the drops. Tubes were loaded in the ROTO ring inside the Real-time PCR instrument. Interpretation of the Ct value by the ROTOR-Gene Q

Real-Time PCR Ct (Cycle threshold): A real-time PCR assay in a positive reaction is

detected by the accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold exceeding the background level. Cycle threshold (Ct) is a semi-quantitative value that can broadly categorize the concentration of bacterial genetic material in a patient or environmental sample following testing by RT PCR as low, medium, or high, for example, indicates approximately how much viral genetic material is in the sample.

Ct levels are inversely proportional to the amount of target nucleic acid in the sample, that is the lower the Ct level, the greater the amount of target nucleic acid in the sample.

A low Ct indicates a high concentration of bacterial genetic material, typically associated with a high risk of infectivity. A high Ct indicates a low concentration of genetic material, which is typically associated with a lower risk of infectivity.

Ct ≤ 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Ct of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid; Ct of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination. [Note]:Ct interpretation was performed according to the manufacturer's instructions where Ct ≤ 29 was considered positive.

3.8.3 Whole Genome Sequencing of selected CTX-M Genes for Hybrid Assembly long- short sequencing

3.8.3.1 Preparing strains for MicrobesNG in Inactivation Buffer for External supplier sequencing by MicrobesNG, UK

The Genome sequencing was conducted by MicrobesNG (https://microbesng.com/)

CTX-M-detected *E. coli* isolates were sent for hybrid long-short sequencing. They were grown on McConkey plus cefoxatime, incubated at 37 °C overnight, and subcultured on

Luria-Bertoni agar at 37 °C overnight. A single colony of the strain was picked up and mixed with 200 ul of sterile buffer Phosphate Buffered Saline (PBS). One colony from an LB plate was inoculated and streaked onto LB agar using a 10ul loop. Streaking was performed to ensure that approximately one-third of the agar plate was covered with a bacterial lawn. The rest was used to streak for single pure colonies. This was used as a purity check and to culture enough bacterial colonies to be harvested later for sequencing. After overnight incubation at 37 °C, a loop was used to transfer all the bacterial culture off the plate. Then, 10 ml PBS was mixed into 15 ml sterile Falcon tubes and vortexed. The optical density of the resuspended cells was measured at OD600nm. Falcon tubes were first centrifuged and resuspended in 1 ml PBS and transferred in an Eppendorf tube for a second centrifugation at 4000 rpm for 5 m. The pellet was transferred onto the barcoded Microbank tubes provided by MicrobesNG and set immediately at room temperature to the MicrobesNG, UK.

The MicrobesNG used to notify me of the arrival of the strains and that Illumina data was available and my current assemblies were running.

To access data projects, MicrobesNG forwarded the hereunder link: https://microbesng.com/portal/projects/0216bdf5-bc8b-4731-9558-90efa05ad72d

This link contained raw data, assemblies, and annotations. The samples will be assigned to a project upon receipt of the tubes.

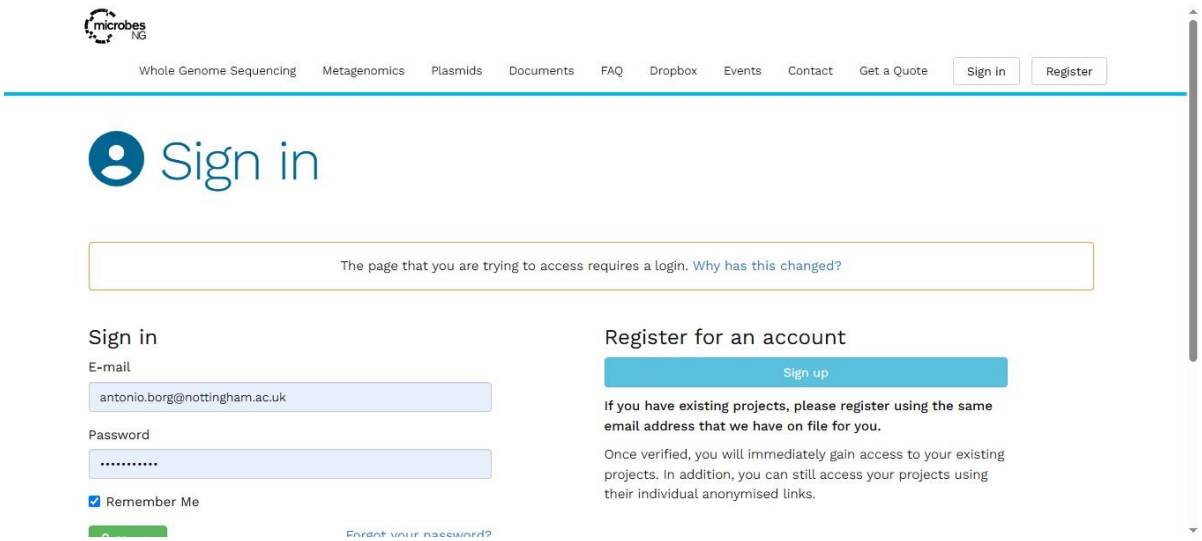


Figure 3.8 Sign in screen for the The MicrobesNG webpage.

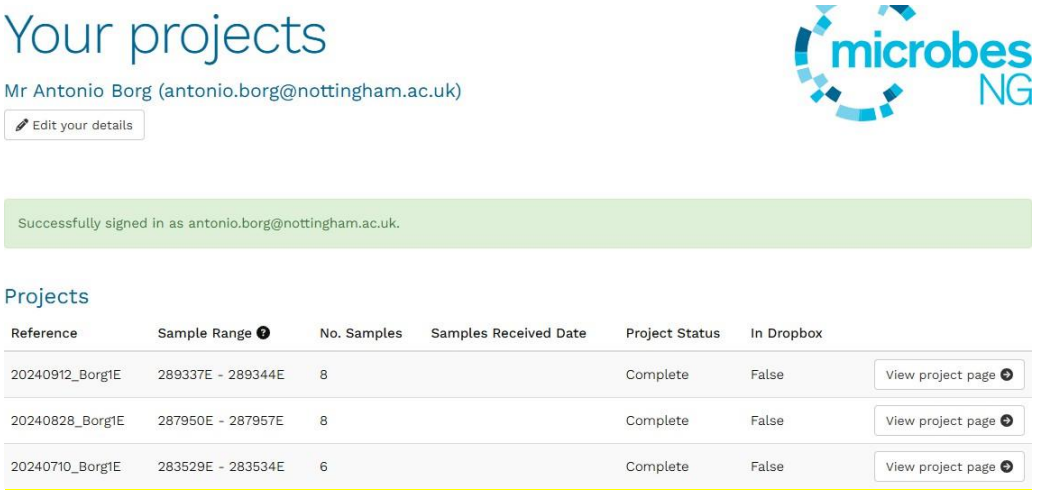


Figure 3.9 Project status the The MicrobesNG webpage.

3.8.3.2 Bioinformatic Analytical Online Tools

Sequences produced using both long- and short-read platforms, NEXTERA XT Illumina Technology and MicrobesNG, have been further analyzed using several bioinformatics programs detailed below. **Centre for Genomic Epidemiology (CGE**)

ResFinder v. 4.4.6

It is a web-based tool developed by the Centre for Genomic Epidemiology (CGE) DTU

to identify antimicrobial resistance genes from whole genome sequences.

CGE is an online platform that allows genome sequence data to be analysed through a central database. It is available at http://www.genomicepidemiology.org/. CGE utilizes rapid analysis algorithms and tools to extract data from Whole Genome Sequencing.

ResFinder is critical in understanding antimicrobial resistance profiles in clinical and environmental isolates.

Another service ResFinder, DTU offers is PlasmidFinder 2.1, a tool designed to detect plasmid replicon types in bacterial genome sequences. By detecting these replicons, one can find whether the plasmid elements of an isolate are essential or HGT, resistance gene determination, and plasmid epidemiology. VirulenceFinder 2.0, another tool available on ResFinder, identifies virulence-associated genes in bacterial genome sequences. VirulenceFinder supports risk assessment and contributes to the underlying of bacterial pathogens.

SerotypeFinder 2.0, available on ResFinder, is another specialized tool to predict the serotype of bacterial isolates based on whole genome sequence data. It focuses primarily on *E. coli*, identifying the somatic O and flagellar H antigens.

Multi-locus Sequence Typing 2.0 (MLST)

MLST was performed on the isolates for sequence type identification by the following methods. The MLST offered by the Centre for Genomic Epidemiology (CGE) is used to quickly analyze whole genome sequence FASTA files to identify the *E. coli* sequence type (ST). For *E. coli* two MLST services were available *Escherichia coli (Escherichia coli #1*(Wirth *et al.*,2006*) and Escherichia coli #2* (Jaureguy *et al.*, 2008)

Plasmid DataBase PLSDB v.2024_05_31_v2

Another online platform is available at https://ccb-microbe.cs.uni saarland.de/plsdb2025

Developed by the chair for Clinical Bioinformatics at Saarland University. It covers

thousands of complete plasmid sequences from NCBI.

The BLAST database is available at https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_446416380, comparing both nucleotide and protein sequences.

CARD – Comprehensive Antibiotic Resistance Database provides data on the molecular basis of antimicrobial resistance. It has a tool called RGI (Resistance Gene Identifier) to find potential resistance genes based on the CAD database. **Advantages of PLSDB over other genome tools like ResFinder**

PLSDB allows users to filter, browse, and explore the plasmid database using multiple metadata fields, such as host organism, plasmid size, isolation, geographical location, presence of specific genes, and visualization of the plasmid.

Accession numbers from the plasmid database can be run using BLAST to identify similarities with other nucleotide or protein sequences.

In contrast, ResFinder is designed to identify antimicrobial resistance genes in user-submitted sequences, offering results based on direct BLAST matches.

Chapter 4: Results

Introduction

This chapter highlights the findings from the detection and characterization of *E. coli* strains. The work described in this study aimed to investigate the presence and similarity of antimicrobial-resistant genes in flies and urinary tract infections (UTIs). It also examined the role of flies in spreading antimicrobial-resistant bacteria and their resistance genes on farms and in their surroundings.

4.1 Overview of Sample Collection and Analysis

A total of 177 fly traps and 90 fresh manure samples were collected across Malta and Gozo between 2018 and 2021. Simultaneously, 70 UTI-related ESBL-*E. coli* strains were obtained from clinical samples. Selective culture using TBX agar supplemented with cefotaxime, disc diffusion-based Antimicrobial Susceptibility Testing (AST), and PCR screening revealed that 42 % of fly samples carried ESBL-producing *E. coli*, with 47 % of these harbouring the $bla_{CTX\text{-}M}$ resistance gene. Among the UTI isolates, 79 % carried the same gene. WGS identified the $bla_{CTX\text{-}M\text{-}15}$ variant as dominant in both environmental and clinical isolates, often carried on IncF-type plasmids and mobilized by the insertion sequence *ISEcp1*. Crucially, both fly and UTI strains shared virulence genes associated with ExPEC and UPEC lineages, suggesting a strong potential for zoonotic transmission. Finally, a comparative analysis was conducted between fly and UTI strains to explore shared resistance mechanisms and virulent patterns.

4.1.1 Aim and Objectives Revisited

The primary aim of this study is to investigate the role of *Musca spp* as potential vectors for the transmission of ESBL-producing *E. coli* from environmental sources to humans with particular focus on strains harbouring the CTX-M resistance gene.

Chapter 4 presents results of both phenotypic and genotypic characteristics of ESBL-producing *E. coli* isolated from environmental fly samples collected from farms and open markets, manure samples and clinical UTI isolates. Sections 4.2-4.3 determine the presence and enumeration of ESBL-producing *E. coli* strain in bovine manure and fly samples isolated on TBX agar supplemented with cefotaxime. In section 4.4 *E. coli* strains from UTI clinical samples associated with ESBL – producing *E. coli* were isolated and prepared from comparative analysis. Reported in section 4.5-4.6 resistant profiles illustrated using disc diffusion data. Accessing the (AST) profile of both sources. As detailed in section 4.7 the presence of CTX-M genes using RT-PCR and to evaluate their distribution between flies collected from farms ,markets and clinical isolates. Addressed in section 4.8-4.10, the investigation of the genetic environment , mobile genetic elements, virulence genes through the WGS in selected isolates. Comparing plasmid types, resistance elements(*ISEcp1*) and shared virulence genes in both fly and UTI.

The following shows results starting with enumeration and isolation of *E. coli* from environmental and clinical sources, followed by phenotypic and genotypic comparisons, to evaluate the mechanical role of houseflies as potential vectors in the spread of ESBL-producing *E. coli* carrying the resistant gene CTX-M and virulence genes linked to human UTI's.

4.2 Enumeration and Detection of *Escherichia coli* in Bovine Manure and Fly samples

E. coli isolates collected from fresh bovine manure and flies were cultured and isolated on Tryptone Bile-X Glucuronide (TBX) agar. TBX agar is made selective for *E. coli* isolation based on the presence of β-Glucuronidase activity, which results in blue-green, typical colonies, and supplemented with cefotaxime (CTX) at a concentration of 1 μg/mL. Manure sample results were expressed as *cfu*/g (x10). Biochemical confirmation of presumptive ESBL-producing *E.coli* was further subjected to biochemical testing. Presumptive *E. coli* isolates were tested for indole production and oxidase activity.

Growth on the CTX supplement indicates that ESBL *E. coli* is resistant to third-generation cephalosporins.

4.2.1 Colony Count and Morphological Identification

Following the ISO standard ISO 16649-2, all samples were analyzed within 24 hours and incubated for 4 to 5 h at 37 °C, followed by 16 to 20 h at 44 °C to increase the selectivity for *E. coli*. In manure samples, ESBL-producing *E. coli* bacteria were quantified, whereas in flies, the presence of ESBL-producing *E. coli* was observed.

Manure samples were used to isolate *E. coli* and identify phenotypic resistance, but they were not further analyzed for the presence of resistance genes by Whole-Genome Sequencing (WGS), but only for the presence of the CTX-M gene by the RT-PCR. This decision was based on the primary aim of the study, which was not to investigate antimicrobial resistance present in animal farms directly or to assess potential antibiotic

misuse by local farmers.

Instead, manure was strategically chosen as a medium to attract flies, which served as the focal point for assessing the potential spread of antimicrobial resistance (AMR) via environmental areas. Therefore, while manure itself may harbour resistance, it may also serve as a reservoir for AMR transmission through flies. Making the direct analysis of resistance genes in manure less critical but somewhat datable.

Table 4.1 Detection and Enumeration of CTX – M Type ESBL-producing *E. coli* Isolated from Bovine Manure Samples collected across Malta and Gozo

Identification No:	***Source***	**Growth on TBX Agar**	***Growth on TBX+CTX***	*Indole*	*Oxidase*	***CTX-M***
Farm Zone G	manure	>300cfμ/ml	*30* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone G	manure	>300cfμ/ml	*10* cfμ/mL	+ pos	-neg	
Farm Zone G	manure	>300cfμ/ml	*NG*			
Farm Zone G	manure	>300cfμ/mL	*NG*			
Farm Zone G	manure	>300cfμ/mL	*NG*			
Farm Zone G	manure	>300cfμ/mL	*NG*			
Farm Zone G	manure	>300cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone A	manure	>300cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*NG*			
Farm Zone A	manure	>300cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*NG*			
Farm 97	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm 248a	manure	>300cfμ/mL	*50* cfμ/mL	+ pos	-neg	CTX-M
Farm 248b	manure	>300cfμ/mL	*50* cfμ/mL	+ pos	-neg	
Farm 230a	manure	200cfμ/mL	*NG*			
Farm 230b	manure	171cfμ/mL	*NG*			
Farm 208	manure	>300cfμ/mL	*NG*			
Farm 223	manure	>300cfμ/mL	*70* cfμ/mL	+ pos	-neg	
Farm 256	manure	>300cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm 167/8	manure	>300cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm 167/7	manure	>300cfμ/mL	*NG*			
Farm Zone	manure	>300cfμ/mL	*NG*			
Farm Zone A	manure	>300cfμ/mL	*NG*			
Farm Zone A	manure	>300cfμ/mL	*30* cfμ/mL	+ pos	-neg	

Table 4.1(continued): CTX-M Type ESBL – producing *E. coli* in Bovine Manure Samples

Farm Zone D	manure	>300cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300cfμ/mL	*NG*			
Farm Zone A	manure	>300cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone D	manure	>300cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm 244	manure	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm 138	manure	>300 cfμ/mL	*NG*			
Farm 45	manure	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm 163 a	manure	>300 cfμ/mL	*NG*			
Farm 163b	manure	>300 cfμ/mL	*NG*			
Farm 256	manure	>300 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*20* cfμ/mL			
Farm Zone G	manure	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone G	manure	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone G	manure	>300 cfμ/mL	*60* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone E	manure	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*60* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone E	manure	>300 cfμ/mL	*40* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300cfμ/mL	*50* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	210 cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	250 cfμ/mL	*20* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*110* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone E	manure	>300 cfμ/mL	*60* cfμ/mL	+ pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*90* cfμ/mL	+ pos	-neg	
Farm Zone G	manure	250 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*80* cfμ/mL	+ pos	-neg	CTX-M
Farm Zone G	manure	>300 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*NG*			
Farm Zone G	manure	>300 cfμ/mL	*NG*			
Farm Zone E	manure	250 cfμ/mL	*10* cfμ/mL	+pos	-neg	
Farm Zone E	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Gozo	manure	>300 cfμ/mL	*NG*			
Farm Gozo	manure	>300 cfμ/mL	*NG*			
Farm Gozo	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Zone C	manure	>300 cfμ/mL	*40* cfμ/mL	+ pos	-neg	
Farm Zone C	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	- neg	
Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone C	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	- neg	
Farm Zone C	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	- neg	

Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone C	manure	>300 cfμ/mL	*50* cfμ/mL	+ pos	-neg	

Table 4.1(continued): CTX-M Type ESBL – producing *E. coli* in Bovine Manure Samples

Farm Zone C	manure	>300 cfμ/mL	*50* cfμ/mL	+ pos	- neg	
Farm Zone C	manure	>300 cfμ/mL	*NRG*			
Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone C	manure	>300 cfμ/mL	*NG*			
Farm Zone A	manure	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*40* cfμ/mL [1]	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*NG*			
Farm Zone A	manure	>300 cfμ/mL	*10* [1]	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*NG*			
Farm Zone A	manure	>300 cfμ/mL	*NG*			
Farm Zone A	manure	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*90* cfμ/mL	+ pos	-neg	
Farm Zone A	manure	>300 cfμ/mL	*NG*			
Farm Zone B	manure	>300 cfμ/mL	*30* cfμ/mL [1]	+ pos	-neg	
Farm Zone B	manure	>300 cfμ/mL	*10* cfμ/mL [1]	+ pos	-neg	
Farm Zone B	manure	>300 cfμ/mL	*10* cfμ/mL [1]	+ pos	-neg	

Table 4.2 Detection and Enumeration of CTX-M Type ESBL – producing *E. coli* from Houseflies (*Musca* spp.) collected from Bovine Farms and Open Markets

Identification No:	***Source***	**Growth on TBX Agar**	***Growth on TBX+CTX***	*Indole*	*Oxidase*	***CTX-M***
Zone G/16	Flies	>300 cfμ/mL	50 cfμ/mL	+ pos	-neg	CTX-M
Zone G/13	Flies	>300 cfμ/mL	*50* cfμ/mL	+ pos	-neg	CTX-M
Zone G/11	Flies	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	CTX-M
Market G/9	Flies	>300 cfμ/mL	*120* cfμ/mL	+ pos	-neg	CTX-M
Market G/7	Flies	>300 cfμ/mL	*180* cfμ/mL	+ pos	-neg	CTX-M
Market G/25	Flies	>300 cfμ/mL	*120* cfμ/mL	+ pos	-neg	CTX-M
Market G/1505	Flies	210 cfμ/mL	*20* cfμ/mL	+ pos	-neg	CTX-M
Zone G/605	Flies	>300 cfμ/mL	*110* cfμ/mL	+ pos	-neg	CTX-M
Zone G/705	Flies	>300 cfμ/mL	*160* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1805	Flies	>300 cfμ/mL	*80* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1905	Flies	>300 cfμ/mL	*130*cfμ/mL	+ pos	-neg	CTX-M
Zone G/1705	Flies	>300 cfμ/mL	*60* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1405	Flies	>300 cfμ/mL	*70* cfμ/mL	+ pos	-neg	CTX-M
Zone G/505	Flies	>300 cfμ/mL	*100* cfμ/mL	+ pos	-neg	CTX-M

Market G/28	Flies	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	CTX-M
Market G/24	Flies	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	CTX-M
Market G/21	Flies	>300 cfμ/mL	*90* cfμ/mL	+ pos	-neg	CTX-M

Table 4.2 (continued) CTX-M Type ESBL -producing *E. coli* in Bovine Farms and Open Markets

Market G/18	Flies	>300 cfμ/mL	*70* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1205	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Zone G/105	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1305	Flies	>300 cfμ/mL	*40* cfμ/mL	+ pos	-neg	CTX-M
Zone G/1105	Flies	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	CTX-M
Zone G/305	Flies	>300 cfμ/mL	*10* cfμ/mL	+ pos	-neg	CTX-M
Zone G/905	Flies	>300cfμ/mL	*10* cfμ/mL	+ pos	-neg	CTX-M
Market G/6	Flies	>300 cfμ/mL	*90* cfμ/mL	+ pos	-neg	CTX-M
Market G/3	Flies	>300 cfμ/mL	*40* cfμ/mL	+ pos	-neg	CTX-M
Market G/1605	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Market G/ 2505	Flies	>300 cfμ/mL	*180* cfμ/mL	+ pos	-neg	CTX-M
Market G/805	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Market G/14	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Market G/2705	Flies	>300 cfμ/mL	*90* cfμ/mL	+ pos	-neg	CTX-M
Zone G/12	Flies	>300 cfμ/mL	*40* cfμ/mL	+ pos	-neg	CTX-M
Zone G/48	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Zone G/331	Flies	>300 cfμ/mL	*30* cfμ/mL	+ pos	-neg	CTX-M
Market G/1005	Flies	>300 cfμ/mL	*20* cfμ/mL	+ pos	-neg	CTX-M

4.3 Urinary Tract Infections

Urinary Tract Infections (UTIs) caused by ESBL *E. coli* strains were not cultured on TBX agar for detection; however, they were first pre-confirmed as ESBL-producing *E. coli* by hospital staff (Table 4.3). These isolates were included in this study for comparison purposes in later phenotypic and genotypic analysis.

Table 4.3 CTX-M ESBL-producing *E. coli* strains sub-cultured from UTI patients samples collected during 2019-2020

Lab No UTI Non Hospital ID No	Sample Box No:	Hospital ID No	Month/ Year of Collection	Month/Year of Subculturing on LB agar	Antimicrobial Susceptibility Testing (AST)	CTX-M RT-PCR	Project No for WGS by MicrobesNG.UK
UTI 62/302	BOX 216	492290 / A2	August 2019	Sept 2019	Oct 2023	Detected	20240710_Borg1E
UTI 63	BOX 216	496554/D7	August 2019	Sept 2019	Oct 2023	Detected	20240912_Borg1E
UTI 64	BOX 216	498547 / F2	August 2019	Sept 2019	Oct 2023	Detected	
UTI 65 / 335	BOX 216	49328 / A9	August 2019	Feb 2020	Oct 2023	Detected	
UTI 67	BOX 231	162591 / A7	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 68	BOX 231	166812 / D7	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 69 / 2205	BOX 231	166394 / D1	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 70 / 2005	BOX 216	499868 / G5	Aug 2019	Sep 2019	Oct 2023	Detected	20240912_Borg1E
UTI 71	BOX 216	498972	Aug 2019	Sep 2019	Oct 2023	Detected	20240912_Borg1E
UTI 76	BOX 230	157400/ E3	Aug 2020	Sep 2019	Oct 2023	Detected	
UTI 77	BOX 231	164225 / B3	Sept 2020	Feb 2021	Oct 2023	Detected	20240912_Borg1E
UTI 79	BOX 231	162591 / A7	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 80	BOX 230	154576 / A1	Sept 2020	Oct 2020	Oct 2023	Detected	20240912_Borg1E
UTI 81	BOX 230	158867 / F3	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 84	BOX 216/21	2644 / I5	Aug 2019	Sep 2019	Oct 2023	Detected	
UTI 86	BOX213/214/215	?I6	Aug 2019	Sep 2019	Oct 2023	Detected	
UTI 87 / 2105	BOX 230	156834/D2	Aug 2020	Oct 2020	Oct 2023	Detected	
UTI 88	BOX213/214/215	?I8	Aug 2019	Sep 2019	Oct 2023	Detected	
UTI 91	BOX 231	16718 / E4	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 92	BOX 231	162702 / A3	Sept 2020	Oct 2020	Oct 2023	Detected	20240912_Borg1E
UTI 93	BOX213/214/215	2417 / I1	Aug 2019	Sep 2019	Oct 2023	Detected	
UTI 94	BOX213/214/215	?I5	Aug 2019	Sep 2019	Oct 2023	Detected	
UTI 95	BOX213/214/215	?I8	Aug 2019	Sept 2019	Oct 2023	Detected	

Lab No UTI Non Hospital ID No	Sample Box No:	Hospital ID No	Month/ Year of Collection	Month/Year of Subculturing on LB agar	Antimicrobial Susceptibility Testing (AST)	CTX-M Detection	Project No for WGS by MicrobesNG.UK
UTI 96	BOX213/214/215	?I2	Aug 2019	Sept 2019	Oct 2019	Detected	
UTI 97	BOX213/24/215	?I3	Aug 2019	Sept 2019	Oct 2019	Detected	20240912_Borg1E
UTI 303	BOX 230	161918/H9	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 305	BOX 230	157129/D6	Sep 2020	Oct 2020	Oct 2023	Detected	
UTI 306	BOX 230	157400/D8	Aug 2020	Sept 2020	Oct 2023	Detected	
UTI 307	BOX 230	162465 / J5	Aug 2020	Sept 2020	Oct 2023	Detected	
UTI 308	BOX 230	156807 / D5	Aug 2020	Sept 2020	Oct 2023	Detected	20240912_Borg1E
UTI 309	BOX 230	162035 / H8	Aug 2020	Sept 2020	Oct 2023	Detected	
UTI 310	BOX 230	16253 / J7	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 311	BOX 230	155453 / C2	Aug 2020	Oct 2020	Oct 2023	Detected	
UTI 313	BOX 231	166394 / D4	Sep 2020	April 2021	Oct 2023	Detected	
UTI 314	BOX 231	916646	Oct 2020	April 2021	Oct 2023	Detected	
UTI 316	BOX 230	528577 / D3	Aug 2020	Oct 2020	Oct 2023	Detected	
UTI 317	BOX 231	164230 / B6	Sept 2020	April 2021	Oct 2023	Detected	
UTI 320	BOX 230	155651 / C1	Aug 2020	Sept 2020	Oct 2023	Detected	
UTI 321	BOX 230	159691/F8	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 323	BOX 230	158060 / E7	Sept 2020	Oct 2020	Oct 2023	Detected	
UTI 325	BOX 231	162461 / B1	Sept 2020	April 2021	Oct 2023	Detected	
UTI 332	BOX 231	165880 / C6	Sept 2020	April 2021	Oct 2023	Detected	
UTI 333	BOX 231	915600 /C1	Sept 2020	April 2021	Oct 2023	Detected	
UTI 337	BOX 231	162579 /A1	Sept 2020	Apil 2021	Oct 2023	Detected	

Upon receipt, the urine samples were first sorted by patient age, sex, and clinical details. The initial screening tool, Urised, is an automated urine microscopy analyzer used to

evaluate the presence of formed foreign particles in the urine specimen, including red blood cells, white blood cells, or crystals. Flagging those with an infection, the samples were subcultured on CLED agar and incubated overnight for 18 to 24 hours. Bacterial growth, if present, was identified using MALDI-TOF MS, and antibiotic-resistant genes were detected using Vitek 2. Finally, confirmation of the CTX-M resistant gene was achieved using a rapid technique, the lateral flow assay (Figure 4.1).

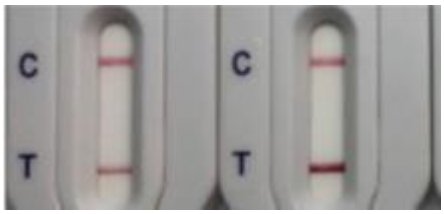


Figure 4.1 Rapid Identification of CTX-M-15 *In* vitro rapid diagnostic test for the detection of CTX-M-14 and CTX-M-15 β-lactamases in bacterial culture *CORIS Bioconcept*

4.3.1 Antimicrobial Susceptibility Testing (AST)

CTX-M enzymes have become the most prevalent ESBLs worldwide and are responsible for a significant proportion of cephalosporin resistance in *E. coli*. The $bla_{CTX\text{-}M}$ β-lactamase confers resistance to third-generation cephalosporins, such as cefotaxime and ceftazidime, as well as other beta-lactam antibiotics, including penicillin and monobactams like aztreonam (ATM). MDR *E. coli* producing ESBL, such as CTX-M, are significant causes of cephalosporin- resistant infections, often resulting in therapeutic failure and serious clinical outcomes.

Cefoxitin (FOX) is a second-generation cephalosporin, CTX is a third–generation cephalosporin, cefquinome (CFQ) is a fourth-generation cephalosporin; amoxicillin-clavulanic acid (AMC) is a beta-lactamase inhibitor combination, ampicillin (AMP) is penicillin and ATM is a monobactam.

Most strains produced the resistant gene *bla* $_{CTX-M}$, a type of ESBL resistance gene, exhibiting resistance to a broad spectrum of beta-lactam antibiotics, including penicillin, third- and fourth-generation cephalosporins, and monobactams, by inactivating the beta-lactam ring (Paterson *et al.*, 2015). Strains that exhibit resistance to β-lactamase inhibitors, such as clavulanic acid, can prevent the inhibition of β-lactamase enzymes, thereby allowing continuous inactivation of β- lactam antibiotics (Munita *et al.*, 2016). When strains are susceptible to AMC and FOX, they are also susceptible to beta-lactamase inhibitors, such as clavulanic acid. The combination of beta-lactam antibiotics with beta-lactamase inhibitors can overcome resistance mediated by CTX-M ESBLs. Clavulanic acid acts synergistically with penicillin and cephalosporins against β–lactamase–producing *E. coli.* Resistance to ampicillin (AMP) typically involves the production of beta-lactamase enzymes that hydrolyze the β-lactam ring, rendering penicillin ineffective (Munita *et al.*, 2016).

Second-generation cephalosporins, although also beta-lactam antibiotics, have a narrower spectrum of activity compared to third-generation antibiotics. All CTX-M strains are resistant to third-generation cephalosporins; however, some may remain susceptible to second- generation cephalosporins, such as FOX, due to differences in their mechanisms of action. These second-generation antibiotics exhibit increased stability against hydrolysis by CTX-M ESBLs. Some strains resistant to third-generation cephalosporins, such as CTX-M, may still be susceptible to 4th generation cephalosporins like CFQ (Dhriti *et al.*, 2022).

4.3.2 Antibiotic Resistance of UTI isolates

UTI ESBL-producing *E. coli* isolates showed the highest antibiotic resistance against ampicillin, cefotaxime, cefpodoxime, ceftazidime, and nitrofurantoin, followed by

cefquinome, aztreonam, and trimethoprim-sulfamethoxazole (TMP-SMX).

The evaluation of the phenotypic resistance profile for the 16 antibiotics and Cefquinome (CFQ) revealed that several UTI *E. coli* isolates were MDR exhibiting a high resistance profile. Nine UTI-associated *E. coli* isolates were grouped based on their antimicrobial susceptibility testing (AST) data, which revealed multiple resistance patterns and taken forward for the Whole Genome Sequencing (WGS). These included suspected $bla_{CTX\text{-}M}$ carriers and strains with reduced susceptibility to ampicillin (AMP), cefotaxime (CTX), aztreonam (ATM), and amoxicillin-clavulanic acid (AMC), as determined by phenotypic resistance evaluation. Out of the nine UTI ESBL-producing *E. coli* isolates with $bla_{CTX\text{-}M}$ resistance genes studied, $bla_{CTX\text{-}M}$, bla_{TEM}, $bla_{OXA\text{-}1}$, and $bla_{CMY\text{-}2}$ were detected in most of the UTI *E. coli* isolates, with $bla_{CTX\text{-}M\text{-}15}$ being the most prevalent CTX-M subtype (66.7%). Only one *E. coli* isolate harboured a Class C AmpC gene $bla_{CMY\text{-}2}$. The CTX-M genes encode enzymes that break down β-lactam antibiotics, including aztreonam, cefotaxime, ceftazidime, and cefquinome, rendering them ineffective. Out of the nine $bla_{CTX\text{-}M}$ resistance genes studied, $bla_{CTX\text{-}M\text{-}15}$ was the predominant ESBL $bla_{CTX\text{-}M}$ gene type in 66.7% of the isolates. Several other studies conducted throughout the world show variable results. In China ESBL bla_{TEM} gene was found as the most predominant ESBL gene, followed by SHV. A study conducted in Canada found that the most prevalent ESBL bla gene was SHV. However, other research studies conducted in various parts of the world, including South America, parts of India, Europe, Spain, and the UK, have revealed the CTX- M gene as the most predominant (Saisi et al., 2019). Six isolates were found to carry $bla_{CTX\text{-}M\text{-}15}$ in association with $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}55}$ and $bla_{CTX\text{-}M\text{-}65}$. Only one isolate was found to carry the CTX-M type, $bla_{CTX\text{-}M\text{-}14}$, in association with $bla_{TEM\text{-}1}$. Only one isolate carried bla_{CMY2}, and another isolate was found to carry only $bla_{CTX\text{-}M\text{-}15}$. Although classified as fluoroquinolones, ciprofloxacin is not a

beta-lactam; however, resistance is sometimes associated with the presence of β-lactamase genes, indicating co-resistance. Carrying resistant mobile genetic elements, such as *intI1* Class 1 integron, is more likely to confer resistance to TMP/SMX, a commonly used treatment for UTIs.

Table 4.4 Antimicrobial Susceptibility Testing (AST) Patterns of CTX-M ESBL – producing *E. coli* isolated from UTI patients

Antibiotic Class	1 Penicillins	2 B-Lactam/ase Inhibi-	3a Second Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3 Fourth Gen Ceph	4 Monobactam	5 Carbapenem	6 Amino-glycosides	7 Tetracyclines	8 Fluoro-quinolones	8 Fluoro-quinolones	9 Quinolones	10 Folate Pathway Inhibi-	10 Folate Pathway Inhibi-	11 Phenicols	12 Nitrofurans	13 Polymyxin	Azithromycin (AZM)
Antibiotic Abb	**AMP**	**AMC**	**FOX**	**CAZ**	**CTX**	**EFT**	**CPD**	**CFQ**	**ATM**	**IPM**	**S10**	**OT**	**CIP**	**ENR**	**NA**	**S3**	**SXT**	**C**	**F**	**CT**	**AZM**
Disc Conc (ug)	**10**	**20 & 10**	**30**	**30**	**30**	**30**	**10**	**30**	**30**	**10**	**10**	**30**	**5**	**5**	**30**	**300**	**1.25 & 23.75**	**30**	**300**	**10**	
Zone Size	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+++	+++	++	++	+	++	+++	+	
CTX – M U62/30 2	0	0	0	0	0		0	20	10	31	15	0	0		0		0	33	21		15
CTX – M U63	0	0	21	0	0		0	0	0	33	0	26	0		0		25	31	20		24
CTX – M U64	0	13	23	0	0		0	0	0	34	9	20	0		0		14	26	22		22

Table 4.4 (continued):AST Patterns of CTX-M ESBL-producing *E. coli* isolated from UTI patients

CTX – M U65/335	0	11	24	12	0		0	0	11	29	0	7	22		19		0	26	20	12
CTX – M U67	0	0	23	9	0		0	7	0	33	13	0	0		0		0	29	21	0
CTX – M U68	0	13	20	9	0		0	0	0	33	12	21	0		0		25	25	21	17
CTX – M U69/2205	0	14	25	15	0		0	11	14	35	15	0	0		0		0	31	25	15
CTX – M U70/2005	0	19	0	25	10		0	15	15	21	0	0	19		0		0	0	21	16
CTX – M U71	0	18	22	11	0		0	8	7	31	0	0	21		19		0	26	22	0
CTX – M ND U72	0	17	25	12	0		0	8	10	34	0	0	0		21		0	0	24	19
CTX – M U76	0	10	23	22	13		0	9	21	30	0	0	10		0		0	0	19	15
CTX – M U77	0	16	23	15	0		0	7	14	34	0	9	0		0		0	27	20	19
CTX – M U78	0	17	20	0	17		0	18	22	34	14	0	0		0		0	30	22	13
CTX – M U79	0	12	24	0	0		0	7	10	35	15	0	0		0		0	33	24	15
CTX – M U80	0	0	11	11	0		0	7	0	36	12	24	0		0		0	30	24	11

Table 4.4 (continued):AST Patterns of CTX-M ESBL-producing *E. coli* isolated from UTI patients

CTX – M U81	0	10	20	24	0		0	11	20	35	16	0	0		0		0	29	22	13
U-B5 1/10	0	10	20	11	0		0	0	12	30	11	0	0		0		0	0	23	13
CTX – M U84	0	12	24	10	20		7	23	10	36	10	0	25		17		0	0	23	16
CTX – M U86	0	15	25	17	0		0	11	14	36	13	0	0		0		0	0	24	17
CTX – M U87/2 105	0	10	25	12	0		0	7	10	15	0	36	0		0		0	31	21	0
CTX – M U88	0	0	25	21	11		0	18	17	34	0	7	13		0		0	0	20	15
CTX – M ND U90	0	10	23	11	0		0	7	12	30	16	8	0		0		26	29	22	17
CTX – M |U91	0	0	21	15	7		0	13	16	28	8	0	20		16		0	0	21	14
CTX – M U92	0	18	31	11	0		0	0	0	36	15	26	0		0		0	32	30	19
CTX – M U93	0	0	24	12	0		0	7	12	32	0	0	28		19		0	29	19	0
CTX – M U94	0	14	20	0	0		0	0	0	32	14	27	0		0		28	31	24	25

Table 4.4 (continued):AST Patterns of CTX-M ESBL-producing *E. coli* isolated from UTI patients

CTX – M U95	0	12	21	12	0		0	9	14	30	0	7	23		19		0	27	19	15
CTX – M U96	0	13	14	11	0		0	7	0	23	0	0	0		14		0	0	16	0
CTX – M U97	0	0	26	11	0		0	0	11	35	16	8	0		0		0	31	23	25
CTX – M U311	0	0	23	15	8		0	10	16	32	11	0	21		17		0	7	21	0
CTX- M U308	0	0	15	17	0		0	14	19	36	0	0	0		0		0	27	21	7
CTX – M U309	0	0	23	17	0		0	10	17	35	0	27	0		0		27	36	23	22
CTX – M U306	0	0	10	0	0		0	0	0	35	15	22	0		0		25	30	20	15
CTX – M U319	0	17	20	22	16		0	19	23	31	13	0	0		0		0	26	21	0
CTX – M U310	0	11	14	0	0		0	0	7	33	9	0	0		0		0	0	17	14
CTX – M U317	0	0	21	0	0		0	13	18	33	13	25	9		0		0	0	23	0
CTX – M U307	0	7	22	10	0		7	0	11	31	16	8	0		0		26	27	23	16

Table 4.4 (continued):AST Patterns of CTX-M ESBL-producing *E. coli* isolated from UTI patients

U321 WGS	0	15	20	12	0		0		0	34	11	0	0		0		23	0	22		18
U316- D3 1/10	0	0	7	0	0		0	8	11	33	14	26	0		0		0	0	22		13
U332	0	12	20	0	0		0	0	0	36	11	0	0		0		0	30	22		10
CTX – M U323	0	9	16	7	0		0	0	0	30	7	0	0		0		0	26	18		12
U334	0	0	0	20	0		0	0	7	29	0	0	0		0		0	20	24		10
CTX – M U303	0	0	22	16	11		0	10	18	34	15	25	0		0		24	32	24		11
U305 - D6 1/10	0	0	21	7	0		0	0	0	33	15	0	0		0		28	10	20		0
U333	0	10	20	11	0		0	0	13	32	15	0	0		0		25	29	22		15
U306?	0	14	19	10	0		0		0	34	14	24	0		0		25	25			16
CTX – M U320	0	0	20	0	0		0	18	20	35	0	0	21		0		0	30	20		7
U325	0	19	0	24	15		0	18	21	33	0	0	23		22		0	27	22		17

Table 4.4 (continued):AST Patterns of CTX-M ESBL-producing *E. coli* isolated from UTI patients

CTX – M U301	0	0	19	23	0		10	17	0	32	11	0	7		16		0	0	19		15
U337	0	20	25	14	7		0	0	10	35	0	0	12		24		0	29	24		16
CTX – M U314	0	11	24	12	0		7	9	13	30	16	9	0		0		27	30	22		17
CTX – M U302/ 62	0	0	0	0	0		0	20	7	32	15	0	0		0		0	32	23		19
CTX – M U313	0	0	14	12	0		0	7	12	32	11	0	0		0		25	22	22		16
U336	0	11	20	12	0		0	9	13	30	16	0	0		0		24	26	21		13
U-E1 1/10	0	7	15	0	0		0	0	8	35	0	24	0		0		0	24	17		14
U- 319 - G7 1/10	0	8	20	28	18		0	20	25	37	16	0	0		0		0	29	24		0
U-D1 1/10		7	0	11	0	0		0	0	7	36	12	0	0		0		0	0	25	

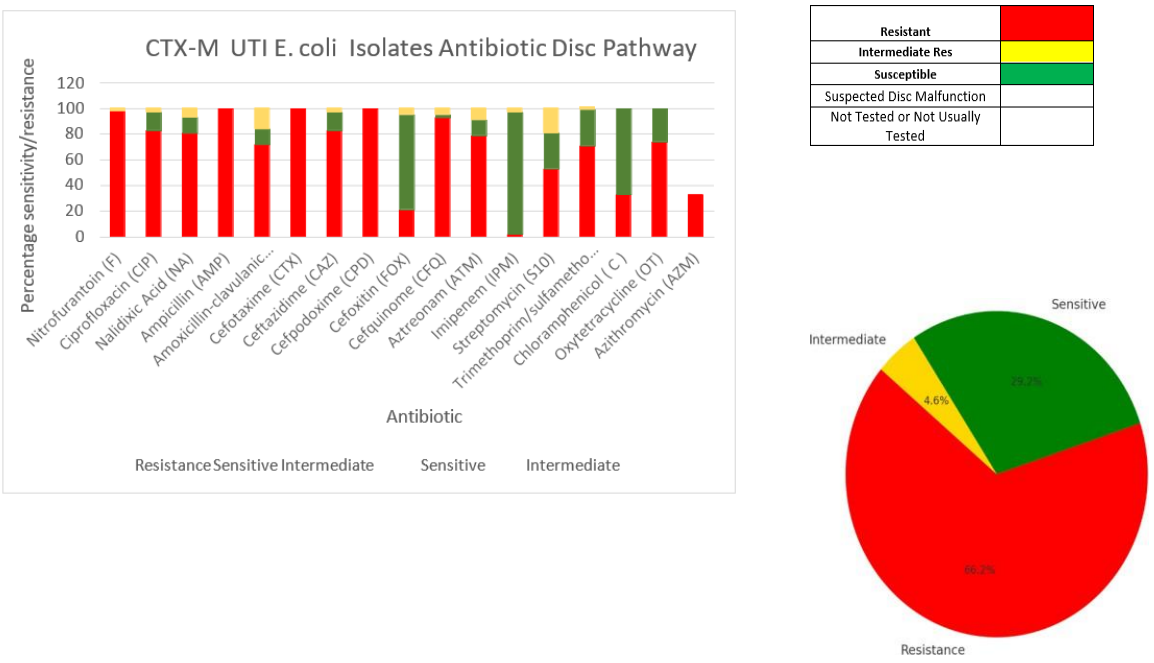


Figure 4.2 Percentages of Resistance, Susceptibility, and Intermediate as observed from the AST pattern for 44 ESBL UTI *E. coli*

Table 4.5 Selected *CTX-M* ESBL-producing *E. coli* strains from UTI patients isolates subjected to Whole Genome Sequencing (WGS)

AST readings	AMP	AMC	FOX	CTX	CFQ	ATM	*bla*$_{CTX-M}$	Sample No: UTI's	For WGS
Interpretation A	R	R	S	R	(S) or (R)	R	Detected	63;64;65;67;68;76; 79;81;84;87;88;91; 93;95;97;303;305; 307;309;311;314; 317;320;332;333	**63;97**
Interpretation B	R	S	S	R	R	R	Detected	71;92;337	**71;92**
Interpretation C	R	R	R	R	R	R	Detected	62;80;96;306;310; 313;316	**62;80**
Interpretation D	R	S	R	R	R	R	Detected	70; 325	**70**
Interpretation E	R	R	I	R	R	R	Detected	308;323	**308**
Interpretation F	R	I	S	R	R	R	Detected	69;77;86;94;321	**77**

4.4 Antibiotic Resistance of Fly isolates

In the present study,75 Fly ESBL-producing *E. coli* isolates obtained houseflies (*Musca domestica*) were studied both phenotypically and genotypically. Antibiotic resistance sensitivity testing (AST) was carried out for 16 antibiotics + CFQ by the disc diffusion method following CLSI guidelines. Whole Genome Sequencing (WGS) hybrid assembly was used to identify isolates harbouring plasmid-mediated resistance genes.

Among the selected 75 housefly ESBL-producing *E. coli*, 35 (81%) were confirmed genotypically to carry the $bla_{CTX\text{-}M}$ resistance gene. These 35 housefly ESBL-producing *E. coli* isolates were subjected to antimicrobial susceptibility testing (AST). These 35 *CTX-M*- identified isolates were grouped according to their AST profile and subsequently selected for sequencing based on the interpretation of their AST profile, as shown in the Table 4.6.

Table 4.6 Antimicrobial Susceptibility Testing (AST) Patterns of CTX-M ESBL – producing *E. coli* isolated from Houseflies (*Musca spp.*)

Antibiotic Class	1 Penicillins	2 B-Lactam/ase In-	3a Second Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3b Third Gen Ceph	3c Fourth Gen Ceph	4 Monobactam	5 Carbapenem	6 Amino-glycosides	7 Tetracyclines	8 Fluoro-quin-	8 Fluoro-quin-	9 Quinolones	10 Folate Pathway	10 Folate Pathway	11 Phenicols	12 Nitrofurans	13 Polymyxin	Azithromycin
Antibiotic Abb.	**AMP**	**AMC**	**FOX**	**CAZ**	**CTX**	**EFT**	**CPD**	**CFQ**	**ATM**	**IPM**	**S10**	**OT**	**CIP**	**ENR**	**NA**	**S3**	**SXT**	**C**	**F**	**CT**	**AZM**
Disc Conc (ug)	**10**	**20 & 10**	**30**	**30**	**30**	**30**	**10**	**30**	**30**	**10**	**10**	**30**	**5**	**5**	**30**	**300**	**1.25 &**	**30**	**300**	**10**	

Table 4.6 (continued): AST Patterns of CTX-M ESBL-producing fly-derived *E. coli* isolates

Zone Size / Isolate	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	+	+	‡	‡	‡	‡	+	‡	‡	+	
Farm CTX-M Fly 2505	0	13	23	0	22		11	24	0	36	0	0	23		22		0	0	24		22
Mar-ket CTX-M Fly 1605	0	0	12	14	0		0	8	12	29	10	0	0		0		0	28	11		17
Farm CTX-M Fly 705	0	0	7	11	0		0	0	7	34	0	0	0		0		0	0	21		13
Farm CTX-M Fly 605	0	0	16	15	0		0	13	15	35	12	0	22		0		0	0	22		15
Mar-ketCTX-M Fly 805	0	0	0	0	0		0	0	0	25	0	0	0		8		0	13	19		15
Farm CTX-M Fly 905	0	18	23	15	0		0	11	13	35	0	7	0		0		0	11	20		20
Farm CTX-M Fly 305	0	0	19	0	10		0	25	0	33	0	0	22		0		0	0	19		16
Farm CTX-M Fly 1105	0	17	25	22			0	16	20	34	0	0	22		20		0	0	23		16
Farm CTX-M Fly 1305	0	17	24	14	0		0	13	11	35	0	0	19		14		0	0	22		0
Farm CTX-M Fly 105	0	18	20	12	12		0	11	11	35	0	0	22		21		0	0	25		19

Table 4.6 (continued): AST Patterns of CTX-M ESBL-producing fly-derived *E. coli* isolates

Farm CTX-M Fly 1205	0	0	9	18	0		0	11	15	32	10	0	25		0		0	0	21		18
Mar- ket CTX -M Fly 1005	0	0	9	9	0		0	0	0	32	0	0	0		0		0	0	20		0
Farm CTX-M Fly 505	0	0	14	11	0		0	14	12	33	0	0	20		0		0	0	21		15
Farm CTX-M Fly 1405	0	19	25	15	11		0	14	14	33	0	0	20		20		0	0	22		17
Mar- ket CTX -M Fly 1505	0	0	18	10	10		0	23	0	32	0	0	21		18		0	0	0		17
Farm CTX-M Fly 1705	0	19	24	12	20		0	23	11	20	0	0	15		0		0	0	24		19

Table 4.6 (continued): AST Patterns of CTX-M ESBL-producing fly-derived *E. coli* isolates

Farm CTX-M Fly 1905	0	17	24	20	15		0	14	15	33	0	0	0		19		0	0	23		0
Farm CTX-M Fly 1805	0	20	25	16	7		0	8	11	34	0	0	25		22		0	28	24		21
Mar-ket CTX-M Fly 2705	0	0	0	12	22		0	24	0	29	0	0	22		23		0	0	21		12
Farm CTX-M Fly 3	0	12	19	0	10		0	0	15	34	8	0	25		19		0	30	0		0
Mar-ket CTX-M Fly 4	0	0	0	0	0		0	11	16	34	0	0	0		0		0	29	22		18
Mar-ket CTX-M Fly 6	0	19	20	21	12		0	12	17	20	0	0	11		22		0	27	22		17
Marke 5at CTX-M Fly 7	0	0	19	0	0		0	7	0	34	11	0	22		15		0	30	20		16
Mar-ket CTX-M Fly 9	0	23	26	23	15		0	11	20	37	0	0	23		23		0	27	26		16

Table 4.6 (continued): AST Patterns of CTX-M ESBL-producing fly-derived *E. coli* isolates

Farm CTX-M Fly 11	0	11	23	7	0		0	0	7	30	16	0	0		0		0	26	21		7
Farm CTX-M Fly 12	0	14	23	0	0		0	0	0	33	10	0	25		19		0	29	22		17
Farm CTX-M Fly 13	0	13	20	20	10		0	11	16	33	9	0	32		20		0	28	22		0
Farm CTX-M Fly 16	0	9	24	0	13		0	17	19	35	0	0	25		20		0	15	24		18
Mar-ket CTX-M Fly 18	0	21	25	21	13		0	16	19	35	7	0	20		19		0	30	0		0
Mar-ket CTX-M Fly 21	0	18	14	18	9		0	12	16	35	0	0	26		21		0	26	23		19
Mar-ket CTX-M Fly 24	0	0	25	0	0		0	7	0	32	0	0	25		0		27	0	21		18
Mar-ket CTX-M Fly 25	0	0	17	13	0		0	10	12	32	12	0	18		0		0	0	20		14

Table 4.6 (continued): AST Patterns of CTX-M ESBL-producing fly-derived *E. coli* isolates

Fam CTX-M Fly 331 (244)	0	0	20	10	7		0	24	12	34	0	0	20		16		0	0	20		21
Farm CTX-M Fly 48	0	11	22	21	0		0	19	15	30	0	0	20		15		0	0	20		0
Mar- ket CTX-M Fly 28	0	13	15	0	0		0	14	0	20	0	0	0		0		0	0	19		0

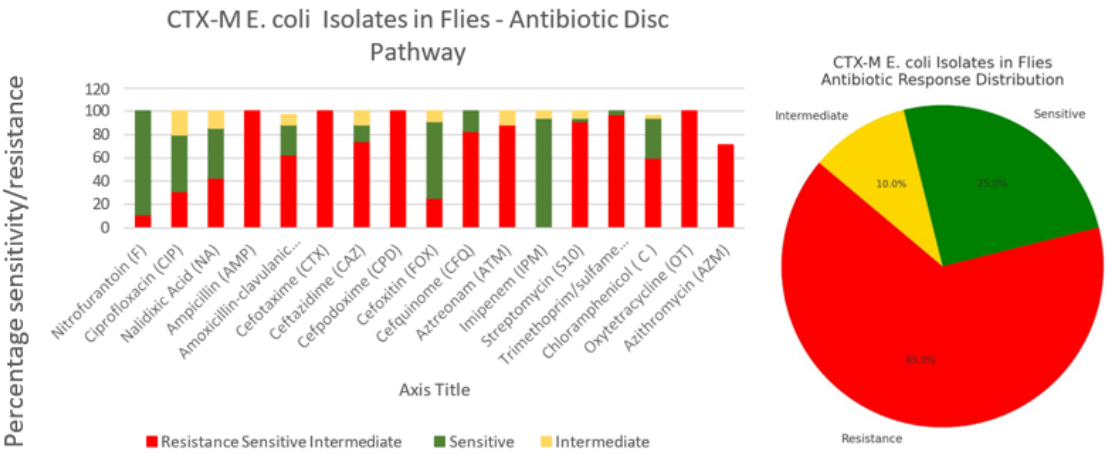


Figure 4.3 Percentages of Resistance, Susceptibility, and Intermediate as observed from the AST pattern for 35 ESBL Housefly *E. coli*

Table 4.7 Selected *CTX-M* ESBL-producing *E. coli* strains from Houseflies isolates subjected to Whole Genome Sequencing (WGS)

AST readings	AM P	AM C	FO X	CT X	CF Q	AT M	blaCTX-M	Sample No:	Date of sampling	Site	WGS
Interpretation A	R	R	S	R	(S) or (R)	R		**F2705**		M*	**F2705 ***
Interpretation B	R	S	S	R	R	R	Present	F105; F905; F1405; F1705; F1805		**Fr*** **Fr*** **Fr*** **Fr*** **Fr***	**F905** **F105** **F1405** **F1705** **F1805**
Interpretation C	R	R	R	R	R	R	Present	**MDH4** : F505: F705; **F805;** **F1005;** **F1605**;		**M*** **Fr*** **Fr*** **M*** **M*** **M***	**4** **F805** **F1005** **F1605**
Interpretation D	R	S	R	R	R	R	Present	N/A	N/A	N/ A	N/A
Interpretation E	R	R	I	R	R	R	Present	MDH: 25;28 F605		**M*** **Fr***	**F605**
Interpretation F	R	I	S	R	R	R	Present	12; F1305**;** **F1905**		**Fr*** **Fr*** **Fr***	**F1905**

*Legend: M – Market, Fr – Farm

4.5 CTX-M Detection by Real-Time PCR

A total of twenty-eight *E. coli* strains from bovine manure, seventy-five *E. coli* strains from houseflies, and fifty-four UTI isolates were analyzed using the Rotor-Gene Q real-time PCR system (QIAGEN, Hilden, Germany) for the detection of *CTX-M* genes. Detection was carried out using the bacterial drug resistance RT-PCR kit (Sacace

Biotechnologies, Italy), which targets genetic elements responsible for resistance to various classes of antibiotics.

Among the twenty-eight bovine manure *E. coli* strains, nine were detected to harbour the resistant gene *CTX-M*. Thirty-five houseflies were detected to carry the *CTX*-M gene, and forty- four UTIs carried the *CTX-M* resistant gene. (See Appendix B for the full dataset and analysis)

4.5.1 Interpretation of the Ct value by the ROTOR-Gene Q

Real-Time PCR Ct (Cycle Threshold): A real-time PCR assay detects a positive reaction by the accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to exceed the threshold, thereby surpassing the background level. The cycle threshold (Ct) is a semi-quantitative value that broadly categorizes the concentration of bacterial genetic material in a patient or environmental sample following testing by RT-PCR as low, medium, or high – for example, it indicates approximately how much DNA genetic material is present in the sample.

Ct levels are inversely proportional to the amount of target nucleic acid in the sample, that is the lower the Ct level, the greater the amount of target nucleic acid in the sample.

A low Ct indicates a high concentration of bacterial genetic material, typically associated with a high risk of infectivity. A high Ct indicates a low concentration of genetic material, which is typically associated with a lower risk of infectivity.

QIAGEN. Rotor-Gene® Q — Pure Detection System. QIAGEN GmbH, Hilden, Germany. https://www.qiagen.com

4.6 Genetic Environment of ESBL Genotype in *E. coli* and its Virulence Genes Detected in samples

4.6.1 Genetic Environment of ESBL Genotype in *E. coli* from UTI human cases

The results presented in Table 4.8 illustrate the widespread distribution of ESBL genes among clinical isolates collected from the Pathology Department at Mater Dei Hospital, Malta. It represents a detailed genomic profile of various ESBL-producing *E. coli* from UTI cases in humans, including isolates from urinary samples collected from both hospitalized and outpatient patients.

From the nine UTI strains, the presence of mobile genetic elements and diverse plasmids underscores the risk of Horizontal Gene Transfer (HGT) and the persistence of multidrug-resistant organisms.

It offers a comprehensive view of the ESBL genotypes identified. Their associated resistance genes and mobile genetic elements facilitate the spread of gene mobility and resistance.

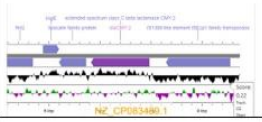
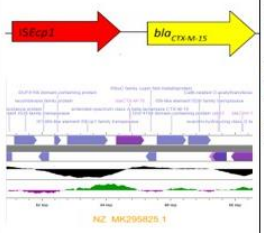
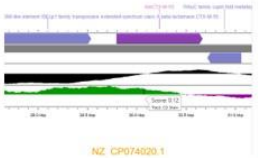
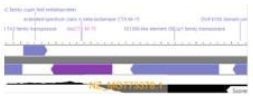
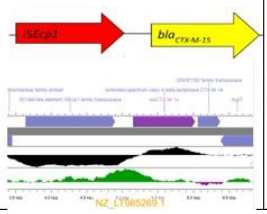
Hypothetically, these genomic profiles cannot be generalized solely based on the fact that resistant pathogens are associated with community- or hospital-acquired infections, such as *E. coli*. The identification and characterization of these resistance gene and mobile gene elements were performed using the Plasmid Database (PLSDB) and BLAST.

bla$_{CTX\text{-}M\text{-}15}$ was the most prevalent and encountered ESBL-resistance gene identified in these UTI strains. The distribution of ***bla***$_{\boldsymbol{CTX\text{-}M\text{-}15}}$ is not unique to Malta, but rather an increasingly global phenomenon that poses a significant threat. The mobile genetic elements associated with ***bla***$_{\boldsymbol{CTX\text{-}M\text{-}15}}$ facilitate the spread (Valenzuela *et al.*, 2023)

ESBLs are responsible for the resistance to third-generation Cephalosporins (Park, 2014).

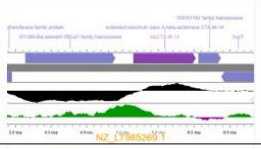
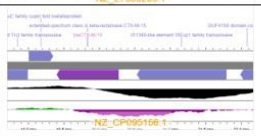
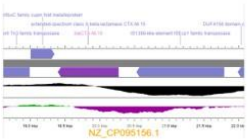
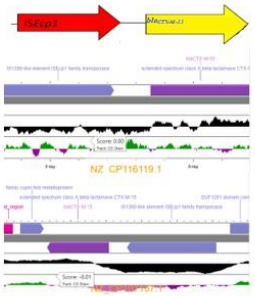
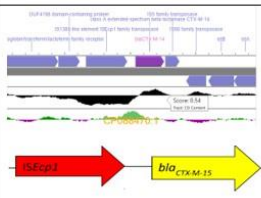
Table 4.8 Whole Genome Sequencing (WGS) findings of CTX-M Type ESBL – producing *E. coli* isolated from UTI patient samples

Table Genetic Environment of ESBL Genotype in *E.coli* from UTI Human cases in Malta

Sample UTI Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of ISEcp1 → blaCTX-M-15	Additional Resistance Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
UTI 62	20240828_BORG 1E	NZ_CP083489.1	*bla*$_{CMY-2}$				
UTI 63	20240912_BORG 1E	NZ_CP107173.1 MK 295825.1 NZ_OR611935.1 ISEcp1 has been truncated from certain Accession numbers but is still intact on others	*bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$		sul1, catB3, aac(6')-Ib-cr5, aac(3)-IIe, AAC(6')-Ib-cr6, aac(3)IIe,dfrA17, aadA5,aph(3'')-Ib, APH(6)-Id,sul2,, tet(A)		IncFIB IncFIA IncFII
UTI 70	20240912_BORG 1E	NZ_CP074020.1 NZ_CP074032.1 NZ_CP102676.1	*bla*$_{CTX-M-55}$, *bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$		lnu(F), aph(3')-Ia,aadA22, aac(3)-IIe,tet(A),linG, aadA23dfrA14, catA2, catII, qacEdelta1, Mrx, rmtB1, tet(A), mph(A), sul1, sul2, aph(6)-Id,aac(3)-IId,aadA5,aph(3'')-Ib,dfrA17,fosA3	*intI1* *intI1*	IncFIB IncFIC
UTI 71	20240912_BORG 1E	NZ_MG773378.1	*bla*$_{CTX-M-15}$		tet(M), APH(3'')-Ib, aph(6)-Id,sul2,floR, aph(6)-Id, dfrA14,aph(3'')-Ib, qnrS1, sul2, tet(A)	*intI1*	IncFIB
UTI 77	20240912_BORG 1E	NZ_MW495061.1 NZ_LT985269.1	*bla*$_{CTX-M-15}$ *bla*$_{TEM-1}$ *bla*$_{CTX-M-14}$		sul2, aph(3'')-Ib, APH (6)-Id,mdtM dfrA5, cvaC,merT,cvaB, merC,tet(A),aph(6)-Id, aph (3')-Ia		IncFIB, IncFIA, IncFIC, IncQ1 Rep_cluster_23 35

Note: Isolate 62 carried *blaCMY-2*, a gene typically associated with overexpression Class C β- lactamase, also known as AmpC.. It also carried bla *CTX-M-15* (Accession No: NZ_CP095128.1 no Insertion Sequence *ISEcp1* present) (Appendix B.2)

Table 4.8 (continued): (WGS) findings of UTI derived CTX-M Type ESBL – producing *E. coli*

							
UTI 80	20240912_BORG1E	NZ_CP095156.1	***bla*$_{CTX-M-15}$** ***bla*$_{TEM-1}$** ***bla*$_{OXA-1}$**		qacEdelta1,mph(A), tet(B),sul1, aac(3)-IId, aac(6')-Ib cr5,, aadA5,dfrA17,catA1,dfrA17, mphA,catB3,tetR, AAC(6')-Ib-cr6,Mrx Virulence Genes iucA,iucB, iucC,iucD,		IncFIB,IncFIA IncFIC
UTI 92	20240912_BORG1E	NZ_CP095156.1 NZ_CP029575.1	***bla*$_{CTX-M-15}$** ***bla*$_{OXA-1}$** ***bla*$_{TEM-1}$** ***bla*$_{CTX-M-15}$** ***bla*$_{TEM-1}$**		qacEdelta1,mph(A),tet(B),sul1,aac(3)-IId, aac(6')-Ib-cr5, aadA5,dfrA17, catA1,mphA,dfrA17,catB3,tetR, AAC(6')-Ib-cr6,Mrx, tet(B),catA1,catB3,tetR, Virulence Genes iucA,iucB, iucC,iucD,	intI1	IncFIB,IncFIA IncFIC IncFIB,IncFIA
UTI 97	20240912_BORG1E	NZ_CP116119.1 NZ_CP107157.1	***bla*$_{CTX-M-55}$** ***bla*$_{CTX-M-15}$**				Rep_cluster_2335 IncI1/B/O
UTI 308	20240912_BORG1E	CP088470.1 CP088491.1	***bla*$_{CTX-M-14}$** ***bla*$_{TEM-1}$**,		aac(3)-IId, aph(6)-Id, APH(3")-Ib, tet(A) Virulence Genes senB		IncFIB,IncFIA IncFII IncFIB,IncFIA IncFIC, IncFII

Nine strains of CTX-M-type ESBL- producing *E. coli* from UTI cases were sequenced using hybrid assemblies and annotations. Genomic identification was performed by uploading FASTA sequences to online tools such as ResFinder, PlasmidFinder, NCBI

BLAST, and CARD. The results are shown in Table 4.8. UTI *E. coli* strains 62, 63, 71, 77, 80, 92, and 97 were shown to carry $bla_{CTX\text{-}M\text{-}15}$, and this was also co-present with other β-lactamase genes, such as $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$, and $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}65}$.

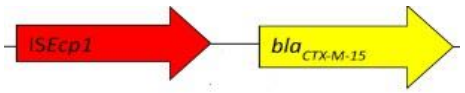


Figure 4.4 illustrates upstream location of ISEcp1 enhances dissemination of plasmid-mediated blaCTX-M β- lactamases

Among the UTI strains, insertion sequences, including *ISEcp1* and I*S26*, were detected and are associated with the genetic environment of $bla_{CTX\text{-}M\text{-}15}$. The insertion sequence IS*Ecp1* was frequently associated with Class 1 integrons, which are involved in the mobilization of $bla_{CTX\text{-}M}$ genes. $bla_{CTX\text{-}M\text{-}15}$ was associated with Class 1 integrons and was most commonly found to be linked with *sul1*, *tet*, *dfrA*, *aadA*, *cat*, and *qac*Delta1, forming the most common gene cassette array. In each case, the classical insertion sequence *ISEcp1* was identified upstream of the $bla_{CTX\text{-}M\text{-}15}$. The upstream location of IS*Ecp1* at the end of plasmid-mediated $bla_{CTX\text{-}M}$ type beta- lactamase-resistance genes typically contributes to their dissemination (Awosile & Agbaje, 2021).

Figure 4.4 illustrates upstream location of *ISEcp1* enhances dissemination of plasmid-mediated $bla_{CTX\text{-}M}$ β- lactamases

The most predominant resistance cassettes among these UTI strains were *dfrA* and *aadA5*. In Awosile and Agbaje (2021), the most frequently associated gene cassettes were the *dfrA* and *aadA5* genes.

The $bla_{CTX\text{-}M}$ beta-lactamases are plasmid-mediated extended-spectrum beta-lactamases (ESBLs) that exhibit resistance to cefotaxime, ceftriaxone, and aztreonam (Karim, Poirel,

Nagarajan, & Nordmann, 2001; Awosile & Agbaje, 2021).

In some *E. coli* UTI isolates, such as UTI 70, 71, and 92, the presence of these mobile genetic elements suggests a strong association between integrons and the dissemination of multidrug resistance. The complex of additional resistance genes conferring resistance to aminoglycosides (*aadA5, aph(6)-Id, aac(3)-IIe)*, tetracyclines (*tet(A), tet(B*)), sulfonamides (*sul 1, sul2*), and the plasmid- mediated quinolones qnrS1. All suggest that the *E. coli* isolates harbouring ESBLs is also resistant to a broad range of antibiotic classes, making the treatment impossible (Kao *et al.*, 2016). Several plasmids, commonly from the IncF family, including IncFIB, IncFIA, IncFIC, and IncFII, were observed in most isolates. Such plasmids play a crucial role in the dissemination of resistance genes through horizontal gene transfer. Plasmids, such as those in the IncF family, are commonly associated with virulence and resistance genes, which raises concerns about their potential clinical impact (Villa *et al.*, 2010; Neffe *et al.*, 2022). Replicons IncFIB, IncFIA, IncFIC, IncFII were the most common plasmid replicon types that horizontally transferred the $bla_{CTX\text{-}M\text{-}15}$ gene (Awosile & Agbaje, 2021). Plasmid clusters, such as Rep_cluster_2335 and Inc1/B/O, were also present. COIRNAI-type or Rep_cluster_2335 plasmids play a crucial role in the dissemination of antimicrobial resistance genes, including the transfer of bla_{KPC}*-type* carbapenemases (Stoesser *et al.*, 2017; Lerminiaux *et al.*, 2023). The *E. coli* isolate U 62 carried $bla_{CMY\text{-}2}$, a gene typically associated with overexpression Class C β- lactamase, also known as AmpC. This gene confers resistance to a wide range of β-lactams, including cephamaycin. (Jacoby, 2009) Here, IS*Ecp1* has been identified upstream of the plasmid-mediated cephalosporins gene $bla_{CMY\text{-}2.}$ It also carried bla $_{CTX\text{-}M\text{-}15}$ (Accession No: NZ_CP095128.1 no Insertion Sequence *ISEcp1* present) (Appendix B.2). Co-harbouring $bla_{CMY\text{-}2}$ and bla $_{CTX\text{-}M\text{-}15}$ would be expected to exhibit resistance to penicillins, β-lactam, β- lactamase inhibitor combinations, third – generation

cephalosporins while generally remaining susceptible to carbapenems unless additional resistance mechanisms are present (Kameyama et al., 2013) However phenotypic AST might provides initial insights, the most accurate detection and characterization of resistance genes is achieved through WGS which allows comprehensiveidentification of plasmid-mediated b-lactamase genes Other *E. coli* isolates, UTI 97 and UTI 308 exhibit multiple ***bla*$_{CTX-M}$** variants and multiple plasmids, indicating sequential acquisition (Bonnet, 2004; Rossolini *et al.*, 2008). This genetic heterogeneity, particularly in isolates from the same patient, indicates the dynamic and evolving nature of resistance elements in clinical findings.

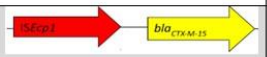
4.6.2 Genetic Environment of ESBL Genotype in *E. coli* from Houseflies

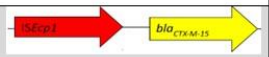
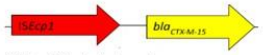
Eleven strains of ESBL *E. coli* from housefly (*Musca domestica*) cases were sequenced, depending on the presence of the resistance gene *CTX-M* and AST pattern, as shown in Table selected *CTX-M* ESBL-producing *E. coli* strains for WGS.

Table 4.9 Whole Genome Sequencing (WGS) findings of CTX-M Type ESBL – producing *E. coli* isolated from Houseflies (*Musca spp.*)

Sample Fly Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of ISEcp1 → $bla_{CTX-M-15}$	Additional Resistance Genes / Virulence Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
905		MW495061.1 CP027485.1	**$bla_{CTX-M-15}$** **$bla_{CTX-M-14}$** **bla_{TEM}**	ISEcp1 → $bla_{CTX-M-15}$ ***ISEcp1 Detected upstream*** Annex	*tet(A), sul2, aph(6)-Id, aph(3")-Ib, strA, strB, APH(6)-Id* Virulence Genes *iucB, iucC, iroN, iucA, iss, iucD, iroD, iroE, iroB, irp2, fyuA, cvaC, cvaB, iss,merC, merT*	Class1 *intI1*	IncFIA, IncFIB, IncFIC, IncQ1
105		CP116911.1	**$bla_{CTX-M-15}$** **bla_{TEM}**	*blaCTX-M-15* ← *ISEcp1* ***ISEcp1 Detected downstream*** Annex	*aph(6)-Id, aph(3")-Ib, tet(A), sul2, mph(A), aph(6)-Id, dfrA14, qnrS1*	Class1 *intI1*	IncFIB

Houseflies (*Musca domestica*) as potential vectors for the spread of ESBL-producing *E. coli* in humans.

Sample Fly Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of *ISEcp1* → *blaCTX-M-15*	Additional Resistance Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
805		CP116911.1	**bla**$_{CTX-M-15}$ **bla**$_{TEM-30}$	*blaCTX-M-15* ← *ISEcp1* ***ISEcp1 Detected downstream*** Annex	*qnrS1, dfrA14, tet(A), sul2, aph(6)-Id, APH(3")-Ib aph(3")-Ib,* Virulence Genes *evgA, evgS capU*	Class1 *intI*	IncFIB
1005		MK878891.1	**bla**$_{CTX-M-3}$ **bla**$_{TEM}$	 ***ISEcp1 Detected upstream*** Annex	*sul2, aph(3")-Ib, aph(6)-Id* Virulence Genes *iucC, iucB, iucA, iucD, iroN, iroE, iroD, iss, cvaC, cvaB, iroC, iroB, merT, merC, ogxA, ogxB*	Class1 *intI1*	IncFIA, IncFIB, IncFIC, IncQ1

Sample Fly Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of ISEcp1 → blaCTX-M-15	Additional Resistance Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
1405		CP116920.1 LR882051.1 KM023153.1	**bla**$_{CTX-M-15}$ **bla**$_{CTX-M-14}$ **bla**$_{TEM-1}$	 ***ISEcp1 Detected upstream*** Annex	tet(X4), floR, tet(A), aph(6)-Id, qnrS1, dfrA14, aph(3")-Ib Virulence Genes evgA, evgS	Class1 *intI1*	IncFIB IncX1 IncX3
1905		CP042872.1	**bla**$_{CTX-M-15}$ **bla**$_{TEM-1}$	*ISEcp1 not Detected* Annex	sul2, tet(A), dfrA14, qnrS1,aph(3")-Ib, aph(6)-Id	Class1 *intI1*	IncX3

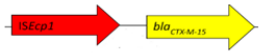
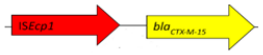
Sample Fly Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of ISEcp1 → blaCTX-M-15	Additional Resistance Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
1805		CP116920.1	**bla**$_{CTX-M-15}$ **bla**$_{TEM}$	 ***ISEcp1 Detected upstream*** Annex	tet(A), aph(6)-Id, qnrS1, dfrA14, aph(3")-Ib, sul2 Virulence Genes evgA, evgS	Class1 *intI1*	IncFIB
4		CP042872.1	**bla**$_{CTX-M-15}$	*ISEcp1 not Detected*	qnrS1, dfrA14, tet(A), sul2, aph(6)-Id, aph(3")-Ib		IncFIB,
1705		CP116930.1 CP010155.1 AP018146.1	**bla**$_{CTX-M-15}$ **bla**$_{TEM-1}$ **bla**$_{NDM-5}$	 ***ISEcp1 Detected upstream*** Annex	aph(3")-Ib, aph(6)-Id, dfrA14,tet(A), qnrS1, floR, qnrS2, sul2 Virulence Genes evgA, evgS	Class1 *intI1*	IncFIB IncX1 IncX3

Table 4.9 (continued)Whole Genome Sequencing (WGS) findings of CTX-M Type ESBL – producing *E. coli* isolated from Houseflies (*Musca spp*.)

Sample Fly Number	Project Number by MicrobesNG	Accession Number BLAST PLSDB v. 2024_05_31_v2	ESBL Genotype	Insertion Sequence Pattern of ISEcp1 → blaCTX-M-15	Additional Resistance Genes	Mobile Genetic Elements	
						Integron /Gene Cassettes	Plasmids
605		AP02248.1	**bla**$_{CTX-M-55}$ **bla**$_{TEM-244}$	**bla**$_{CTX-M-15}$ ← *IS26* **Downstream** Annex	floR, qnrS2, aph(3")-Ia, Virulence Genes FosA3		IncX1

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The results shown in Table 4.9, Houseflies CTX-M-Type ESBL-producing *E. coli* numbers: 905, 105, 805, 1405, 1905, 1805, 4, 1705, and 1605 were shown to carry bla $_{CTX-M-15}$, and this was also co-present with other β-lactamase genes, such as bla $_{OXA-1}$, bla $_{TEM-1}$, *bla*$_{CTX-M-14}$, *bla*$_{NDM-5}$. Only Fly 4 carried by itself bla$_{CTX-M-15}$. Houseflies number 605 and 1005 were shown to harbour bla $_{CTX-M-55}$ and bla$_{CTX-M-3}$ respectively.

Several plasmids, commonly from the IncF family, including IncFIB, IncFIA, IncFIC, and were observed in most isolates. Such plasmids play a crucial role in the dissemination of resistance genes through horizontal gene transfer. Plasmids, such as those in the IncF family, are commonly associated with virulence and resistance genes, which raises concerns about their potential clinical impact (Villa *et al.*, 2010; Neffe *et al.*, 2022). A geographical study focusing on the distribution of CTX-M-15 revealed that the IncFIB, IncFIA, IncFIC, and IncFII replicons are the most common plasmid replicon types that horizontally transfer the *bla*$_{CTX-M-\ 15}$ gene (Awosile & Agbaje, 2021).

Among the housefly strains, insertion sequences, including *ISEcp1*, I*S26* and *ISEc9* were detected and are associated with the genetic environment of *bla*$_{CTX-M-15}$ and bla$_{CTX-M-55}$

The insertion sequence IS*Ecp1* located upstream was frequently associated with *Class 1 integrons*, which are involved in the mobilization of bla_{CTX-M} genes (Awosile & Agbaje, 2021) $bla_{CTX-M-15}$ was associated with *Class 1 integrons* and was most commonly found to be linked with *sul2, , tet(A), dfrA14, aph(6)-Id, aph(3'')-Ib,qnrS1, floR, qnrS2,*forming the most common gene cassette array. In each case, the classical insertion sequence *ISEcp1* was identified upstream of the $bla_{CTX-M-15}$. The upstream location of IS*Ecp1* at the end of plasmid-mediated bla_{CTX-M} type β-lactamase-resistance genes typically contributes to their dissemination (Awosile & Agbaje, 2021).

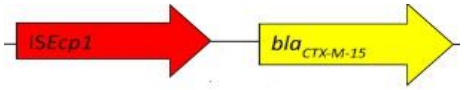


Figure 4.5 illustrates upstream location of *ISEcp1* enhances dissemination of plasmid-mediated bla_{CTX-M} β- lactamases

The complex of additional resistance genes conferring resistance to aminoglycosides (*aadA5, aph(6)-Id, aac(3)-IIe*), tetracyclines (*tet(A), tet(B)*), sulfonamides (*sul1, sul2*), and the plasmid- mediated quinolones qnrS1. All suggest that the *E. coli* isolates harbouring ESBLs is also resistant to a broad range of antibiotic classes, making the treatment impossible (Kao *et al.*, 2016). Fly 1705 *E. coli* strain exhibits multiple variants and multiple plasmids.

4.7 Virulence Genes Detected in Samples

7.7.1 Virulence Genes Detected in UTI Human Cases

E. coli is the primary causative agent of Urinary tract infection (UTI) in humans, particularly affecting women, children, and immunocompromised elderly patients.

Chapter 3 on UTI iden- tifies and explains the *E. coli* strains involved in Uropathogenic *Escherichia. coli* (UPEC), which carry a variety of virulence-associated genes that enable colonization of the urinary tract, evasion of host immune responses, and persistence within the host. Genomic data from human UTI cases reveal a diverse range of virulence genes across various

E. coli strains. Notably, genes such as *iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD,* and *iroE* pose a significant challenge to the survival of invading pathogens. The Aerobactin *iucA, iucB, iucC, iucD*) and Salmochelin (*iroN, iroB, iroC, iroD, iroE*) systems are often plasmid-mediated and enhance Horizontal Gene Transfer (HGT) among other bacterial populations. Additionally, genes such as *cvA* and *cvB* play a crucial role in inhibiting the growth of competing bacterial strains in the urinary tract. Meanwhile, toxins like *senB* worsen inflammation and lead to tissue injury. The presence of virulence genes on plasmids co-associated with antimicrobial resistance genes, such as bla *CTX-M*, poses a significant public health concern, and the plasmid replicon of the IncF family harbouring multiple virulence and resistance genes, making treatment extremely challenging when UPEC possesses them.

4.7.2 Virulence Genes detected in *E. coli* Fly strains.

Domestic flies are recognized as mechanical vectors of pathogenic bacteria, and recent findings have highlighted their potential role in disseminating virulence–associated and antimicrobial resistance genes through plasmid-mediated transfer.

In this study, multiple virulence genes, including siderophore–associated genes (*iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD*), iron acquisition genes (*irp2, fyuA*), and serum survival genes (*iss*), were identified on plasmids of fly-derived *E. coli* isolates. Notably,

these genes were predominantly associated with IncF-type replicons (IncFIA, IncFIB, IncFIC), which are commonly found in pathogenic Enterobacteriaceae.

The co-occurrence of virulence genes on IncF plasmid replicons alongside ESBL resistance genes, bla $_{CTX\text{-}M\text{-}15}$, suggests that the possibility of co-selection exists, where antibiotic pressure may indirectly promote the spread of virulence genes.

The detection of virulence genes such as *iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD,* and *iss* on IncF type plasmids in *E. coli* from domestic flies highlights the potential of these insects as an environmental reservoir for the distribution of pathogenic strains. IncF replicons are widely recognized for their role in facilitating Horizontal Gene Transfer (HGT), particularly in clinical settings. The co-localization of these virulence genes, associated with resistance genes ESBL, on the same plasmids raises concerns about the high-risk dissemination of these resistant genes.

Chapter 5: Discussion

5.1 Introduction

Discussing the hypothetical link between antibiotic resistance genes in ESBL-producing *E. coli* strains and domestic houseflies (*Musca spp*) in relation to urinary tract infection (UTI) human cases may reveal the role of domestic flies as overlooked vectors in the spread of antimicrobial resistance, contributing to UTI cases in humans. The continuous rise of multidrug-resistant bacteria is considered one of the top global health threats (World Health Organization, WHO, (2020). Antibiotic resistance is one of the most vital threats to public health and livestock. This crisis has been attributed to various causes, including the overuse and misuse of these agents, extensive use in livestock, and inadequate hygiene standards. ESBL-producing *E. coli* has gained significant interest in the medical community worldwide. This highlights the role of flies in the transmission of resistance genes, posing a substantial threat to public health. This exposure may represent an underrecognized transmission route contributing to the treatment of urinary tract infections in humans. The mobility of flies enables them to be more efficient in transmitting resistance genes between animals and humans. The increasing prevalence of antibiotic-resistant *E. coli* strains in urinary tract infections has become a significant public health concern. This threat is well documented in *E. coli* isolates from human patients (Doi *et al.*, 2016). Domestic flies (*Musca domestica*) have long been considered as pests; in fact, they are vectors associated with contaminated environmental reservoirs containing antibiotic-resistant *E. coli*, which can be transferred to vulnerable human communities, such as UTI patients.

Schaumberg *et al.* (2016) suggested in their study that flies carry human-like strains, and Awosile *et al.* (2022) reported that this resistance was also spread through humans,

animals, and the environment. The purpose of conducting this research in an urban area with high density of bovine farms located approximately 2 km from human dwellings or other human activities like open markets selling fruits and ready-to eat vegetables, was to investigate the potential role of flies as potential vectors for the transmission between livestock, the environment and human associated settings.

The manure was collected over a period four months, from May to August 2018, during which the average daily maximum temperature in Malta reached and exceeded 25 °C to 35 °C. In May, Malta's average daily maximum temperature is 25 °C or even slightly higher. As the weather started to warm up, several flies on farms and in the surrounding areas, especially near manure piles, begin to multiply periodically. Under such favourable conditions of dry climate, the flies become more active and multiply more quickly in the presence of rich, contaminated waste, such as fresh manure flies themselves are attracted to the cow's udder which offers warmth, moisture and organic matter; milk which will increase the risk of bacterial transfer during feeding or resting behaviour. Notably, local farmers reported a seasonal increase in bovine mastitis, which requires prolonged treatment, especially during the summer. To address these concerns, questions have been raised:

- Can mastitis be caused by poor hygiene?
- Can flies cause bacterial infection?
- Can flies act as vectors and carry Antimicrobial-resistant bacteria like ESBL-producing *E. coli*?

The fact that mastitis was challenging to treat during the summer, despite the farms maintaining high standards of cleanliness, raised concerns. However, some studies highlight manure as a primary environmental source of AMR and emphasize the role of HGT in the spread of resistance genes (Lima *et al.*, 2020)

From a microbiological perspective, this study's phenotypic analysis of bovine manure on TBX agar supplemented with cefotaxime (CTX) revealed low colony-forming unit (*CFU*) counts of ESBL-producing *E. coli,* which may explain the treatment challenges. Consequently, prolonged treatment for mastitis was necessary. Additionally, the presence of flies remained a concern. Unlike traditional rural farming systems, the proximity of flies to urban areas creates a unique environmental and public health concern in the spread of AMRs. Flies are known as mechanical vectors of pathogenic bacteria, which can travel several kilometres, facilitating the spread of ESBL-producing *E. coli* strains and other antibiotic-resistant bacteria from bovine manure to fresh products. Additionally, shared environmental reservoirs, such as wastewater, dust, and soil, may also contribute to the persistence of resistance genes across different locations. Since flies are found in both farm and urban settings, they are ideal candidates for studying the transmission of AMR. Such a study aims to identify whether flies captured from farms, open markets, and urban areas exhibit similar resistance patterns, suggesting active movement of resistance genes between different locations. However, the attempt to capture flies from our National Hospital environment is hindered due to the institution's high hygienic standards and strict infection control measures, which minimize the presence of flies. Given the strict EU Directives imposed on local farmers to prevent the misuse of antibiotics in livestock animals, together with the continuous implementation of the Malta National Plan on Antimicrobial Resistance, aligned with the EU One Health Action Plan against antimicrobial resistance (AMR), this report hypothesizes that these strict EU regulations and national policies have contributed to the low prevalence of ESBL-producing *E. coli* strains detected in manure.

This discrepancy, observed in this current study of the low counts of ESBL-producing *E. coli* in bovine manure on TBX agar with CTX, showed a low prevalence of *CTX-M* by

RT-PCR in the 28 manure ESBL-producing *E. coli* samples, where only 32 % harboured the *CTX-M* gene Supporting the notion that manure may not be the primary medium at high levels of ESBL- producing *E.coli* harbouring *CTX-M* . However, this does not suggest that the primary environmental reservoir for the spread of ESBL-producing *E.coli* harbouring *CTX-M* is necessarily manure itself, but rather that flies may carry resistance genes from other environmentally contaminated sources. Unless a possible explanation could arise from environmental and microbial factors influencing *CTX-M* detection in manure and even low ESBL-producing *E. coli* strains. Microbial competition may have played a crucial role in the microbial ecosystem, as it does with other competing bacteria or the presence of lactic acid bacteria, which, due to their antimicrobial properties, suppress the growth of ESBL-producing *E. coli* in the manure samples (Ibrahim *et al.*, 2021). The continued presence of AMR genes in flies raises important questions regarding the persistence of past antibiotic use.

Despite global and national efforts to minimize antibiotic misuse in healthcare services and veterinary medicine, its presence in urban flies suggests that external environmental factors may be contributing to the dissemination of AMR.

5.2 Tracing the Spread of AMR Genes: Are Environmental Vectors the Missing Link?

Furthermore, this study revealed that the molecular detection of fly isolates shared similar resistance genes, mobile genetic elements, and virulence genes with those found in clinically associated resistance genes of ESBL-producing *E. coli CTX-M* UTI strains from human cases. Further reinforcing and strengthening the hypothetical link that flies may serve as vectors for the dissemination of resistance genes to UTI patients (Schaumburg *et*

al., 2016). Flies captured from open markets were found to harbour resistance genes, suggesting that agricultural farm purposes do not solely drive antimicrobial resistance (AMR). However, AMRs therefore represent a broader environmental phenomenon and a serious global health threat (Pestinaci *et al.*,2015). This local study has significant public health implications, particularly when involving food safety risks and measures, as flies carrying ESBL-producing *CTX-M E. coli* strains could contaminate fresh food products like fruits and ready-to-eat vegetables from the market stalls. These food raw items continuously expose consumers to resistant bacteria through either food consumption or direct contact, leading to resistant infections that can sometimes be difficult to treat.

Antibiotic resistance is one of the most vital threats to public health, and it has been attributed to various causes, including the overuse and misuse of these agents. *E. coli* isolates harbouring *CTX-M* type ESBL have gained significant interest in the medical community worldwide (Halldórsdóttir *et al.*, 2024). The increasing prevalence of antibiotic-resistant *E. coli* strains in urinary tract infections has become a significant public health concern. This threat is well documented in *E. coli* isolates from human patients (Doi *et al.*, 2016). Locally, no studies have been published on the transmission of AMRs by domestic flies, demonstrating that houseflies can act as vectors for ESBL-producing *E. coli* harbouring additional resistance genes, replicon-type plasmids, mobile genetic elements, and virulence genes. Recent studies have highlighted that environmental sources, such as houseflies, may contribute to the spread of antibiotic resistance genes to humans (Shaumbug *et al.*,2016).

The findings of this study demonstrate that domestic flies can harbour and disseminate *E. coli* isolates harbouring *CTX-M* type ESBL, thereby bridging the environmental source and human infections. In this analysis, *E. coli* isolates harbouring *CTX-M* type ESBL were isolated from a significant proportion of houseflies caught from bovine farms, open

markets near urban areas where bovine farms are located, and UTI human cases. Notably, Schaumburg *et al.* (2016) found *E. coli* to be one of the common ESBL-producing resistance genes in flies, and the risk that flies spread resistance genes from livestock to humans. Confirming that ESBL-producing
E. coli genes from flies share similar genetic behaviour with human clinical isolates, pointing out a common source of transmission route of genetic background between flies and human cases (Schaumberg *et al.*, 2016).

The findings of this current study are consistent with those of Schaumberg *et al.* (2016), who concluded that flies can carry similar genetic background resistance genes in both urban and natural environments. Wetzket *et al.* (2019) found diverse ESBL-producing *E. coli* in urban houseflies, including clinically associated strains. Such mechanical transmission is plausible, given that the ecology of houseflies, which frequently move between contaminated areas, carries resistant microbes on their exoskeleton and in their gut. They are coprophagic, feeding on a wide range of materials, especially in contaminated environments that harbour resistance genes, which they absorb and deposit on surfaces such as food or other areas that people can come into contact with (Kook *et al.*, 2025). Consequently, for this study, the method employed by Blaak *et al.* (2014) was approached, and more recently, Shing *et al.* (2025) reported no significant differences in the prevalence of resistance genes between the external surfaces and the gut contents of the fly. Supporting the notion that both compartments can serve as reservoirs of antibiotic resistance that has a crucial role in the epidemiology of many infectious diseases (Hald *et al.*, 2008) Notably, in the study no significant differences were observed in the resistance genes carried by flies originating from bovine farms commonly associated with bovine manure and those collected from open markets. Further to the previous report on the

selected sampling area in the Southeast of Malta, the highest percentages of the resistant gene *CTX-M* were isolated from flies captured on farms (n= 21), while the lowest were found in flies captured from open markets (n= 14). Furthermore, Whole-Genome Sequencing (WGS) revealed that both studies, representing different environmental sources, exhibit identical resistance profiles, including bla*CTX-M-15*. This mirrors how flies are ubiquitous in the environment and capable to spread pathogenic bacteria harbouring resistance from one place to another (Ying *et al.*, 2022). This suggests that flies act as mechanical vectors harbouring CTX-M-type ESBL- producing *E. coli* resistance genes which have been picked from contaminated sources and subsequently introducing into the environment where humans can acquire infectious diseases or make treatment for certain infections impossible to heal.

Given Malta's small geographical size, high population density, and human dwellings close to urban areas, the spatial separation between livestock farms and other contained waste areas, such as landfills, is limited, facilitating the movement of flies across residential areas.

This proximity enables flies to easily cross borders from rural, contaminated areas to other environmental sources. These support structures that fly serve as an underappreciated reservoir and potential vehicle for resistant pathogens (Nayducher *et al.*, 2023). This has an important implication since communities are continuously exposed to multi-drug resistance *E. coli* through common vectors like houseflies. From the microbiological interpretations, both isolates identified from flies and UTI human cases, both displayed ESBL genotypes comparable to each other, in particular ESBL bla*CTX-M-15*, co-associated with other ESBL genotypes, mobile genetic elements, and virulence factors.

Bla*CTX-M-15* showed a significant predominance and identified in both niches. This parallel raises concern and is alarming since bla*CTX-M-15* has become the dominant CTX-M-type

ESBL- producing *E. coli* posing a significant threat to Public Health (Yu *et al.*, 2023). The presence of bla$_{CTX\text{-}M\text{-}15}$ in flies suggests that these vehicles may acquire and harbour highly virulent, human-associated *E. coli* strains from the surrounding environment or even vice versa. Wetzker *et al.* (2019) demonstrated that flies can carry ESBL-producing *E. coli* with clinically resistant genes.

Comparing Wetzker *et al.* (2019) to the current study highlights significant genetic similarities between CTX-M-type ESBL-producing *E. coli* strains from other human UTIs and houseflies; providing strong evidence that flies may serve as reservoirs and vectors for AMRs. This genetic analysis revealed that the predominant ESBL gene in both environmental and clinical sources was bla$_{CTX\text{-}M\text{-}15.}$ A key resistance determinant against third-generation Cephalosporins.

Bla$_{CTX\text{-}M}$ is the predominant ESBL resistance gene among other ESBLs, including TEM, OXA- 1, and NDM-4. Nearly all fly isolates and UTI CTX-M-type ESBL-producing *E. coli* strains carry bla$_{CTX\text{-}M}$, with bla$_{CTX\text{-}M\text{-}15}$ being the most prevalent.

Bevan *et al.* (2017) described bla$_{CTX\text{-}M\text{-}15}$ as the most widely distributed ESBL resistance gene worldwide, and Zanger *et al.* (2025) note that bla$_{CTX\text{-}M\text{-}15}$ is the most common among humans, animals, and other environmental reservoirs. Awosile *et al.* (2021) concluded that bla$_{CTX\text{-}M\text{-}15}$ is ' ubiquitous' in a particular region.

Despite not being directly exposed to a hospital environment, farmers may acquire these resistance genes through other environmental sources. In parallel, farmers working near livestock or other workers working in waste management environments are continuously being exposed to antibiotic-resistant bacteria, including genes commonly found in nosocomial *E. coli* strains. A prospective cohort study conducted by Cambridge University Hospitals NHS Foundation Trust investigated how *E. coli* carrying plasmids

are transmitted in hospitals. However, to date, no studies have directly linked farm antimicrobial use to cases of AMR in humans. In other words, there is no identification from farm to fork, for example, antimicrobial- resistant *Salmonella spp* in humans. Humans can acquire Salmonellosis from a variety of additional sources, including the consumption of vegetables, international travel, and exposure to wildlife. The *E. coli* isolates from UTI cases, including both hospitalized patients and outpatients, exhibit genetic features characteristic of hospital-acquired nosocomial strains. Notably, a high prevalence of ESBL genes was observed, including other bla variants, particularly bla $_{CTX\text{-}M\text{-}15}$ and various CTX-M type variants, such as *CTX-M-14*, *CTX-M-55*, and *CTX-M-65* with some co-associated with *bla OXA-1* and *bla TEM*. This combination of ESBLs is a hallmark of globally disseminated nosocomial *E. coli* clones. Additionally, several UTI isolates harboured multiple ESBL genes simultaneously. Plasmid-mediated quinolone resistance determinants were frequently detected in conjunction with these ESBL genes. However, since these UTI strains were collected from both clinical settings, this indicates that individuals do not necessarily acquire these resistance genes solely from the hospital. Instead, the presence of these associated genes in patient cases suggests that such genes may already be circulating in the community or even acquired through other environmental sources, not excluding houseflies as potential vectors. Commonly, nosocomial *E. coli* resistance genes are associated with hospital-acquired infections. An individual discharged from the hospital after receiving treatment may continue to carry and shed multidrug-resistant *E. coli*, including strains that harbour bla$_{CTX\text{-}M\text{-}15}$. These carriers can continue to contribute and transmit these resistant genes to other household contacts, food handlers, or public spaces. Vulnerable people, such as the elderly and immunocompromised, may be particularly at risk. These strains can persist in the gastrointestinal tract or be transferred via contaminated surfaces, especially under poor

hygienic conditions (Huddleston, 2014). The current study highlights houseflies (*Musca domestica*) as potential vectors in the environmental dissemination of antimicrobial resistance (AMR), particularly in relation to the presence of resistance genes associated with hospital- acquired infections. The Whole Genome Sequence readings of *E. coli* isolates from fly samples revealed the presence of multiple extended–spectrum β-lactamase ESBL genes, including bla$_{CTX\text{-}M\text{-}15}$, bla$_{CTX\text{-}M\text{-}14}$, and bla$_{TEM}$, all of which are frequently implicated in resistance infections in clinical settings, notably the detection of bla$_{NDM\text{-}5}$ in fly isolate 1705, which is of critical concern and alarming. Additional resistance genes, such as *qnrS1, mph(A), sul1/2, tet(A), and aac(6')-Ib-cr*, were also identified and carried on plasmids (Appendix B), as well as other mobile genetic elements that facilitate Horizontal Gene Transfer (HGT). The presence of virulence factors and disinfectants resistance markers such as *qacEΔ1*, further suggests that flies may serve as a bridging agent between clinical and agricultural environments. The dissemination of resistance genes in both housefly and UTI isolates was largely driven by mobile genetic elements, upwards located insertion sequences *ISEcp1* to bla$_{CTX\text{-}M\text{-}15}$, *Class 1 integrons* and various replicon-type plasmids IncF type (Appendices A). Plasmids mediated in both sources carried a diverse range of plasmid replicon types. The most common were IncF plasmid (IncFIA, IncFIB, IncFIC), which are all responsible for carrying ESBL genes and other resistance determinants**.** While the presence of resistance genes bla$_{CTX\text{-}M\text{-}15}$ poses a concern to Public Health, virulence genes detected in flies also pose a significant threat. The presence of virulence genes is worrisome. Many UTI isolates can be classified as *Uropathogenic E. coli* (*UPEC*), a subtype of *Extraintestinal pathogenic E. coli (ExPEC),* causing urinary tract infection (Rezatofighi *et al.* (2021). *UPEC* poses a multitude of virulence genes factors in order to facilitate infections and enable them to colonize, invade and damage the urinary tract and in severe cases can cause sepsis and even kidney failure

(Bien *et al.*, 2012). The presence of virulence genes identified in fly samples raises a potential concern of zoonotic transmission. Genomic data from human UTI cases reveal a diverse range of virulence genes across various *E. coli* strains. Notably, genes such as *iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD*, and *iroE* pose a significant challenge to the survival of invading pathogens. The Aerobactin (*iucA, iucB, iucC, iucD*) and Salmochelin (*iroN, iroB, iroC, iroD, iroE*) systems are often plasmid-mediated and enhance Horizontal Gene Transfer (HGT) among other bacterial populations (Tarchouna *et al.*, 2013). Additionally, genes such as *cvA* and *cvB* play a crucial role in inhibiting the growth of competing bacterial strains in the urinary tract. At the same time, toxins like *senB* exacerbate inflammation and lead to tissue injury. Interestingly same genes are frequently identified in Avian Pathogenic *E. coli* (APEC) suggesting a set of virulence traits across host species (Zhao *et al.*, 2009). The presence of virulence genes on plasmids co-associated with antimicrobial resistance genes, such as bla *CTX-M*, poses a significant public health concern, and the plasmid replicon of the IncF family harbouring multiple virulence and resistance genes, making treatment extremely challenging when *UPEC* possesses them (Hossain *et al.*, 2020) Domestic flies are recognized as mechanical vectors of pathogenic bacteria, and recent findings have highlighted their potential role in disseminating virulence–associated and antimicrobial resistance genes through plasmid-mediated transfer. In this current study, multiple virulence genes, including siderophore–associated genes (*iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD*), iron acquisition genes (*irp2, fyuA*), and serum survival genes (*iss*), were identified on plasmids of fly-derived *E. coli* isolates. Notably, these genes were predominantly associated with IncF- type replicons (IncFIA, IncFIB, IncFIC), which are commonly found in pathogenic Enterobacteriaceae. The co-occurrence of virulence genes on IncF plasmid replicons alongside ESBL resistance genes, bla$_{CTX\text{-}M\text{-}15}$, suggests that the possibility of co-selection

exists, where antibiotic pressure may indirectly promote the spread of virulence genes (Negeri *et al.*, 2023). The detection of virulence genes such as *iucA, iucB, iucC, iucD, iroN, iroB, iroC, iroD,* and *iss* on IncF type plasmids in *E. coli* from domestic flies highlights the potential of these insects as an environmental reservoir for the distribution of pathogenic strains (Paixão *et al*., 2016) IncF replicons are widely recognized for their role in facilitating Horizontal Gene Transfer (HGT), particularly in clinical settings. The virulence genes identified in this study, which are present in fly strains, particularly those associated with iron acquisition, serum resistance, toxins, and regulatory systems, can cause severe infections in humans. For example, a gut-colonizing *E. coli* strain might acquire a plasmid with *iucA, iucB, iucC, iucD,* giving it a substantial survival advantage in iron-inhibited environments like the urinary tract (Robison *et al.*, 2018). Colistin is widely used for pork production in China (Wanget *et al.*, 2018). Colistin is a last-resort antibiotic for treating carbapenem-resistant Enterobacteriaceae (CRE), including bacteria like *E. coli*. mcr-1 has now been detected in most countries worldwide, suggesting the widespread circulation of AMR bacteria in the environment. Nonetheless, the presence of AMR on farms is shaped by a complex combination of factors (Ransom *et al.*, 2024). However, this remains a significant gap in how antimicrobials are being transferred in the environment. These findings underscore the importance of considering houseflies within the broader context of epidemiological surveillance for antimicrobial resistance and the dissemination of virulence genes. Furthermore, hospitals are public domains that receive a high volume of daily visitors, including family and friends of patients who have been admitted. These can either carry asymptomatic carriers of nosocomial pathogens. This dynamic challenges the traditional boundaries between hospital–acquired and community-acquired infections, highlighting the need for awareness, post-discharge hygiene education, and broader surveillance strategies to prevent the silent spread of

antimicrobial resistance beyond hospital walls. However, this remains a significant gap in how antimicrobials are being transferred in the environment. Therefore, flies should not only be considered nuisance pests but also a key player in the One Health Approach. Antimicrobial resistance (AMR) is widely recognised as a complex involving humans, livestock animals, and other environmental reservoirs. Specific potential environmental vectors of resistance transmission are not mentioned in some studies. Observation of a recently published paper title 'One Health Approach to Tackling Antimicrobial Resistance' (Woolhouse, 2024) with reference to 'The antimicrobial resistance ecosystem' illustrates key routes through which AMR spreads via direct or indirect transmission from food, water, and sewage. However, it doesn't mention flies as potential vectors, despite the fact that the study has documented their role in transmitting pathogenic bacteria that harbour resistance strains. Flies, particularly those frequenting waste and livestock environments, have been shown to carry and disseminate antimicrobial-resistant bacteria. However, most studies do not highlight flies as potential vectors of AMR transmission, despite evidence suggesting that they can carry and transfer resistant bacteria**.** Apart from livestock manure, waste contamination, and migratory birds can also play a crucial part in the indirect dissemination of multidrug- resistant ESBL-producing *E. coli*. Shah *et al.* (2022) reported the presence of antibiotic resistance from migratory birds with bla CTX-M and bla TEM genes. Bird migration in Malta holds particular relevance, as the island's geographic position serves as a key stopover for migratory birds, facilitating their passage between Europe and Africa, or vice versa. During this season of bird migration, bird droppings are often deposited across urban agricultural areas. These can harbour resistance genes and provide another food source for flies to feed on and breed in. The presence of CTX-M-15 in this study is not ubiquitous to Malta but part of a global pattern. Malta cannot be directly compared to other countries, particularly those where individual regions are

larger than the entire Maltese territory. Focusing on flies to UTI cases suggests a possible indirect zoonotic route, and flies could acquire ESBL-associated UPEC from the sanitary environment and then transfer them to humans. The concern arising from this study none of the environmental *E. coli* acquired resistance but dealing with pathogenic *E. coli* strains causing diseases and harbouring ESBL CTX-M resistance genes. Patients infected with such strains often experience complications that make treatment ineffective with standard antibiotics. Ultimately requiring the use of last resort drugs such as carbapenems (Eslami *et al*., 2025). In Malta, on hot days, during which the average daily maximum temperature in Malta reached and exceeded 35 °C. In open markets, domestic flies were observed to be attracted to most high-sugar-content perishable summer fruits, such as peaches, plums, melons, and figs. They seek sugary substances and feed more efficiently on ripe and fermented fruits. Another factor that attracted flies to trucks parked in open markets selling vegetables and fruits was the presence of condensation, or water droplets, on the fruits and vegetables. Condensation typically occurs when fruits and vegetables are removed from the refrigerator and taken to the stalls at ambient temperatures. Flies tend to congregate in moist areas to stay hydrated; condensation on fruits might be enough for the flies. Flies are only one piece of the puzzle in the spread of CTX-M-type ESBL-producing *E. coli* strains are not only related to clinical environments but are increasingly recognized as being implemented into the environmental ecosystem. Climate change, high temperatures all promote bacterial growth carrying resistance genes and transferred from one species to another via Horizontal Gene Transfer (HGT). Other factors contributing to the dissemination of AMR include human mobility, either for short-term or long stays, which facilitate the spread of infections, including AMR (Singh *et al.*,2024). The influx of foreign individuals from regions where antibiotic use in livestock animals is widespread and poorly regulated. Improper waste treatment, land fill management of

sewage discharge into the sea, marine environment as a potential reservoir for AMRs which all further complicate the control while contributing to this global problem (Gambino *et al.*, 2022; Singh *et al.*,2024).

5.3 Conclusion

The results of this research will contribute to a new Health Approach to AMR control, emphasising the need for integrated strategies that address AMR across animal, human and environmental health sectors while supporting Malta's National AMR Strategic Plan 2025-2028 which focuses on surveillance, antimicrobial stewardship, infection prevention and public awareness to mitigate the spread of resistance in both clinical and environmental settings. Furthermore, it explores whether flies primarily acquired resistance genes from different sources or locations here in Malta or through other factors contributing to the spread of AMRs, such as migratory birds.

5.4 Future Work

While sequencing remains the golden standard for profiling antimicrobial-resistant genes, its prohibitive costs limit its use to reporting relative abundances. To enhance future research, the use of quantitative PCR to target specific resistance genes associated with commonly used antibiotics should be considered. By using quantitative PCR to detect and measure the absolute abundance of specific resistance genes, researchers can compare AMR levels across different environments and sample types. This method is not only used across farms but can be applied to other environmental sources. The same approach is

used to study antimicrobial resistance genes (AMR) in livestock animals and their products. However, it can also be extended to other environmental sources, especially to travellers coming from countries where antibiotics are not regulated.

References

A Strategy and Action Plan for the Prevention and Containment of Antimicrobial Resistance in Malta (2020–2028).

Aarestrup, F. M. (2006). Antimicrobial Resistance in Bacteria of Animal Origin. Washington, DC: ASM Press., Ch.1–10.

Abdus Salam, Md., Yusuf Al-Amin, Md., Tabassoom Salam, M., Singh Pawar, J., Akhter, N., Rabaan, A. A., & Alqumber, M. A. A. (2023). Antimicrobial resistance: A growing serious threat for global public health. Healthcare (Basel), 11(13), 1946.

Abe, C. M., Salvador, F. A., Falsetti, I. N., Vieira, M. A. M., Blanco, J., Blanco, J. E., Blanco, M., Machado, A. M. O., Elias, W. P., Hernandes, R. T., & others. (2008). Uropathogenic Escherichia coli (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunology & Medical Microbiology, 52(3), 397–406.

Adedeji, W. A. (2016). The treasure called antibiotics. Annals of Ibadan Postgraduate Medicine, 14(2), 56–57.

Ahmed, S. K., Hussein, S., Qurbani, K., Ibrahim, R. H., Fareeq, A., Mahmood, K. A., & Mohamed, M. G. (2024). Antimicrobial resistance: Impacts, challenges, and future prospects. Journal of Medicine, Surgery, and Public Health, 2, Article 100081.

Al Sulayyim, H. J., Ismail, R., Al Hamid, A., & Ghafar, N. A. (2022). Antibiotic resistance during COVID-19: A systematic review. International Journal of Environmental Research and Public Health, 19(19), 11931.

Alegbeleye, O. O., Singleton, I., & Sant'Ana, A. S. (2018). Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review. Food Microbiology, 73, 177–208.

Alghamdi, S. A. A. (2023). From urinary tract infection patients in Al-Baha, Saudi

Arabia. Micro, 11(12), 2820.

Alves, T. dos S., Lara, G. H. B., Maluta, R. P., Ribeiro, M. G., & Leite, D. da S. (2018). Carrier flies of multidrug-resistant Escherichia coli as potential dissemination agent in dairy farm environment. Science of The Total Environment, 633, 1345–1351. DOI: https://doi.org/10.1016/j.scitotenv.2018.03.265

Ambler, R. P. (1980). The structure of beta-lactamases. Philosophical Transactions of the Royal Society of London. B. Biological Sciences, 289(1036), 321–331.

Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. The Lancet, 399(10325), 629–655.

Apata, D. F. (2009). Antibiotic resistance in poultry. International Journal of Poultry Science, 8, 404–408.

Appau, A. A. A., & Ofori, L. A. (2024). Antibiotic resistance profile of *E. coli* isolates from lettuce, poultry manure, irrigation water, and soil in Kumasi, Ghana. International Journal of Microbiology, 2024, 6681311. DOI: https://doi.org/10.1155/2024/6681311

Arnold, K. E., Laing, G., McMahon, B. J., Fanning, S., Stekel, D. J., Pahl, O., Coyne, L., Latham, S. M., & McIntyre, K. M. (2024). The need for One Health systems-thinking approaches to understand multiscale dissemination of antimicrobial resistance. The Lancet Planetary Health, 8(2), e124–e133. DOI: https://doi.org/10.1016/S2542-5196(23)00249-2

Awache, I., & Farouk, A. A. (2016). Bacteria and fungi associated with houseflies collected from cafeteria and food centres in Sokoto. FUW Trends in Science & Technology Journal, 1(1), 123–125.

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M. K. F., & Baloch, Z.

(2018). *Antibiotic resistance: A rundown of a global crisis. Infection and Drug Resistance, 11,* 1645–1658. https://doi.org/10.2147/IDR.S173867

Awosile, B. B., & Agbaje, M. (2021). Genetic environments of plasmid-mediated blaCTX-M- 15 beta-lactamase gene in Enterobacteriaceae from Africa. Microbiology Research, 12(2), 383–394. DOI: https://doi.org/10.3390/microbiolres12020026

Ayodele Oluwaseun Ajayi, Olajide Joseph Akinjogunla, Adebowale Toba Odeyemi & Akinyomade Oladipo Owolabi (2024). Role of plasmids in antibiotic resistance in clinical infections and implications for epidemiological surveillance: a review, All Life, 17:1, 2350414, DOI: 10.

Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: Causes and control strategies. Antimicrobial Resistance & Infection Control, 6, 47.

Balaraman, V., Drolet, B. S., Mitzel, D. N., Wilson, W. C., Owens, J., Gaudreault, N. N., ... Nayduch, D. (2021). Mechanical transmission of SARS-CoV-2 by house flies. Parasites & Vectors, 14(1), 214. DOI: https://doi.org/10.1186/s13071-021-04703-8

Barola, S., Grossman, O. K., & Abdelhalim, A. (2024). Urinary tract infections in children. StatPearls. Last updated January 11, 2024.

Barreiro, C., Albano, H., Silva, J., & Teixeira, P. (2013). Role of flies as vectors of foodborne pathogens in rural areas. Microbiology, 2013, Article 718780.

Bassett, E. J., Keith, M. S., Armelagos, G. J., Martin, D. L., & Villanueva, A. R. (1980). Tetracycline-labelled human bone from ancient Sudanese Nubia (A.D. 350). Science, 209(4464), 1532–1534.

Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M. H., & Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. Environmental Research, 169, 483–493.

Bennett, P. M. (2008). Plasmid-encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology, 153(Suppl 1), S347–S357.

Berg, R. D. (1996). The indigenous gastrointestinal microflora. Trends in Microbiology, 4, 430–435.

Bertelloni, F., Bresciani, F., Cagnoli, G., Scotti, B., Lazzerini, L., Marcucci, M., ... Ebani, V. V. (2023). House flies (*Musca domestica*) from swine and poultry farms carrying antimicrobial resistant Enterobacteriaceae and Salmonella. Veterinary Sciences, 10(2), 2.

Bevan, E. R., Jones, A. M., & Hawkey, P. M. (2017). Global epidemiology of CTX-M β-lactamases: Temporal and geographical shifts in genotype. Journal of Antimicrobial Chemotherapy, 72(8), 2145–2155.

Bien, J., Sokolova, O., & Bozko, P. (2012). Role of uropathogenic Escherichia coli virulence factors in development of urinary tract infection and kidney damage. International Journal of Nephrology, 2012, 681473.

Blaak, H., Hamidjaja, R. A., van Hoek, A. H. A. M., de Heer, L., de Roda Husman, A. M., & Schets, F. M. (2014). Detection of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli on flies at poultry farms. Applied and Environmental Microbiology, 80(1), 239–246. DOI: https://doi.org/10.1128/AEM.02616-13

Bobate, S., Mahalle, S., Dafale, N. A., & Bajaj, A. (2023). Emergence of environmental antibiotic resistance: Mechanism, monitoring and management. Environmental Advances, 13, 100409. DOI: https://doi.org/10.1016/j.envadv.2023.100409

Bonnet, R. (2004). Growing group of extended-spectrum β-lactamases: The CTX-M enzymes. Antimicrobial Agents and Chemotherapy, 48(1), 1–14. DOI: https://doi.org/10.1128/AAC.48.1.1-14.2004

Bono, M. J., Leslie, S. W., & Reygaert, W. C. (2023). Uncomplicated urinary tract infections. StatPearls. Last updated November 13, 2023.

Brunton, L. L., Knollmann, B. C., & Hilal-Dandan, R. (2018). Goodman & Gilman's: The Pharmacological Basis of Therapeutics (13th ed.). McGraw-Hill Education.

Burmeister, A. R. (2015). Horizontal gene transfer. Evolution, Medicine, and Public Health, 2015(1), 193–194.

Bush, K. (2010). Bench-to-bedside review: The role of β-lactamases in antibiotic-resistant Gram-negative infections. Critical Care, 14(3), 224.

Bush, K., & Bradford, P. A. (2016). Beta-lactams and beta-lactamase inhibitors: An overview. Cold Spring Harbor Perspectives in Medicine, 6(8), a025247. DOI: https://doi.org/10.1101/cshperspect.a025247

Campos, A. C. da C., Couto, N., Andrade, N. L. da S., Friedrich, A. W., Rosa, A. C. de P., Damasco, P. V., ... & Rossen, J. W. A. (2020). Virulence and resistance properties of *E. coli* isolated from urine samples of hospitalized patients in Rio de Janeiro, Brazil – The role of mobile genetic elements. International Journal of Medical Microbiology, 310(8), 151453. DOI: https://doi.org/10.1016/j.ijmm.2020.151453

Canton, R., & Coque, T. M. (2006). The CTX-M beta-lactamase pandemic. Current Opinion in Microbiology, 9, 466–475.

Canton, R., Gonzalez-Alba, J. M., & Galan, J. C. (2012). CTX-M enzymes: Origin and diffusion. Frontiers in Microbiology, 3, 110.

Carattoli, A. (2008). Animal reservoirs for extended-spectrum beta-lactamase producers. Clinical Microbiology and Infection, 14, 117–123.

Carattoli, A. (2013). Plasmids and the spread of resistance. International Journal of Medical Microbiology, 303, 298–304.

Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy, 52(2), 159–161.

Cassar, T., & Mifsud, D. (2024). *Insects of the Maltese Islands*. Malta: Entomological Society of Malta.

Castanheira, M., Simner, P. J., & Bradford, P. A. (2021). Extended-spectrum β-lactamases: An update on their characteristics, epidemiology and detection. JAC-Antimicrobial Resistance, 3(3).

Cella, E., Giovanetti, M., Benedetti, F., Scarpa, F., Johnston, C., Borsetti, A., Ceccarelli, G., Azarian, T., Zella, D., & Ciccozzi, M. (2023). *Joining forces against antibiotic resistance: The One Health solution.* Pathogens, 12(9), 1074. https://doi.org/10.3390/pathogens12091074

Centers for Disease Control and Prevention (CDC). (2019). [No additional citation information provided.]

Chaiwong, T., Srivoramas, T., Sueabsamran, P., Sukontason, K., Sanford, M., & Sukontason, K. (2014). The blow fly, Chrysomya megacephala, and the house fly, *Musca domestica*, as mechanical vectors of pathogenic bacteria in Northeast Thailand. Tropical Biomedicine, 31(2), 336–346.

Chan, M. (2012, March 14). Resistance to antibiotics could bring the end of modern medicine as we know it, WHO claims. The Telegraph.

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: Risk factors, therapeutic strategies, and alternative treatments — A review. Asian-Australasian Journal of Animal Sciences, 33(11), 1699–1713.

Cimitile, M. (2009). Worried about antibiotics in your beef? Vegetables may be no better. Sci Am January, 6.

Claus, D. (1992). A standardized Gram staining procedure. World Journal of Microbiology and Biotechnology, 8, 451–452.

Cook, K., Premchand-Branker, S., Nieto-Rosado, M., Portal, E. A. R., Li, M., Orbegozo Rubio, C., ... & Sands, K. (2025). Flies as carriers of antimicrobial resistant (AMR) bacteria in Nigerian hospitals: A workflow for surveillance of AMR bacteria carried by arthropod pests in hospital settings. Environment International, 196, 109294.

Cottone, C. B., Szalanski, A. L., McKay, T., & Steelman, C. D. (2014). Molecular detection of Campylobacter spp. and *E. coli* O157:H7 carried by filth flies (Diptera: Muscidae) associated with turkey production facilities. Midsouth Entomologist, 7(1), 1–7.

Czajkowski, K., Broś-Konopielko, M., & Teliga-Czajkowska, J. (2021). Urinary tract infection in women. Przegląd Menopauzalny, 20(1), 40–47. DOI: https://doi.org/10.5114/pm.2021.105382

Danaei, B., Sarmastzadeh, T., Khalili, F., Yazarlou, F., Centis, R., D'Ambrosio, L., ... & Nasiri, M. J. (2023). The battle against colistin-resistant *E. coli* and the need for a One Health approach. New Microbes and New Infections, 54, 101161. DOI: https://doi.org/10.1016/j.nmni.2023.101161

Datta, N., & Hedges, R. W. (1971). Compatibility groups among fi-R factors. Nature, 234(5323), 222–223.

Davies, J. (2006). Are antibiotics naturally antibiotics? Journal of Industrial Microbiology and Biotechnology, 33, 496–499.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews, 74(3), 417–433.

Delveen, R. I., Dodd, C. E. R., Stekel, D. J., Meshioye, R. T., Diggle, M., Lister, M., & Hobman, J. L. (2022). Multidrug-resistant ESBL-producing *E. coli* in clinical

samples from the UK.

DHSC (Department of Health and Social Care). (2019). The UK's second five-year National Action Plan (NAP) aims to build on progress made since 2019 and contributes to the UK's 20-year vision to contain, control, and mitigate antimicrobial resistance (AMR) by 2040.

Doctor, J. (2020) *Musca domestica* Retrieved from https://animaldiversity.org/accounts/*Musca*_domestica/ [accessed July 2023]

Doi, Y., et al. (2016). Antimicrobial resistance in Escherichia coli from the human population: A global overview. Antimicrobial Agents and Chemotherapy, 60(7), 3900–3908.

dos Santos Alves, T., Lara, G. H. B., Maluta, R. P., Ribeiro, M. G., & da Silva Leite, D. (2018). Carrier flies of multidrug-resistant Escherichia coli as potential dissemination agents in dairy farm environments. Science of The Total Environment, 633, 1345–1351. DOI: https://doi.org/10.1016/j.scitotenv.2018.03.235

Du, L., & Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems: A review. Agronomy for Sustainable Development, 32, 309–327.

Džidic, S., Šušković, J., & Kos, B. (2008). Antibiotic resistance mechanisms in bacteria: Biochemical and genetic aspects. Food Technology and Biotechnology, 46, 11–21.

Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., & Burkholder, P. R. (1947). Chloromycetin, a new antibiotic from a soil actinomycete. Science, 106(2757), 417.

Emödy, L., Kerényi, M., & Nagy, G. (2003). Virulence factors of uropathogenic Escherichia coli. International Journal of Antimicrobial Agents, 22(Suppl 2), S29–S33.

Eslami, M., Safaripour, A., Banihashemian, S. Z., Nikjoo Niaragh, S., Hemmati, M. A.,

Shojaeian, A., & Oksenych, V. (2025). Innovative antibiotic therapies for carbapenem- resistant Gram-negative bacterial infections: Clinical efficacy, safety, and comparative studies. Microorganisms, 13(2), 295. DOI: https://doi.org/10.3390/microorganisms13020295

European Centre for Disease Prevention and Control. (2018, November 6). 33,000 people die every year due to infections with antibiotic-resistant bacteria. Retrieved from https://www.ecdc.europa.eu/en/news-events/33000-people-die-every-year-due-infections-antibiotic-resistant-bacteria [accessed July 2023]

Fernandes, J. B. C., Zanardo, L. G., Galvão, N. N., Carvalho, I. A., Nero, L. A., & Moreira, M. A. S. (2011). Escherichia coli from clinical mastitis: Serotypes and virulence factors. Journal of Veterinary Diagnostic Investigation, 23(6), 1146–1152.

Fernandez, G. (2022). Turning the juggernaut. Lancet Planetary Health, 6(2), E75.

Fernández, L., & Hancock, R. E. W. (2012). Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clinical Microbiology Reviews, 25(4), 661–681.

Flores-Mireles, A. L., Walker, J. N., Caparon, M., & Hultgren, S. J. (2015). Urinary tract infections: Epidemiology, mechanisms of infection, and treatment options. Nature Reviews Microbiology, 13(5), 269–284.

Flores-Oropeza, M. A., Ochoa, S. A., Cruz-Córdova, A., Chavez-Tepecano, R., Martínez-Peñafiel, E., Rembao-Bojórquez, D., ... & Xicohtencatl-Cortes, J. (2024). Comparative genomic analysis of uropathogenic Escherichia coli strains from women with recurrent urinary tract infection. Frontiers in Microbiology, 14, 1340427.

Förster, M., Klimpel, S., & Sievert, K. (2008). The house fly (*Musca domestica*) as a potential vector of metazoan parasites caught in a pig-pen in Germany. Veterinary

Parasitology, 160(1–2), 163–167. DOI: https://doi.org/10.1016/j.vetpar.2008.10.087

Foxman, B. (2014). Urinary tract infection syndromes: Occurrence, recurrence, bacteriology, risk factors, and disease burden. Infectious Disease Clinics of North America, 28(1), 1–13.

Francia, M. V., et al. (2004). Classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiology Reviews, 28(1), 79–100.

Fu, M., Gong, Z., Zhu, Y., Li, C., Zhou, Y., Hu, L., et al. (2023). Inappropriate antibiotic prescribing in primary healthcare facilities in China: A nationwide survey, 2017–2019. Clinical Microbiology and Infection, 29.

Gales, A. C., et al. (2019). Environmental and clinical isolates of Escherichia coli and their resistance to antimicrobial agents. Journal of Antimicrobial Chemotherapy, 74(2), 407– 412.

Galhano, B. S. P., Ferrari, R. G., Panzenhagen, P., de Jesus, A. C. S., & Conte-Junior, C. A. (2021). Antimicrobial resistance gene detection methods for bacteria in animal-based foods: A brief review of highlights and advantages. Microorganisms, 9(5), 923.

Gambino, D., Savoca, D., Sucato, A., Gargano, V., Gentile, A., Pantano, L., ... & Alduina, R. (2022). Occurrence of antibiotic resistance in the Mediterranean Sea. Antibiotics, 11(3), 332. DOI: https://doi.org/10.3390/antibiotics11030332

Gaynes, R. (2017). The discovery of penicillin—New insights after more than 75 years of clinical use. Emerging Infectious Diseases, 23(5), 849–853.

Geng, J., Liu, X., Wang, J., & Li, S. (2022). Accumulation and risk assessment of antibiotics in edible plants grown in contaminated farmlands: A review. Science of The Total Environment, 853.

Ghimpeteanu, Q. M., Pogurschi, E. N., & Popa, M. E. (2022). Antibiotic use in livestock and residues in food – A public health threat: A review. Foods, 11, 1430.

Gioia, G., Freeman, J., Sipka, A., Santisteban, C., Wieland, M., Alanis Gallardo, V., ... & Moroni, P. (2022). Pathogens associated with houseflies from different areas within a New York State dairy. JDS Communications, 3(4), 285–290. DOI: https://doi.org/10.3168/jdsc.2021-0200

Global burden of bacterial antimicrobial resistance 1990 – 2021: a systematic analysis with forecasts to 2050. The Lancet, Volume 404, Issue 10459, 1199 – 1226

Gniadkowski, M., Schneider, I., Palucha, A., et al. (1998). Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: Identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase. Antimicrobial Agents and Chemotherapy, 42(4), 827–832.

Gomes, F., & Henriques, M. (2016). Control of bovine mastitis: Old and recent therapeutic approaches. Current Microbiology, 72(4), 377–382.

Google. (2025). *Map of southeastern Malta showing Wied il-Għajn and surrounding districts* [Map]. Google Maps. https://www.google.com/maps

Graham, J. P., Price, L. B., Evans, S. L., Graczyk, T. K., & Silbergeld, E. K. (2009). Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Science of The Total Environment, 407(8), 2701–2710. DOI: https://doi.org/10.1016/j.scitotenv.2008.11.056

Gustafson, R. H., & Bowen, R. E. (1997). Antibiotic use in animal agriculture. Journal of Applied Microbiology, 83, 531–541.

Hald, B., Skovgård, H., Pedersen, K., & Bunkenborg, H. (2008). Influxed insects as vectors for Campylobacter jejuni and Campylobacter coli in Danish broiler houses. Poultry Science, 87, 1428–1434.

Halldórsdóttir, A. M., Hrafnkelsson, B., Einarsdóttir, K., & Kristinsson, K. G. (2024). Prevalence and risk factors of extended-spectrum beta-lactamase producing *E. coli*

causing urinary tract infections in Iceland during 2012–2021. European Journal of Clinical Microbiology & Infectious Diseases, 43(9), 1689–1697. DOI: https://doi.org/10.1007/s10096-024-04882-z

Hang Yin, J., Kelly, P. J., & Wang, C. (2022). Flies as vectors and potential sentinels for bacterial pathogens and antimicrobial resistance: A review. Veterinary Sciences, 9(6), 300.

Hart, C. A., & Percival, A. (1982). Resistance to cephalosporins among gentamicin-resistant Klebsiellae. Journal of Antimicrobial Chemotherapy, 9, 275–286.

Heiden, S. E., Kurz, M. S. E., Bohnert, J., Bayingana, C., Ndoli, J. M., Sendegeya, A., ... & Schaufler, K. (2020). Flies from a tertiary hospital in Rwanda carry multidrug-resistant Gram-negative pathogens including ESBL-producing *E. coli* ST131. Antimicrobial Resistance & Infection Control, 9, 34. DOI: https://doi.org/10.1186/s13756-020-0696-y

Hemmatinezhad, B., Ommi, D., Hafshejani, T. T., & Khamesipour, F. (2015). Molecular detection and antimicrobial resistance of Pseudomonas aeruginosa from houseflies (*Musca domestica*) in Iran. Journal of Venomous Animals and Toxins Including Tropical Diseases, 21, 18. DOI: https://doi.org/10.1186/s40409-015-0021-z

Holmes, A. H., Moore, L. S. P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., ... & Piddock, L. J. V. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. The Lancet, 387(10014), 176–187.

Hornish, R. E., & Kotarski, S. F. (2002). Cephalosporins in veterinary medicine – Ceftiofur use in food animals. Current Topics in Medicinal Chemistry, 2(7), 717–731.

Hossain, M., Tabassum, T., Rahman, A., et al. (2020). Genotype–phenotype correlation of β- lactamase-producing uropathogenic Escherichia coli (UPEC) strains from Bangladesh. Scientific Reports, 10, 14549.

House Fly (*Musca domestica* L.) Attraction to Insect Honeydew. (2015). PLoS ONE, 10(5), e0126205.

Huddleston, J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes. Infection and Drug Resistance, 7, 167–176. DOI: https://doi.org/10.2147/IDR.S48820

Hughes, V. M., & Datta, N. (1983). Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature, 302, 725–726.

Hung, K. Y., Michailides, T. J., Millar, J. C., Wayadande, A., Gerry, A. C., & Lazzari, C. R. (2015). [Details missing from original reference].

Husna, A., Rahman, M. M., Badruzzaman, A. T. M., Sikder, M. H., Islam, M. R., Rahman, M. T., ... & Ashour, H. M. (2023). Extended-spectrum β-lactamases (ESBL): Challenges and opportunities. Biomedicines, 11(11), 2937.

Hussain, H. I., Aqib, A. I., Seleem, M. N., Shabbir, M. A., Hao, H., Iqbal, Z., ... & Li, K. (2021). Genetic basis of molecular mechanisms in β-lactam resistant Gram-negative bacteria. Microbial Pathogenesis, 158, 105040. DOI: https://doi.org/10.1016/j.micpath.2021.105040

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present, and future. Current Opinion in Microbiology, 51, 72–80.

Ibrahim, D. R., Dodd, C. E., Stekel, D. J., Meshioye, R. T., Diggle, M., Lister, M., & Hobman, J. L. (2023). Multidrug-resistant ESBL-producing *E. coli* in clinical samples from the UK. Antibiotics, 12(1), 169.

Ibrahim, S. A., Ayivi, R. D., Zimmerman, T., Siddiqui, S. A., Altemimi, A. B., Fidan, H., ... & Bakhshayesh, R. V. (2021). Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. Foods, 10(12), 3131. DOI: https://doi.org/10.3390/foods10123131

Jacoby, G. A., Strahilevitz, J., & Hooper, D. C. (2014). Plasmid-mediated quinolone resistance. Microbiology Spectrum, 5, 10.

Jadeja, N. B., & Worrich, A. (2022). From gut to mud: Dissemination of antimicrobial resistance between animal and agricultural niches. Environmental Microbiology, 24(8), 3290–3306.

Jancel, T., & Dudas, V. (2002). Management of uncomplicated urinary tract infections. Western Journal of Medicine, 176(1), 51–55.

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., ... & Brisse, S. (2008). Phylogenetic and genomic diversity of human bacteremic Escherichia coli strains. BMC Genomics, 9, 560. DOI: https://doi.org/10.1186/1471-2164-9-560

Jones, G. M. (1999, September 22). Proper dry cow management critical for mastitis control. The Cattle Site. Retrieved from https://www.thecattlesite.com/articles/696/proper-dry-cow-management-critical-for-mastitis-control (Accessed on 10/02/25)

Joyner, C., Mills, M. K., & Nayduch, D. (2013). Pseudomonas aeruginosa in *Musca domestica* L.: Temporospatial examination of bacterial population dynamics and house fly antimicrobial responses. PLoS ONE, 8(11), e79224. DOI: https://doi.org/10.1371/journal.pone.0079224

Kahlmeter, G., & Poulsen, H. O. (2012). Antimicrobial susceptibility of Escherichia coli from community-acquired urinary tract infections in Europe: The ECO·SENS study revisited. International Journal of Antimicrobial Agents, 39(1), 45–51.

Kameyama, M., Chuma, T., Yabata, J., Tominaga, K., Iwata, H., & Okamoto, K. (2013). Prevalence and epidemiological relationship of CMY-2 AmpC β-lactamase and CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* isolates from

broiler farms in Japan. *Journal of Veterinary Medical Science, 75*(8), 1009–1015. https://doi.org/10.1292/jvms.12-0453

Kao, C. Y., Wu, H. M., Lin, W. H., et al. (2016). Plasmid-mediated quinolone resistance determinants in quinolone-resistant Escherichia coli isolated from patients with bacteremia in a university hospital in Taiwan, 2001–2015. Scientific Reports, 6, 32281. DOI: https://doi.org/10.1038/srep32281

Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic Escherichia coli. Nature Reviews Microbiology, 2(2), 123–140.

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of Anaesthesiology, Clinical Pharmacology, 33(3), 300–305.

Karim, A., Poirel, L., Nagarajan, S., & Nordmann, P. (2001). Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. FEMS Microbiology Letters, 201(2), 237–241. DOI: https://doi.org/10.1111/j.1574-6968.2001.tb10762.x

Katzung, B. G., Vanderah, T. W., Trevor, A. J., Masters, S. B., & Kruidering-Hall, M. (2023). Basic and Clinical Pharmacology (16th ed.). McGraw-Hill.

Khamesipour, F., Lankarani, K. B., Honarvar, B., et al. (2018). A systematic review of human pathogens carried by the housefly (*Musca domestica* L.). BMC Public Health, 18, 1049. DOI: https://doi.org/10.1186/s12889-018-5934-3

Khan, H. A. A., Akram, W., Iqbal, J., & Naeem-Ullah, U. (2015). Thiamethoxam resistance in the house fly, *Musca domestica* L.: Current status, resistance selection, cross-resistance potential and possible biochemical mechanisms. PLoS ONE, 10(5), e0126722.

Klugman, K., Goldstein, F., Kohno, S., & Baquero, F. (1997). The role of fourth-

generation cephalosporins in the treatment of infections caused by penicillin-resistant streptococci. Clinical Microbiology and Infection, 3(Suppl. 1), S48–S60.

Kobayashi, M., Sasaki, T., Saito, N., Tamura, K., Suzuki, K., Watanabe, H., & Agui, N. (1999). Houseflies: Not simple mechanical vectors of enterohemorrhagic Escherichia coli O157:H7. American Journal of Tropical Medicine and Hygiene, 61(4), 625–629. DOI: https://doi.org/10.4269/ajtmh.1999.61.625

Kocsis, B., Gulyás, D., & Szabó, D. (2022). Emergence and dissemination of extraintestinal pathogenic high-risk international clones of Escherichia coli. Life, 12(12).

Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: From targets to networks. Nature Reviews Microbiology, 8(6), 423–435. DOI: https://doi.org/10.1038/nrmicro2333

Laguipo, A. B. B. (2021, April 22). House flies can carry SARS-CoV-2 up to 24 hours after exposure, study finds. News-Medical.net. Retrieved from https://www.news-medical.net/news/20210422/House-flies-can-carry-SARS-CoV-2-up-to-24-hours-after-exposure-study-finds.aspx [accessed July 2023]

Lee, J.-H. (2019). Perspectives towards antibiotic resistance: From molecules to population. Journal of Microbiology, 57(3), 181–184.

Lerminiaux, N., Mitchell, R., Bartoszko, J., Davis, I., Ellis, C., Fakharuddin, K., ... & Mataseje, L. (2023). Plasmid genomic epidemiology of blaKPC carbapenemase-producing Enterobacterales in Canada, 2010–2021. Antimicrobial Agents and Chemotherapy, 67(12), e00860-23. DOI: https://doi.org/10.1128/aac.00860-23

Levine, J. F. (1987). Vancomycin: A review. Medical Clinics of North America, 71(6), 1135– 1145.

Lima, T., Domingues, S., & Da Silva, G. J. (2020). Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. Veterinary Sciences, 7(3), 110.

Livermore, D. L. (1995). Beta-lactamases in laboratory and clinical resistance. Clinical Microbiology Review, 8, 557–584.

Lobanovska, M., & Pilla, G. (2017). Penicillin's discovery and antibiotic resistance: Lessons for the future? Yale Journal of Biology and Medicine, 90(1), 135–145.

Ludden, C., Coll, F., Gouliouris, T., Restif, O., Blane, B., Blackwell, G. A., ... & Peacock, S. J. (2021). Defining nosocomial transmission of Escherichia coli and antimicrobial resistance genes: A genomic surveillance study. The Lancet Microbe, 2(9), e472–e480. DOI: https://doi.org/10.1016/S2666-5247(21)00117-8

Mahdizade Ari, M., Dashtbin, S., Ghasemi, F., Shahroodian, S., Kiani, P., Bafandeh, E., ... & Darbandi, A. (2023). Nitrofurantoin: Properties and potential in treatment of urinary tract infection: A narrative review. Frontiers in Cellular and Infection Microbiology, 13, 1148603. DOI: https://doi.org/10.3389/fcimb.2023.1148603

Malekzadegan, Y., Khashei, R., Sedigh Ebrahim-Saraie, H., et al. (2018). Distribution of virulence genes and their association with antimicrobial resistance among uropathogenic Escherichia coli isolates from Iranian patients. BMC Infectious Diseases, 18, 572.

Madhouse Heaven. (2025). *Fresh fish and seafood for sale at Marsaxlokk market* [Photograph]. *The Next Crossing.* https://thenextcrossing.com/marsaxlokk-market-sunday *Image retrieved in 2024 from Google Images*

Malta National Park. (2023). *Farmer's market* [Photograph]. Malta National Park. https://maltanationalpark.mt

Manyi-Loh, C., Mamphweli, S., Meyer, E., & Okoh, A. (2018). Antibiotic use in

agriculture and its consequential resistance in environmental sources: Potential public health implications. Molecules, 23(4), 795.

Maron, D. F., Smith, T. J. S., & Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey.

Marti, R., Scott, A., Tien, Y.-C., et al. (2013). The impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil, and on vegetables at harvest. Applied and Environmental Microbiology, 79(18).

Martin, K. E. (2013). Treatment of ESBL-producing Escherichia coli lower urinary tract infection. Pharmacy Practice in Focus: Health Systems, 2(6).

Medeiros, A. A. (1997). Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clinical Infectious Diseases, 24(Suppl), S19–S45. Melican, K., Sandoval, R. M., Kader, A., Josefsson, L., Tanner, G. A., Molitoris, B. A., & Richter-Dahlfors, A. (2011). Uropathogenic Escherichia coli P and type 1 fimbriae act in synergy in a living host to facilitate renal colonization leading to nephron obstruction. PLoS Pathogens, 7(2), e1001298. DOI: https://doi.org/10.1371/journal.ppat.1001298

Mellmann, A., Köck, R., & Becker, K. (2016). A geospatial analysis of flies and the spread of antimicrobial resistant bacteria. International Journal of Medical Microbiology, 306(7), 566–571.

Mendonça, J., Guedes, C., Silva, C., Sá, S., Oliveira, M., Accioly, G., ... & Fernandes, R. (2022). New CTX-M group conferring β-lactam resistance: A compendium of phylogenetic insights from biochemical, molecular, and structural biology. Biology, 11(2), 256.

Metwaly, A. M., Ghoneim, M. M., Eissa, I. H., Elsehemy, I. A., Mostafa, A. E., Hegazy,

M. M., ... & Dou, D. (2021). Traditional ancient Egyptian medicine: A review. Saudi Journal of Biological Sciences, 28(10), 5823–5832.

Moellering, R. C., Jr. (1983). Rationale for use of antimicrobial combinations. The American Journal of Medicine, 75(2A), 4–8.

Mueller, M., & Tainter, C. R. (2023, July 13). Escherichia coli infection. In StatPearls. StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK557578/ [accessed July 2023]

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. Microbiology Spectrum, 4(2), 10.1128/microbiolspec.VMBF-0016-2015. DOI: https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

Murillo, A. C., Cox, D., & Mullens, B. A. (2018). Longevity of fly baits exposed to field conditions. Journal of Economic Entomology, 111(2), 967–973. DOI: https://doi.org/10.1093/jee/toy016

Murvosh, C. M., & Thaggard, C. W. (1966). Ecological studies of the house fly. Annals of the Entomological Society of America, 59(3), 533–547. DOI: https://doi.org/10.1093/aesa/59.3.533

Muteeb, G., Rehman, M. T., Shahwan, M., & Aatif, M. (2023). Origin of antibiotics and antibiotic resistance, and their impacts on drug development: A narrative review. Pharmaceuticals (Basel), 16(11), 1615.

Nathan, C., & Cars, O. (2014). Antibiotic resistance—Problems, progress, and prospects. The New England Journal of Medicine, 371, 1761–1763.

National Committee for Clinical Laboratory Standards. (2000). Performance standards for antimicrobial susceptibility testing. Tenth Informational Supplement (Aerobic Dilution), M100-S10. Wayne, PA: NCCLS.

National Statistics Office Malta. (2023, January 7). *Census of Agriculture - 2020 Report*.

https://nso.gov.mt/themes_publications/agricensus-2020/

Nayduch, D., & Burrus, R. G. (2017). Flourishing in filth: House fly–microbe interactions across life history. Annals of the Entomological Society of America, 110(1), 6–18. DOI: https://doi.org/10.1093/aesa/saw083

Nayduch, D., & Burrus, R. G. (2022). House flies as vectors for antimicrobial-resistant bacteria: A threat to public health? Current Opinion in Insect Science, 49, 31–36. DOI: https://doi.org/10.1016/j.cois.2021.12.004

Nayduch, D., & Joyner, C. (2013). Expression of antimicrobial peptide genes in the house fly (*Musca domestica* L.) larval midgut in response to bacterial feeding. Developmental & Comparative Immunology, 39(3), 295–303. DOI: https://doi.org/10.1016/j.dci.2012.10.007

Nayduch, D., Noblet, G. P., & Stutzenberger, F. J. (2002). Vector potential of house flies for *Campylobacter jejuni*. American Journal of Veterinary Research, 63(2), 211–215. DOI: https://doi.org/10.2460/ajvr.2002.63.211

Nelson, R. E., Hatfield, K. M., Wolford, H., Samore, M. H., Scott, R. D., Reddy, S. C., ... & Jernigan, J. A. (2021). National estimates of healthcare costs associated with multidrug- resistant bacterial infections among hospitalized patients in the United States. Clinical Infectious Diseases, 72(Supplement_1), S17–S26. DOI: https://doi.org/10.1093/cid/ciaa1581

Nesporova, K., Cerny, M., & Maixnerova, M. (2023). Review: Plasmid-mediated resistance to beta-lactams—A narrative review. Antibiotics, 12(5), 779. DOI: https://doi.org/10.3390/antibiotics12050779

Ngbede, E. O., Raji, M. A., Kwanashie, C. N., Kwaga, J. K. P., Odugbo, M. O., Kumbish, P. R., ... & Adikwu, A. A. (2020). Risk factors for multidrug-resistant Escherichia coli in Nigerian poultry farms. Veterinary Medicine and Science, 6(4), 899–908.

DOI: https://doi.org/10.1002/vms3.308

Nikaido, H. (2009). Multidrug resistance in bacteria. Annual Review of Biochemistry, 78, 119–146. DOI: https://doi.org/10.1146/annurev.biochem.78.082907.145923

Nikaido, H., & Pagès, J.-M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiology Reviews, 36(2), 340–363. DOI: https://doi.org/10.1111/j.1574-6976.2011.00290.x

Nordmann, P., Naas, T., & Poirel, L. (2011). Global spread of Carbapenemase-producing Enterobacteriaceae. Emerging Infectious Diseases, 17(10), 1791–1798. DOI: https://doi.org/10.3201/eid1710.110655

Nwafia, I. N., Ohanu, M. E., Ebede, S. O., & Ozumba, U. C. (2019). Molecular detection and sequencing of beta-lactamase genes responsible for ESBL-producing Escherichia coli and Klebsiella pneumoniae isolates from abattoirs in Enugu State, Nigeria. Annals of Clinical Microbiology and Antimicrobials, 18, 6. DOI: https://doi.org/10.1186/s12941-019-0292-0

O'Neill, J. (2016). Tackling drug-resistant infections globally: Final report and recommendations. The Review on Antimicrobial Resistance. Retrieved from https://amr-review.org [accessed July 2023]

Odwar, J. A., Kikuvi, G. M., Kariuki, J. N., Kariuki, S., & Onyango, D. M. (2014). Risk factors for infection with extended-spectrum beta-lactamase producing Enterobacteriaceae in patients admitted at a hospital in Kenya. BMC Infectious Diseases, 14, 373. DOI: https://doi.org/10.1186/1471-2334-14-373

Ojer-Usoz, E., González, D., Vitas, A. I., & Leiva, J. (2014). Detection of extended-spectrum β-lactamase producing Escherichia coli in raw meat samples from Navarra, Spain. Microbial Drug Resistance, 20(5), 437–441. DOI: https://doi.org/10.1089/mdr.2013.0156

Olaitan, A. O., Morand, S., & Rolain, J.-M. (2014). Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. Frontiers in Microbiology, 5, 643. DOI: https://doi.org/10.3389/fmicb.2014.00643

Oliver, S. P., Murinda, S. E., & Jayarao, B. M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: A comprehensive review. Foodborne Pathogens and Disease, 8(3), 337–355. DOI: https://doi.org/10.1089/fpd.2010.0730

Onwugamba F.C., Fitzgerald J.R., Rochon K., Guardabassi L., Alabi A., Kühne S., Grobusch M.P., Schaumburg F. (2018) *The role of "filth flies" in the spread of antimicrobial resistance.* Travel Medicine and Infectious Disease, 22:8-17.

Opintan, J. A., Newman, M. J., Arhin, R. E., Donkor, E. S., Gyansa-Lutterodt, M., & Mills- Pappoe, W. (2015). Laboratory-based nationwide surveillance of antimicrobial resistance in Ghana. Infection and Drug Resistance, 8, 379–389. DOI: https://doi.org/10.2147/IDR.S85542

Otter, J. A., Mutters, N. T., Tacconelli, E., Gikas, A., Holmes, A. H., & Working Group 1 of the Healthcare Infection Society & ESGAP. (2015). Controversies in guidelines for the control of multidrug-resistant Gram-negative bacteria in EU hospitals. Clinical Microbiology and Infection, 21(12), 1057–1066. DOI: https://doi.org/10.1016/j.cmi.2015.09.021

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., ... & Kluytmans, J. (2011). Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerging Infectious Diseases, 17(7), 1216–1222. DOI: https://doi.org/10.3201/eid1707.110209

Google. (2025). *Map of southeastern Malta showing Wied il-Għajn and surrounding districts* [Map]. Google Maps. https://www.google.com/maps

Padfield, N. (2021, August 2). *Musca domestica* (House fly). Animal Diversity Web. Panda, A. K., Barman, N. N., Das, S. K., & Sarma, D. K. (2014). Isolation and characterization of Escherichia coli from house flies (*Musca domestica*) with special reference to their public health importance. Veterinary World, 7(2), 82–86. DOI: https://doi.org/10.14202/vetworld.2014.82-86

Panoramic view of Marsaxlokk fishing village in southeastern Malta, known for its traditional *luzzu* fishing boats and weekly open-air market. *From Visit Malta (2023), "Marsaxlokk fishing village" [Photograph], Visit Malta. https://www.visitmalta.com.*

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. Clinical Microbiology Reviews, 31(4), e00088-17. DOI: https://doi.org/10.1128/CMR.00088-17

Perry, J. A., & Wright, G. D. (2013). The antibiotic resistance "mobilome": Searching for the link between environment and clinic. Frontiers in Microbiology, 4, 138. DOI: https://doi.org/10.3389/fmicb.2013.00138

Peymani, A., Naserpour Farivar, T., Nikmanesh, B., & Farahani, A. (2017). Prevalence and antimicrobial resistance pattern of ESBL producing Escherichia coli isolates from patients with urinary tract infection. Archives of Clinical Infectious Diseases, 12(1), e40820. DOI: https://doi.org/10.5812/archcid.40820

Piddock, L. J. V. (2006). Multidrug-resistance efflux pumps? Not just for resistance. Nature Reviews Microbiology, 4(8), 629–636. DOI: https://doi.org/10.1038/nrmicro1464

Pitout, J. D. D., & Laupland, K. B. (2008). Extended-spectrum β-lactamase-producing Enterobacteriaceae: An emerging public-health concern. The Lancet Infectious Diseases, 8(3), 159–166. DOI: https://doi.org/10.1016/S1473-3099(08)70041-0

Pitout, J. D. D., & Nordmann, P. (2005). Emergence of Enterobacteriaceae producing extended- spectrum β-lactamases (ESBLs) in the community. Antimicrobial Agents and Chemotherapy, 49(8), 3261–3266. DOI: https://doi.org/10.1128/AAC.49.8.3261- 3266.2005

Poirel, L., Madec, J.-Y., Lupo, A., Schink, A.-K., Kieffer, N., Nordmann, P., & Schwarz, S. (2018). Antimicrobial resistance in Escherichia coli. Microbiology Spectrum, 6(4). DOI: https://doi.org/10.1128/microbiolspec.ARBA-0026-2017

Poirel, L., Naas, T., & Nordmann, P. (2010). Diversity, epidemiology, and genetics of class D β-lactamases. Antimicrobial Agents and Chemotherapy, 54(1), 24–38. DOI: https://doi.org/10.1128/AAC.01512-09

Poirel, L., Potron, A., & Nordmann, P. (2012). OXA-48-like carbapenemases: The phantom menace. Journal of Antimicrobial Chemotherapy, 67(7), 1597–1606. DOI: https://doi.org/10.1093/jac/dks121

Poirel, L., Walsh, T. R., Cuvillier, V., & Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. Diagnostic Microbiology and Infectious Disease, 70(1), 119–123. DOI: https://doi.org/10.1016/j.diagmicrobio.2010.12.002

Ponce-Alonso, M., Sáez de la Fuente, J., Rincón-Carlavilla, A., et al. (2021). Evolution of antimicrobial resistance in a hospital during the COVID-19 pandemic: A genomic and epidemiological perspective. Clinical Microbiology and Infection, 27(12), 1772.e1– 1772.e5. DOI: https://doi.org/10.1016/j.cmi.2021.07.009

Prescott, J. F. (2000). Antimicrobial drug use in food animals: The global perspective. Animal Biotechnology, 11(1), 115–122. DOI: https://doi.org/10.1080/10495390009525809

Qian, X., Gu, J., Sun, W., Wang, X.-J., & Su, J.-Q. (2018). Diversity, abundance, and

persistence of antibiotic resistance genes in various types of animal manure following industrial composting. Journal of Hazardous Materials, 344, 716–722. DOI: https://doi.org/10.1016/j.jhazmat.2017.11.020

Ranjan, A., Shaik, S., Mondal, A., Nandanwar, N., Hussain, A., Semmler, T., ... & Ahmed, N. (2016). Molecular epidemiology and genome dynamics of New Delhi metallo-β-lactamase-producing extraintestinal pathogenic Escherichia coli strains from India. Antimicrobial Agents and Chemotherapy, 60(11), 6795–6805. DOI: https://doi.org/10.1128/AAC.01179-16

Rees, J., Stokes, H. W., Smith, M. A., & Roy Chowdhury, P. (2021). Mobile genetic elements carry a secret cargo: Functional and regulatory genes embedded within antibiotic resistance gene cassettes. Current Genetics, 67, 71–80. DOI: https://doi.org/10.1007/s00294-020-01106-0

Regev-Yochay, G., Raz, M., Dagan, R., et al. (2004). Association between carriage of antibiotic-resistant Streptococcus pneumoniae and the use of antibiotics by children: A prospective cohort study. The Lancet, 363(9420), 855–863. DOI: https://doi.org/10.1016/S0140-6736(04)15797-0

Riley, L. W. (2014). Pandemic lineages of extraintestinal pathogenic Escherichia coli. Clinical Microbiology and Infection, 20(5), 380–390. DOI: https://doi.org/10.1111/1469- 0691.12646

Roberts, M. C. (2003). Acquired tetracycline and macrolide-lincosamide-streptogramin resistance genes and their dissemination. Antonie van Leeuwenhoek, 84, 111–122. DOI: https://doi.org/10.1023/A:1026015918503

Rolain, J.-M., Parola, P., & Cornaglia, G. (2010). New Delhi metallo-β-lactamase (NDM-1): Towards a new pandemia? Clinical Microbiology and Infection, 16(12), 1699–1701. DOI: https://doi.org/10.1111/j.1469-0691.2010.03385.x

Rossolini, G. M., Arena, F., Pecile, P., & Pollini, S. (2014). Update on the antibiotic resistance crisis. Current Opinion in Pharmacology, 18, 56–60. DOI: https://doi.org/10.1016/j.coph.2014.09.006

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., ... & Mevius, D. J. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. Journal of Antimicrobial Chemotherapy, 73(5), 1121–1137. DOI: https://doi.org/10.1093/jac/dkx488

Russo, T. A., & Johnson, J. R. (2000). Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC. Journal of Infectious Diseases, 181(5), 1753–1754. DOI: https://doi.org/10.1086/315418

Sáez-López, E., Guiral, E., & Soto, S. M. (2022). The epidemiology of Escherichia coli urinary tract infections. Microorganisms, 10(9), 1851.

Sáez-López, E., Guiral, E., Fernández-Orth, D., et al. (2016). Characterization of Uropathogenic Escherichia coli from pregnant and non-pregnant women. International Journal of Environmental Research and Public Health, 13(12), 124. DOI: https://doi.org/10.3390/ijerph13121124

Saisi, H., Makobe, C., Kangongo, M., & Kariuki, S. (2019). Prevalence of CTXM, SHV, TEM and OXA genes among extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* from Mukuru Slum, Kenya. *Advances in Microbiology, 9*(10), 785–797. https://doi.org/10.4236/aim.2019.910052

Sakineh, A., Yousef, M., Mohammad Reza, A., et al. (2019). Distribution of virulence factors in uropathogenic Escherichia coli isolated from women with urinary tract infections. African Journal of Microbiology Research, 13(1), 1–9.

Salehi, T. Z., Badouei, M. A., Ghazisaeedi, F., et al. (2014). Virulence factors and antimicrobial resistance properties of uropathogenic Escherichia coli isolates from

Iranian hospital patients. Tropical Biomedicine, 31(1), 143–149.

Salinas, L., Cárdenas, P., Johnson, T. J., et al. (2021). Environmental spread of extended-spectrum beta-lactamase (ESBL) producing Escherichia coli in rural Ecuador. Environmental Health Perspectives, 129(6), 067005. DOI: https://doi.org/10.1289/EHP8650

Santos, M. N. P. dos, Silva, T. R. da, Amato, C. B., & Zanetti, M. O. (2021). Extended-spectrum β-lactamases: Classification, epidemiology and detection. Revista do Instituto de Medicina Tropical de São Paulo, 63, e69. DOI: https://doi.org/10.1590/S1678-9946202163069

Sawant, A. A., Sordillo, L. M., Jayarao, B. M. (2005). A survey on antibiotic usage in dairy herds in Pennsylvania. Journal of Dairy Science, 88(8), 2991–2999. DOI: https://doi.org/10.3168/jds.S0022-0302(05)72979-9

Schmid, D., Fretz, R., Winter, P., Mann, M., Höger, G., Stöger, A., ... & Allerberger, F. (2007). Outbreak of acute gastroenteritis in an Austrian boarding school, 2006. Wiener Klinische Wochenschrift, 119(7–8), 236–241.

Schroeder, C. M., Zhao, C., DebRoy, C., Torcolini, J., Zhao, S., White, D. G., ... & Meng, J. (2002). Antimicrobial resistance of Escherichia coli O157 isolated from humans, cattle, swine, and food. Applied and Environmental Microbiology, 68(2), 576–581. DOI: https://doi.org/10.1128/AEM.68.2.576-581.2002

Schwarz, S., Loeffler, A., & Kadlec, K. (2017). Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine. Advances in Veterinary Dermatology, 8, 31– 42.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., & Zhu, K. Y. (2013). Towards the elements of successful insect RNAi. Journal of Insect Physiology, 59(12), 1212–1221. DOI:

https://doi.org/10.1016/j.jinsphys.2013.08.014

Seiffert, S. N., Hilty, M., Perreten, V., & Endimiani, A. (2013). Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: An emerging problem for human health? Drug Resistance Updates, 16(1–2), 22–45. DOI: https://doi.org/10.1016/j.drup.2012.12.001

Shaheen, B. W., Nayak, R., Foley, S. L., Kweon, O., Deck, J., Park, M., & Boothe, D. M. (2011). Molecular characterization of resistance to extended-spectrum cephalosporins in clinical isolates of Escherichia coli from dogs in the United States. Antimicrobial Agents and Chemotherapy, 55(2), 566–575. DOI: https://doi.org/10.1128/AAC.00995-10

Shakya, P., Barrett, P., Diwan, V., Stålsby Lundborg, C., Marrone, G., & Joshi, A. B. (2013). Antibiotic resistance among Escherichia coli isolates from stool samples of children aged 3 to 14 years from Ujjain, India. BMC Infectious Diseases, 13, 477. DOI: https://doi.org/10.1186/1471-2334-13-477

Sharma, A., Chandraker, S., Patel, V. K., & Ramteke, P. (2009). Antibacterial activity of medicinal plants against pathogens causing complicated urinary tract infections. Indian Journal of Pharmaceutical Sciences, 71(2), 136–139.

Shehabi, A. A., & Bulos, N. K. (1998). Resistance patterns and plasmid profiles of Escherichia coli isolated from children with diarrhoea in Jordan. Journal of Diarrhoeal Diseases Research, 16(3), 195–200.

Shrestha, B., Tada, T., Shrestha, S., Ohara, H., Kirikae, T., & Sherchand, J. B. (2017). Emergence of an extensively drug-resistant Escherichia coli co-producing NDM-5 and OXA-181 in Nepal. International Journal of Infectious Diseases, 57, 107–108. DOI: https://doi.org/10.1016/j.ijid.2017.01.036

Silbergeld, E. K., Graham, J., & Price, L. B. (2008). Industrial food animal production,

antimicrobial resistance, and human health. Annual Review of Public Health, 29, 151– 169. DOI: https://doi.org/10.1146/annurev.publhealth.29.020907.090904

Singh, P., & Prakash, A. (2021). Review on multidrug-resistant bacteria and their roles in environmental contamination. Environmental Science and Pollution Research, 28(36), 50484–50496. DOI: https://doi.org/10.1007/s11356-021-14769-7

Singh, S. P., & Kapoor, V. (2022). Antibiotics: Classification and mechanisms of action with emphasis on antibiotic resistance. Acta Scientific Microbiology, 5(9), 42–47.

Skariyachan, S., Taskeen, N., Ganta, M., & Venkata Krishna, B. (2018). Recent perspectives on the virulent factors and treatment options for multidrug-resistant Escherichia coli infections. 3 Biotech, 8, 512. DOI: https://doi.org/10.1007/s13205-018-1493-2

Smith, H. W. (1969). Transfer of resistance factors from animal and human strains of Escherichia coli to resident strains in the human intestine. The Lancet, 294(7622), 1174– 1176. DOI: https://doi.org/10.1016/S0140-6736(69)92214-4

Smith, J. L., Fratamico, P. M., & Gunther, N. W. (2007). Extraintestinal pathogenic Escherichia coli. Foodborne Pathogens and Disease, 4(2), 134–163. DOI: https://doi.org/10.1089/fpd.2007.0087

Sniegocki, T., Gbylik-Sikorska, M., & Posyniak, A. (2021). A review on the development of antimicrobial resistance in the environment and its mitigation by antibiotics used in veterinary medicine. Journal of Veterinary Research, 65(4), 289–298. DOI: https://doi.org/10.2478/jvetres-2021-0040

Snyder, A. B., & Worobo, R. W. (2014). Microbial safety of fresh produce. Annual Review of Food Science and Technology, 5, 263–285. DOI: https://doi.org/10.1146/annurev-food- 030713-092753

Solomkin, J. S., Jenkins, J. M., & Nichol, K. L. (2007). Resistance in the management of

intra-abdominal infections. Infectious Disease Clinics of North America, 21(3), 547–564. DOI: https://doi.org/10.1016/j.idc.2007.05.008

Sommer, M. O. A., Dantas, G., & Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. Science, 325(5944), 1128– 1131. DOI: https://doi.org/10.1126/science.1176950

Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., & Ercolini, D. (2016). Coexistence of lactic acid bacteria and potential spoilage microbiota in a dairy processing environment. Applied and Environmental Microbiology, 82(16), 4672–4681. DOI: https://doi.org/10.1128/AEM.01172-16

Stepanović, S., Vuković, D., Hola, V., et al. (2007). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS, 115(8), 891–899. DOI: https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

Stock, I. (2010). Natural antimicrobial susceptibility patterns and biochemical profiles of Escherichia coli strains isolated from animal and human sources. Journal of Medical Microbiology, 59(8), 975–986. DOI: https://doi.org/10.1099/jmm.0.019646-0

Su, J. Q., Wei, B., Xu, C. Y., et al. (2012). Antibiotic resistome in a large-scale healthy human gut microbiota deciphered by metagenomic approach. Environmental Microbiology, 14(1), 109–119. DOI: https://doi.org/10.1111/j.1462-2920.2011.02589.x

Sunde, M., & Norström, M. (2006). The use of tetracyclines in Norwegian animal farming and its impact on antimicrobial resistance. Journal of Antimicrobial Chemotherapy, 58(1), 161–166. DOI: https://doi.org/10.1093/jac/dkl181

Sunset over mountains. (n.d.). [Photograph]. Google Images. Retrieved November 2024, from https://images.google.com

Tadesse, D. A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M. J., & McDermott, P.F. (2012). Antimicrobial drug resistance in Escherichia coli from humans and food animals, United States, 1950–2002. Emerging Infectious Diseases, 18(5), 741–749. DOI: https://doi.org/10.3201/eid1805.111153

Takkar, S., Shrivastava, D., & Kumar, N. (2020). Antibiotic resistance: A global concern. International Journal of Research in Medical Sciences, 8(9), 3257–3262. DOI: https://doi.org/10.18203/2320-6012.ijrms20203450

Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., ... & Ghali, W. A. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: A systematic review and meta-analysis. The Lancet Planetary Health, 1(8), e316–e327. DOI: https://doi.org/10.1016/S2542-5196(17)30141-9

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010). The population genetics of commensal Escherichia coli. Nature Reviews Microbiology, 8(3), 207–217. DOI: https://doi.org/10.1038/nrmicro2298

Thanner, S., Drissner, D., & Walsh, F. (2016). Antimicrobial resistance in agriculture. mBio, 7(2), e02227-15. DOI: https://doi.org/10.1128/mBio.02227-15

The Review on Antimicrobial Resistance. (2014, December). *Antimicrobial resistance: Tackling a crisis for the health and wealth of nations.* HM Government & Wellcome Trust.

Thomson, K. S. (2010). Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. Journal of Clinical Microbiology, 48(4), 1019–1025. DOI: https://doi.org/10.1128/JCM.00212-10

Torres, E. G., & Reveles, K. R. (2021). Antimicrobial resistance: More than an infectious disease problem. Journal of the American College of Clinical Pharmacy, 4(10),

1234– 1243. DOI: https://doi.org/10.1002/jac5.1505

Tschudin-Sutter, S., Lucet, J. C., Mutters, N. T., & Stewardson, A. J. (2020). Environmental contamination of patient rooms with extended-spectrum β-lactamase-producing Enterobacterales: Significant but manageable. Current Opinion in Infectious Diseases, 33(4), 353–358. DOI: https://doi.org/10.1097/QCO.0000000000000662

Tshisevhe, V. S., Lekota, K. E., & Sekwadi, P. (2022). Extended spectrum β-lactamase-producing Enterobacteriaceae in livestock and the environment: A review. Veterinary World, 15(4), 900–910. DOI: https://doi.org/10.14202/vetworld.2022.900-910

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., ... & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences, 112(18), 5649–5654. DOI: https://doi.org/10.1073/pnas.1503141112

Van der Bij, A. K., & Pitout, J. D. D. (2012). The role of international travel in the worldwide spread of multiresistant Enterobacteriaceae. Journal of Antimicrobial Chemotherapy, 67(9), 2090–2100. DOI: https://doi.org/10.1093/jac/dks191

van Duin, D., & Paterson, D. L. (2016). Multidrug-resistant bacteria in the community: Trends and lessons learned. Infectious Disease Clinics of North America, 30(2), 377–390. DOI: https://doi.org/10.1016/j.idc.2016.02.004

Van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., & Aarts, H. J. (2011). Acquired antibiotic resistance genes: An overview. Frontiers in Microbiology, 2, 203. DOI: https://doi.org/10.3389/fmicb.2011.00203

Varga, C., Ferro, P. J., Sherwood, J. S., & Scott, H. M. (2009). Assessing environmental load of antimicrobial-resistant enteric bacteria from waste application sites in

agriculture. Applied and Environmental Microbiology, 75(2), 794–800. DOI: https://doi.org/10.1128/AEM.01798-08

Ventola, C. L. (2015). The antibiotic resistance crisis: Part 1: Causes and threats. Pharmacy and Therapeutics, 40(4), 277–283.

Ventola, C. L. (2015). The antibiotic resistance crisis: Part 2: Management strategies and new agents. Pharmacy and Therapeutics, 40(5), 344–352.

Vittecoq, M., Laurens, C., Brazier, L., Durand, P., Elguero, E., Arnal, A., ... & Renaud, F. (2017). VIM-1 carbapenemase-producing Escherichia coli in gulls from southern France. Ecology and Evolution, 7(4), 1224–1232. DOI: https://doi.org/10.1002/ece3.2707

Vora, P., Johner, K. A., & Callaghan, M. (2023). Uropathogenic Escherichia coli. StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK470472/ [accessed July 2024]

Wagner, D., & Hewlett, A. (2011). Survival of bacterial pathogens on paper and bacterial retrieval from paper to hands: Preliminary results. American Journal of Infection Control, 39(2), e1–e2. DOI: https://doi.org/10.1016/j.ajic.2010.10.010

Wang, C., Li, M., Tian, Y., et al. (2023). Global prevalence, risk factors and outcomes of extended-spectrum β-lactamase-producing Enterobacteriaceae in urinary tract infections: A meta-analysis. Journal of Global Antimicrobial Resistance, 33, 95–105. DOI: https://doi.org/10.1016/j.jgar.2023.02.015

Wang, J., Wang, S., Shen, J., et al. (2016). Use of antimicrobial agents in China and the potential for stewardship: A cross-sectional study. The Lancet Infectious Diseases, 16(7), 757– 765. DOI: https://doi.org/10.1016/S1473-3099(16)30094-8

Wellington, E. M. H., Boxall, A. B. A., Cross, P., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. The

Lancet Infectious Diseases, 13(2), 155–165. DOI: https://doi.org/10.1016/S1473-3099(12)70317-1

WHO (World Health Organization). (2017). Global action plan on antimicrobial resistance. Retrieved from https://www.who.int/publications/i/item/9789241509763 [accessed July 2023]

WHO (World Health Organization). (2020). Antimicrobial resistance. Retrieved from https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance [accessed July 2023]

WHO (World Health Organization). (2023). Antimicrobial resistance and the COVID-19 pandemic: A double threat. Retrieved from https://www.who.int/news/item/18-11-2023-antimicrobial-resistance-and-the-covid-19-pandemic [accessed July 2023]

Wiles, T. J., Kulesus, R. R., & Mulvey, M. A. (2008). Origins and virulence mechanisms of uropathogenic Escherichia coli. Experimental and Molecular Pathology, 85(1), 11– 19. DOI: https://doi.org/10.1016/j.yexmp.2008.03.007

Williams, N. T. (2014). The benefits of probiotics. Pharmacy and Therapeutics, 39(8), 536– 543.

Wong, A. (2019). Unknown risk of antibiotic resistance in the environment: A call for research and action. Applied and Environmental Microbiology, 85(13), e00645-19. DOI: https://doi.org/10.1128/AEM.00645-19

Woodford, N., & Livermore, D. M. (2009). Infections caused by Gram-positive bacteria: A review of the global challenge. Journal of Infection, 59(Suppl 1), S4–S16. DOI: https://doi.org/10.1016/S0163-4453(09)70004-1

Woolhouse, M.E.J. (2024). One Health approaches to Tackling Antimicrobial Resistance. *Science in One Health*,3, 100082. DOI: https://doi.org/10.1016/j.soh.2024.100082

Wright, G. D. (2007). The antibiotic resistome: The nexus of chemical and genetic diversity. Nature Reviews Microbiology, 5(3), 175–186. DOI: https://doi.org/10.1038/nrmicro1614

Wu, D., Huang, W., Yu, W., et al. (2023). Epidemiology and molecular characterization of multidrug-resistant Escherichia coli from urinary tract infections: A multicenter study in China. Frontiers in Microbiology, 14, 1116714. DOI: https://doi.org/10.3389/fmicb.2023.1116714

Xia, X., Meng, J., Zhao, S., et al. (2011). Antimicrobial resistance of Salmonella isolates from retail meats in the United States. Foodborne Pathogens and Disease, 8(4), 495–501. DOI: https://doi.org/10.1089/fpd.2010.0715

Yang, X., Wu, Q., Zhang, J., et al. (2019). Prevalence, antibiotic resistance, and genetic diversity of Escherichia coli isolated from retail raw meats in China. Frontiers in Microbiology, 10, 497. DOI: https://doi.org/10.3389/fmicb.2019.00497

Yao, Y., Lazaro-Perona, F., Bernabeu, S., et al. (2023). Whole-genome analysis of multidrug- resistant Escherichia coli from wastewater treatment plants in Spain. Microorganisms, 11(2), 401. DOI: https://doi.org/10.3390/microorganisms11020401

Yao, Y., Xie, Q., Zhang, L., et al. (2022). Characterization of antimicrobial resistance in Escherichia coli isolated from environmental and clinical sources. Antibiotics, 11(5), 610. DOI: https://doi.org/10.3390/antibiotics11050610

Yilmaz, M., Elaldi, N., Akalin, S., et al. (2009). The role of extended-spectrum beta-lactamases in nosocomial infections. Journal of Infection in Developing Countries, 3(5), 340– 345. DOI: https://doi.org/10.3855/jidc.240

Yoo, H. J., Ha, J. Y., & Lee, H. J. (2023). Environmental spread of antimicrobial resistance

and the role of wastewater in resistance transmission. Environmental Research, 220, 115117. DOI: https://doi.org/10.1016/j.envres.2022.115117

Zaidi, M. B., McDermott, P. F., Fedorka-Cray, P. J., et al. (2006). Antimicrobial-resistant bacterial infections from food-producing animals. Foodborne Pathogens and Disease, 3(4), 346–355. DOI: https://doi.org/10.1089/fpd.2006.3.346

Zhang, Q. Q., Ying, G. G., Pan, C. G., et al. (2015). Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance. Environmental Science & Technology, 49(11), 6772– 6782. DOI: https://doi.org/10.1021/acs.est.5b00729

Zhang, X. X., Zhang, T., & Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. Applied Microbiology and Biotechnology, 82, 397–414. DOI: https://doi.org/10.1007/s00253-008-1829-z

Zhao, S., Blickenstaff, K., Glenn, A., et al. (2009). Antimicrobial resistance in Escherichia coli from retail meats and its association with resistance in humans. Journal of Antimicrobial Chemotherapy, 64(4), 778–785. DOI: https://doi.org/10.1093/jac/dkp287

Zhou, L. J., Ying, G. G., Liu, S., et al. (2013). Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in South China. Science of The Total Environment, 452–453, 365–376. DOI: https://doi.org/10.1016/j.scitotenv.2013.02.050

Zhou, Y., Ma, W., Yang, X., et al. (2021). Molecular characterization of ESBL-producing Escherichia coli from urinary tract infections in China. Frontiers in Microbiology, 12, 712332. DOI: https://doi.org/10.3389/fmicb.2021.712332

Zhu, Y. G., Gillings, M., Simonet, P., et al. (2017). Microbial mass movements: Monitoring antibiotic resistance genes in the environment. Environmental Science &

Technology, 51(10), 5259–5270. DOI: https://doi.org/10.1021/acs.est.7b00364

Zou, M., Keelara, S., Thakur, S. (2021). Multidrug resistance and virulence characteristics of Escherichia coli from swine and farm workers in North Carolina. Journal of Global Antimicrobial Resistance, 27, 186–192. DOI: https://doi.org/10.1016/j.jgar.2021.08.007

Zowawi, H. M., Balkhy, H. H., Walsh, T. R., & Paterson, D. L. (2013). β-Lactamase production in key Gram-negative pathogen isolates from the Arabian Peninsula. Clinical Microbiology Reviews, 26(3), 361–380. DOI: https://doi.org/10.1128/CMR.00096-12

Zowawi, H. M., Harris, P. N., Roberts, M. J., et al. (2015). The emerging threat of multidrug- resistant Gram-negative bacteria in urology. Nature Reviews Urology, 12(10), 570– 584. DOI: https://doi.org/10.1038/nrurol.2015.199

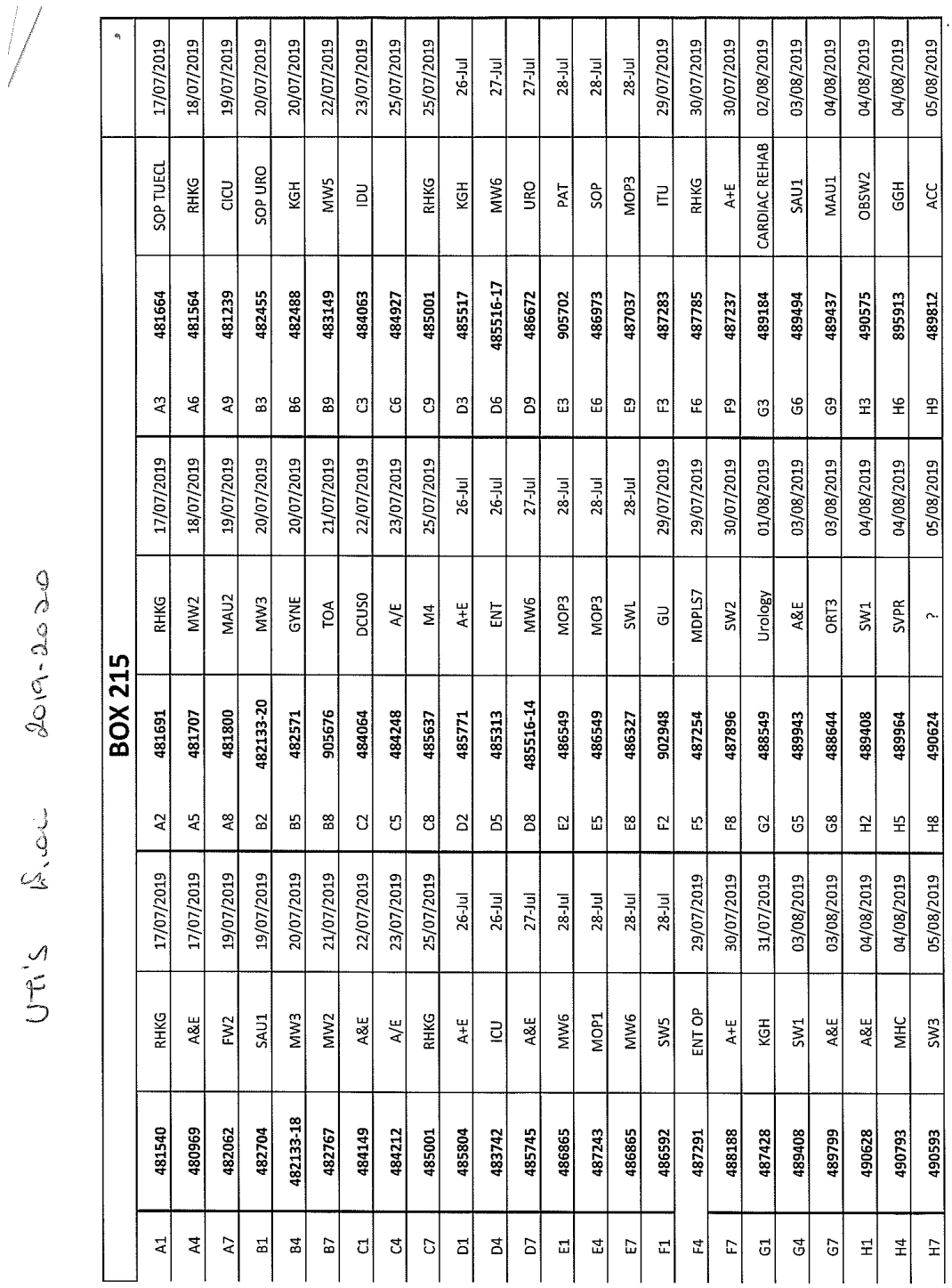
Zowawi, H. M., Paterson, D. L., & Walsh, T. R. (2015). Extended-spectrum β-lactamases and carbapenemases in the Middle East. Lancet Infectious Diseases, 15(9), 1001–1013. DOI: https://doi.org/10.1016/S1473-3099(15)00152-7

Zowawi, H. M., Sartor, A. L., Sidjabat, H. E., et al. (2014). Molecular epidemiology of carbapenem-resistant Enterobacteriaceae in the Gulf Cooperation Council states: Dominance of OXA-48 and NDM producers. Antimicrobial Agents and Chemotherapy, 58(6), 3085–3090. DOI: https://doi.org/10.1128/AAC.02050-13

Appendix A: Sample Collection Identification, Lab Kits, Protocols and Standard Operating Procedures

A.1 Sample Collection Identification Sites (Attached already)

A.1.1 Patient UTI Box Numbers



UTI's March 2019-20 20

BOX 215

A1	**481540**	RHKG	17/07/2019	A2	**481691**	RHKG	17/07/2019	A3	**481664**	SOP TUECL	17/07/2019
A4	**480969**	A&E	17/07/2019	A5	**481707**	MW2	18/07/2019	A6	**481564**	RHKG	18/07/2019
A7	**482062**	FW2	19/07/2019	A8	**481800**	MAU2	19/07/2019	A9	**481239**	CICU	19/07/2019
B1	**482704**	SAU1	19/07/2019	B2	**482133-20**	MW3	20/07/2019	B3	**482455**	SOP URO	20/07/2019
B4	**482133-18**	MW3	20/07/2019	B5	**482571**	GYNE	20/07/2019	B6	**482488**	KGH	20/07/2019
B7	**482767**	MW2	21/07/2019	B8	**905676**	TOA	21/07/2019	B9	**483149**	MW5	22/07/2019
C1	**484149**	A&E	22/07/2019	C2	**484064**	DCUS0	22/07/2019	C3	**484063**	IDU	23/07/2019
C4	**484212**	A/E	23/07/2019	C5	**484248**	A/E	23/07/2019	C6	**484927**		25/07/2019
C7	**485001**	RHKG	25/07/2019	C8	**485637**	M4	25/07/2019	C9	**485001**	RHKG	25/07/2019
D1	**485804**	A+E	26-Jul	D2	**485771**	A+E	26-Jul	D3	**485517**	KGH	26-Jul
D4	**483742**	ICU	26-Jul	D5	**485313**	ENT	26-Jul	D6	**485516-17**	MW6	27-Jul
D7	**485745**	A&E	27-Jul	D8	**485516-14**	MW6	27-Jul	D9	**486672**	URO	27-Jul
E1	**486865**	MW6	28-Jul	E2	**486549**	MOP3	28-Jul	E3	**905702**	PAT	28-Jul
E4	**487243**	MOP1	28-Jul	E5	**486549**	MOP3	28-Jul	E6	**486973**	SOP	28-Jul
E7	**486865**	MW6	28-Jul	E8	**486327**	SWL	28-Jul	E9	**487037**	MOP3	28-Jul
F1	**486592**	SW5	28-Jul	F2	**902948**	GU	29/07/2019	F3	**487283**	ITU	29/07/2019
F4	**487291**	ENT OP	29/07/2019	F5	**487254**	MDPLS7	29/07/2019	F6	**487785**	RHKG	30/07/2019
F7	**488188**	A+E	30/07/2019	F8	**487896**	SW2	30/07/2019	F9	**487237**	A+E	30/07/2019
G1	**487428**	KGH	31/07/2019	G2	**488549**	Urology	01/08/2019	G3	**489184**	CARDIAC REHAB	02/08/2019
G4	**489408**	SW1	03/08/2019	G5	**489943**	A&E	03/08/2019	G6	**489494**	SAU1	03/08/2019
G7	**489799**	A&E	03/08/2019	G8	**488644**	ORT3	03/08/2019	G9	**489437**	MAU1	04/08/2019
H1	**490628**	A&E	04/08/2019	H2	**489408**	SW1	04/08/2019	H3	**490575**	OBSW2	04/08/2019
H4	**490793**	MHC	04/08/2019	H5	**489964**	SVPR	04/08/2019	H6	**895913**	GGH	04/08/2019
H7	**490593**	SW3	05/08/2019	H8	**490624**	?	05/08/2019	H9	**489812**	ACC	05/08/2019

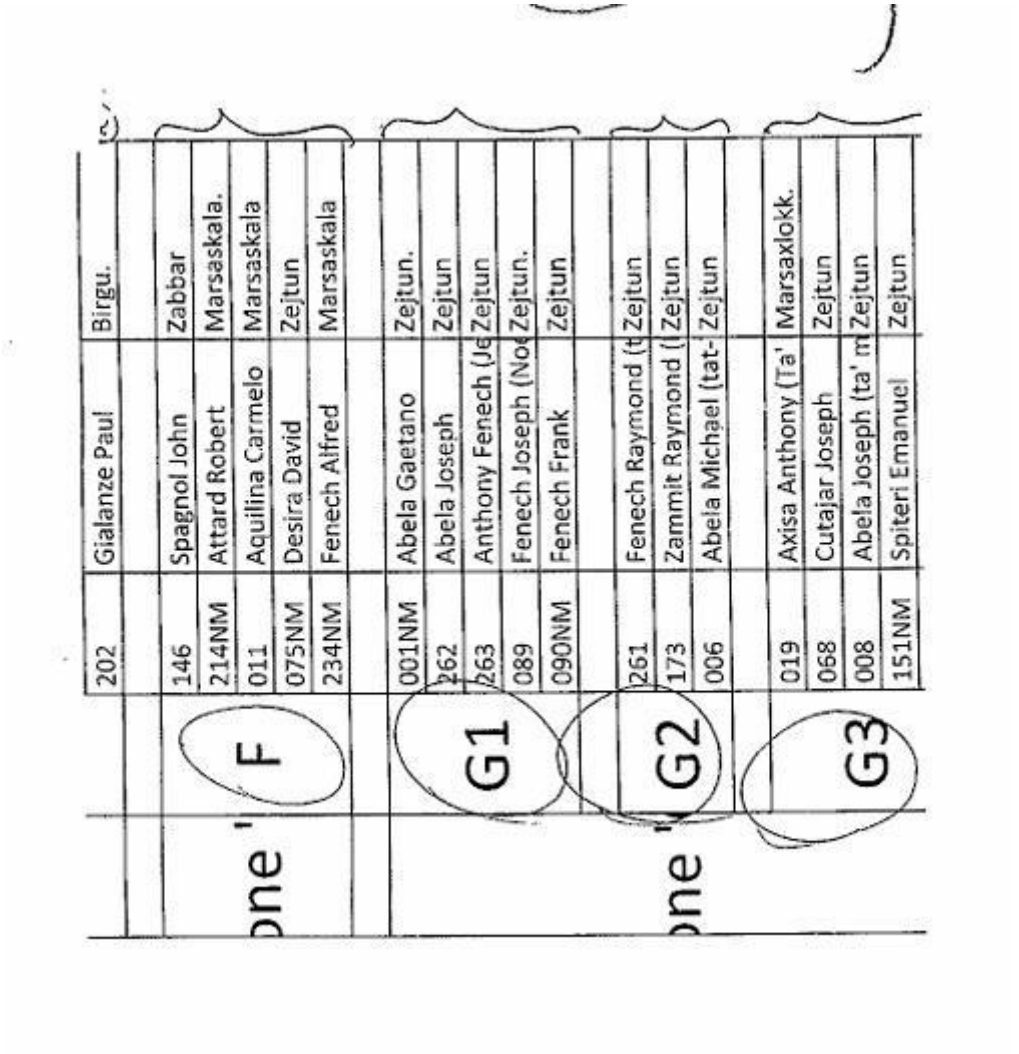
BOX 230

A1	**154576**	URO2	20/08/2020	a2	**154282**	OC Haem	20/08/2020	A3	**528919**	GGH	21/08/2020
A4	**154672**	ITU	21/08/2020	A5	**154675**	ITU	21/08/2020	A6	**154577**	URO2	22/08/2020
A7	**155020**	MW9	22/08/2020	A8	**155092**	RHKG	22/08/2020	A9	**155188**	SVPR	23/08/2020
B1	**155431**	Dental	23/08/2020	B2	**155490**	RHKG	23/08/2020	B3	**155223**	SW1	23/08/2020
B4	**155538**	SAU1	23/08/2020	B5	**915269**	GGH	24/08/2020	B6	**155506**	RHKG	24/08/2020
B7	**528947**	GGH	24/08/2020	B8	**155895**	A & E	24/08/2020	B9	**155745**	RHCGP	24/08/2020
C1	**155651**	OBS2	24/08/2020	C2	**155453**	SW2	24/08/2020	C3	**156288**	SVPR	26/08/2020
C4	**155587**	SW3	26/08/2020	C5	**156363**	ITU	27/08/2020	C6	**156528**	ITU	27/08/2020
C7	**155977**	MIU4	27/08/2020	C8	**156223**	A&E	27/08/2020	C9	**156704**	SVPR	28/08/2020
D1	**156475**	TOA	28-Aug	D2	**156834**	SOP	28-Aug	D3	**528577**	GGH	28-Aug
D4	**916253**	TOA	28-Aug	D5	**156807**	TOA	29-Aug	D6	**157129**	A&E	29-Aug
D7	**893294**	TOA	30/08/2020	D8	**157400**	RENAL	30/08/2020	D9	**158079**	TVU	30-Aug
E1	**158080**	TVU	30-Aug	E2	**158081**	TVU	30-Aug	E3	**157400**	RENAL	31/08/2020
E4	**157601**	SW5	31/08/2020	E5	**158372**	MOP 3/ NEURO2	31/08/2020	E6	**158309**	PLASTICS	01/09/2020
E7	**158060**	MOP1	01/09/2020	E8	**913944**		01/09/2020	E9	**913944**		01/09/2020
F1	**158264**	MW2	02/09/2020	F2	**158281**	A+E	02/09/2020	F3	**158867**	A&E	03/09/2020
F4	**159182**	SVPR	04/09/2020	F5	**159302**	A&E	05/09/2020	F6	**160078**	A&E	05/09/2020
F7	**912631**	pathology	05/09/2020	F8	**159691**	svpr	05/09/2020	F9	**159620**	NMW	06/09/2020
G1	**159907**	PAED DAY CARE	06/09/2020	G2	**159664**		06/09/2020	G3	**160074**	MAU3	07/09/2020
G4	**160543**	CSW	08/09/2020	G5	**160173**	WNDR	08/09/2020	G6	**914087**	GGH	08/09/2020
G7	**160876**	ORT	09/09/2020	G8	**160808**	ITU	09/09/2020	G9	**912657**	TOA	10/09/2020
H1	**161053**	RHKG	10/09/2020	H2	**161251**	A & E	10/09/2020	H3	**161015**	MIU	10/09/2020
H4	**160970**	MW6	10/09/2020	H5	**160619**	ITU (KPNEM)	10/09/2020	H6	**161061**	MW7	11/09/2020
H7	**161086**	SVPR	11/09/2020	H8	**162035**	MOP1	12/09/2020	H9	**161918**	SVPR	12/09/2020
J1	**161828**	A&E	12/09/2020	J2	**161589**	ORT1	12/09/2020	J3	**162408**	SVPR	13/09/2020
J4	**162636**	MW3	13/09/2020	J5	**162465**	A&E	14/09/2020	J6	**162164**	MOP3	14/09/2020
J7	**162523**	BHC	14/09/2020	J8	**162307**	SAMOC	14/09/2020	J9	**162125**	ITU	14/09/2020

Patients UTI ESBL E.coli June 2018/2019 - 2020.

BOX 213											
A1	**904735**	TOA	24/05/2019	A2	**455108**	N/A	24/05/2019	A3	**455689-13**	MW9	25/05/2019
A4	**455689-18**	MW9	25/05/2019	A5	**455169**	URO2	25/05/2019	A6	**455128**	A and E	25/05/2019
A7	**455165**	Oncology	25/05/2019	A8	**456126**	KGH	25/05/2019	A9	**456020**	PHC	26/05/2019
B1	**455055**	GGH	26/05/2019	B2	**456306**	MAU2	26/05/2019	B3	**456345**	OBSW2	26/05/2019
B4	**455674**	PATH CLINIC	26/05/2019	B5	**456201**	NPICU	27/05/2019	B6	**455979**	MIU	27/05/2019
B7	**457237**	MW5	28/05/2019	B8	**457265**	A&E	28/05/2019	B9	**457271**	KGH	28/05/2019
C1	**456367**	DSNY	28/05/2019	C2	**457119**	NPICU	29/05/2019	C3	**457349**	A&E	29/05/2019
C4	**457992**	SWA	29/05/2019	C5	**457916**	SVPR	29/05/2019	C6	**457706**	SVPR	30/05/2019
C7	**457981**	NPICU	30/05/2019	C8	**458150**	MAU2	30/05/2019	C9	**458423**	FHC	30/05/2019
D1	**457119**	NPICU	30/05/2019	D2	**458558**	A&E	30/05/2019	D3	**459017**	RHKG	31/05/2019
D4	**459005**	SW4	01/06/2019	D5	**459022**	OBS WARD	01/06/2019	D6	**523710**	GGH	01/06/2019
D7	**899265**	TOA	01/06/2019	D8	**459122**	MAU1	02/06/2019	D9	**523727**	GGH	02/06/2019
E1	**460015**	OBS	02/06/2019	E2	**459990**	RHKG	03/06/2019	E3	**460157**	ITU	03/06/2019
E4	**459956**	OBSW2	03/06/2019	E6	**460436**	SVPR	04/06/2019	E7	**523744**	GGH	04/06/2019
E8	**460435**	SVPR	04/06/2019	E9	**461015**	MW8	05/06/2019	F1	**461507**	A&E	07/06/2019
F2	**461932**	SW1	07/06/2019	F3	**461991**	A&E	07/06/2019	F4	**462307**	MW3	08/06/2019
F5	**462469-6**	MW3	08/06/2019	F6	**462469-9**	MW5	08/06/2019	F7	**461941**	ITU	08/06/2019
F8	**462321**	SVPR	09/06/2019	F9	**463123**	A and E	09/06/2019	G1	**463135**	NMW	09/06/2019
G2	**463429**	ITU	10/06/2019	G3	**462963**	MW8	11/06/2019	G4	**464300**	SW4	11/06/2019
G5	**463725**	MW4	11/06/2019	G6	**463114**	A&E	11/06/2019	G7	**463119**	COP	11/06/2019
G8	**464358**	A&E	13/06/2019	G9	**465045**	OC HAEM	14/06/2019	H1	**465049**	OC HAEM	14/06/2019
H2	**465047**	OC HAEM	14/06/2019	H3	**465394**	MAU1	14/06/2019				
H4	**465924**	ORT1	15-May	H5	**465772**	St Joseph ward 3	15/06/2019	H6	**466771**	RHKG	16/06/2019
H7	**466730**	MW1	17/06/2019	H8	**466497**	SW2	17/06/2019	H9	**466542**	A&E	17/06/2019

A.1.2 Farm Zones



		Farm	Name	
	A1	260	Calleja Richard	Maghtab
		045	Calleja Michael	Maghtab
		259	Calleja Matthew	Maghtab
		223NM	Micallef Patrick	Maghtab
	A2	092	Galea Carmel (Stev	Maghtab
		241	Galea Charles (Dav	Maghtab
		208	Vella Carmelo (Chri	Maghtab
one '		233NM	Camilleri Carmelo	Mosta
	A3	220NM	Vella George	Salina
		163	Vella Stephen	Salina
		167	Xuereb Emanuel	Salina
		168NM	Xuereb Jonathan	Salina
	A4	157NM	Vella Brian	San Gwann
		160NM1	Salvu Mizzi	San Gwann
		164	Vella Calcidon	San Gwann
	B	256	St. Rita Farm Co. Lt	Qormi
one '		034	Brincat Carmel	Qormi
		212	Casha Luciano	Qormi
		203	Borg George (R)	Hal-Farrug
	C1	248	Borg Christian	Qrendi
		249	Borg Joseph	Qrendi
		138NM	Schembri Giuseppi	Qrendi
one '		097NM1	Bezzina Ronald	Qrendi
		230	Gatt Joseph	Qrendi
		244	Azzopardi Louis (W	Qrendi
		130	Sacco Paul	Qrendi
	C2	236	Abela Michael	Zurrieq
		206	Caruana Felicia	Zurrieq
	D	247	Azzopardi Raymond	Dingli
one '		020NM	Kristinu Azzopardi &	Rabat
		022	Azzopardi Simon	Rabat
		095	Gatt Anthony	Rabat
	E1	079	Ellul Victor (imbaqd	Ghaxaq
		005NM	Abela Silvan	Ghaxaq
		010	Abela Victor	Ghaxaq
one '		226	Abela Bernard	Ghaxaq

A.2 Kits

A.2.1 QIAamp DNA mini kit - QIAcube® User Manual

https://www.qiagen.com/us/resources/resourcedetail?id=f7d77c6e-0479-4b2b-a2e0-5ca747114e34&lang=en

Version 1.3 March 2018

QIAcube® User Manual

QIAGEN®

Sample & Assay Technologies

A.2.2 SACACE Biotechnologies

https://sacace.com/manuals.htm

Sacace

BIOTECHNOLOGIES

Bacterial Drug resistance

Handbook

Real Time PCR kits for detection of genetic elements responsible for bacterial resistance to various antibiotic drugs
(Cephalosporins, Carbapenems, Glycopeptides, Penicillins, Mac.rolides)

A.3 Scheme of work of Good Quality Media Preparation and Strain Preparation

A.3.1 Preparation of Microbiological Culture Media and Reagents

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PROCEDURE — Preparation of Microbiological Culture Media and Reagents	Page 1 of 8

1.0 INTRODUCTION

1 .1 Many microbiological culture media (hereunder referred to as 'medium' or 'media' accordingly) are available commercially in complete dehydrated form and are also quality control (QC) checked by the manufacturer. These media may or may not include the addition of supplements.

1 .2 In some cases, the medium is formulated in-house using basic ingredients.

1 .3 Occasionally, commercially available ready-to-use bottled media are utilized.

2.0 PURPOSE

2.1 To ensure that all in-house produced media and reagents are prepared, stored and handled using good laboratory practices since media is one of the factors affecting the reliability and validity of the test results obtained.

3.0 SCOPE

3.1 Media and reagents prepared at the MPHL.

3.2 This procedure does not apply to commercially available ready-to-use bottled media.

4.0 RESPONSIBILITY

4.1 Commercially available dehydrated Media: Media Preparation Officers (M.P.O.'s)

4.2 Media formulated using basic ingredients and Reagents: M.P.O.'s

4.3 Miscellaneous Duties complementary to preparation of media and reagents: M.P.O.'s as per (MPQ-PRG-OOI)

4.3 Commercially available ready-to-use Media: Laboratory Officers (L.O.'s) using it. L.O.'s are to melt, temper, record the media used in the 'Stock Records' (RS-175) and store the kit insert/literature as per, 'Writing In-House Management System Documents' (GPR-PCD-002).

4.4 Request for Preparation of Media: L.c.'s

5.0 FREQUENCY

5.1 On request

6.0 ASSOCIATED DOCUMENTS

6.1 'Miscellaneous Duties for Media Preparation Officers' (MPQ-PRG-OOI)
6.2 'Writing In-House Management System Documents' (GPR-PCD-002)
6.3 'Performance Monitoring of Balances' (CPM-PCD-OOI)
6.4 'Media Preparation and QC Manual' for media prepared from basic ingredients
6.5 'Monitoring of Water Quality' (MPQ-PCD-OOI)
6.6 'QC of Microbiological Culture Media' (MPQ-PCD-003)

6.7 'Operation and Maintenance of Autoclaves (MPHL-STROI, MPHL-STR02 and MPHL-STR08)' (EQPPCD-MB-006)
6.8 'Operation and Maintenance of the Weighing Station (MPHL-BSEOI)' (EQP-PCD-MB-020)
6.9 'Operation and Maintenance of the Automatic Media Preparator (MPHL-AMPOI (EQP-PCD-MB-021)
6.10 'Operation and Maintenance of Media Dispensers' (EQP-PCD-MB-023)
6.1 1 'Request for Preparation of Media Form' (RS-MB-068)
6.12 'Microbiological Culture Media Production Information' (RS-MB-069)
6.13 'Media Production Form' (RS-MB-070)
6.14 'Stock Records' (RS-175)
6.1 5 'Media Production Form - BOB, ITC, PB, RPF, -rsc' (FRS-MB-211)
6.1 6 'Reagents Production Information' (RS-MB-220)
6.1 7 'Reagents Production Form' (RS-MB-222)
6.1 8 Kit Inserts — Current Versions' file
6.1 9 'Media and Reagent Labels' file
6.20 'Material Safety Data Sheets' of relevant media, chemicals and antibiotics

7.0 SAFETY CONSIDERATIONS

7.1 Normal microbiology laboratory precautions apply.

7.2 A number of media ingredients and supplements may be hazardous to health and care should be taken when handling. In order to avoid accidents and to comply with any appropriate regulations, the supplier's material safety data sheets are to be referred to as necessary.

7.3 Avoid actions which can lead to inhalation of dust/fumes.

8.0 EQUIPMENT

8.1 Usual laboratory equipment and in addition:

8.1 . 1 Top pan balance capable of weighing to 0.01 g
8.1 .2 Top pan balance capable of weighing to 0.0001 g
8.1 .3 0.2 gm sterile filter membranes
8.1 .4 5, 20 ml sterile syringes
8.1 .5 Hot-plate stirrer
8.1 .6 pH meter
8.1.7 Autoclave
8.1 .8 Waterbath at 47 - 50 °c
8.1 .9 Automatic Media Preparator
8.1 .1 0 Media Dispensers
8.1 .1 1 Roller Mixer
8.1 .1 2 Label Dispenser
8.1 .1 3 Hot Air Blower

9.0 PROCEDURE

Microbiology - Public Health Laboratory	
PROCEDURE - Preparation of Microbiological Culture Media and Reagents	Page 3 of 8

9.1 REQUEST FOR PREPARATION OF MEDIA AND REAGENTS

9.1 .1 L.O.'s shall ensure that there are adequate stocks of media and reagents in excess of the amount required for the programmed activities.

9.1 .2 L.O.'s shall record their request for the preparation of media/reagents (indicating the type, quantity and if necessary how urgent it is required) on the 'Request for Preparation of Media Form' (RS-MB-068) located in the Common Area or the Media Preparation Lab. 1 .

9.1 .3 M.P.O.'s shall go through the list of media/reagents being requested daily and liaise with the L.O. making the request as necessary.

9.2 WATER QUALITY

Note 1 : The quality of water used can have a critical bearing on the performance of the completed media.

9.2.1 Monitor the quality of the water used for media and reagent preparation as per, 'Monitoring of Water Quality' (MPQ-PCD-OOI).

9.3 GLASSWARE

9.3.1 Any flasks used to heat, boil or sterilize media must be made of heat resistant borosilicate glass e.g. Pyrex.

9.3.2 Rinse all the required glassware with distilled/deionised water prior to use.

9.3.3 After use, thoroughly scrub glassware containing residue of media using a wire brush and cleaning agent (e.g. Decon 90). Care must be taken to remove any traces of cleaning agent as this can hinder any efforts made to produce good quality media.

9.4 PREPARATION OF MEDIA

9.4.1 In order to improve the reproducibility of results, use commercially available dehydrated media where available, Follow manufacturer's instructions, unless otherwise indicated in the procedures compiled in the 'Media Preparation and QC Manual'.

9.4.2 For media prepared from basic ingredients, follow the appropriate procedure held in the 'Media Preparation and QC Manual'.

9.4.3 Record each batch of medium prepared in the 'Media Production Form' (RS-MB-070) or 'Media Production Form - BOB, ITS, PB, RPF, TSC' (FRS-MB-21 1) accordingly.

9.5 RECONSTITUTION OF MEDIA

Note 2: Care must be taken when calculating solution strengths and volumes to ensure the correct formulation of the final medium.

9.5.1 Weighing out the Powder

9.5.1 .1 Before weighing any powders, monitor the balance to be used as per, 'Performance Monitoring of Balances' (CPM-PCD-OOI).

9.5.1 .2 Use a top pan balance with an accuracy of ± 0.01 g suitable for weighing quantities of 1 g or more. Weigh smaller quantities (< 1 g) on an analytical balance with an accuracy of ± 0.0001 g.

9.5.1 .3 Use the weighing station when any powders or chemicals are weighed to prevent contaminating the work environment as per, 'Operation and Maintenance of the Weighing Station (MPHL-BSEOI)' (EQPPCD-MB-020).

9.5.1 .4 Weigh out dehydrated media, chemicals and ingredients separately and carefully into separate weighing boats using a clean, dry implement such as a stainless steel spoon. Do not use caked media.

9.5.1 .5 Replace closures on dehydrated medium containers as soon as weighing is complete.

9.5.2 Mixing of Powders with Water

9.5.2.1 Measure the required volume of distilled/deionised water using a measuring cylinder.

9.5.2.2 Use a clean glass conical or round-bottomed flask, approximately double the volume of the medium to be prepared.

9.5.2.3 Pour approximately one third of the volume of the water required into the flask, add the powder slowly whilst constantly swirling, and then add the remaining water, taking care to wash down any powder adhering to the sides of the flask.

9.5.2.4 For preparation of media using the calibrated automatic media preparator, refer to the procedure 'Operation and Maintenance of the Automatic Media Preparator (MPHL-AMPOIA)' (EQP-PCD-MB-

9.6 pH & VOLUME ADJUSTMENT, DISSOLVING AND DISPENSING OF MEDIUM

9.6.1 pH Adjustment

9.6.1 .1 Adjust the pH of the medium, if necessary with 1 M HCl and/or 1M NaOH, accordingly, using the calibrated pH meter as per its operation and maintenance procedure, currently in use in the Media Preparation 1 .

9.6.1 .2 Mix well and re-check the pH of the prepared medium.

Note 3: The media that require pH adjustment before sterilization are listed in 'Microbiological Culture Media Production Information' (RS-MB-069). pH adjustment may be different for media of different brands.

9.6.2 Dissolving Agar Media prior to Sterilisation

9.6.2.1 Heat and mix agar media using a hot plate and stirrer. Care must be taken to prevent scorching and overheating of the medium.

Note 4: Carry out regular checks to determine whether the agar has dissolved during heating. This is done by tilting the medium in the flask and allowing it to stream slowly down the sides to detect particles of undissolved agar.

9.6.3 Dispensing Broths & Volume Adjustment prior to Sterilization

9.6.3.1 Some broths are dispensed in volumes specified in the 'Microbiological Culture Media Production Information' (RS-MB-069), before sterilization. This is carried out using calibrated media dispensers as per 'Operation and Maintenance of Media Dispensers' (EQP-PCD-MB-023).

9.6.3.2 Since during sterilization a small volume of medium will be lost, a slightly larger volume should be dispensed before sterilisation.

Note 5: The media that require volume adjustment before sterilization are listed in the 'Microbiological Culture Media Production Information' (RS-MB-069).

9.7 STERILIZATION AND TEMPERING OF MEDIA

9.7.1 Sterilization of Media

9.7.1 .1 Attach autoclave indicator tape to the media containers or basket so as to distinguish between sterilized and non-sterilized media thereby precluding the inadvertent use of the latter.

9.7.1 .2 Broths dispensed in Bijoux bottles are to be tightly closed during the sterilization process. Broths dispensed in greater volumes should not have their caps sealed during autoclaving.

9.7.1.3 Sterilize media as per, 'Operation and Maintenance of Autoclaves (MPHL-STROI, MPHL-STR02 and MPHL-STR08)' (EQP-PCD-MB-006) or as per, 'Operation and Maintenance of the Automatic Media Preparator (MPHL-AMPOI)' (EQP-PCD-MB-021 y accordingly.

Note 6: Most media are sterilized by the application of a suitable autoclave process (usually 121 °c for 15 min at 1 .1 bar). However, refer to the manufacturer's instructions for further information. E.g. boil media using the autoclaves or the automatic media preparator (same conditions) at 100 °c for 2 min and at 100 °c for 1 min respectively.

9.7.1 .4 Certain solid media (e.g. XLD) contain heat sensitive constituents which preclude their being autoclaved.

9.7.2 Tempering of Media

9.7.2.1 After sterilization, temper the hot molten agar in a waterbath, to 47 — 50 °c.

Note 7: It is very important that molten media is tempered to this temperature as otherwise this can result in the denaturation and a reduction in the selective/differential properties of the medium when heat sensitive supplements are added.

9.7.2.2 The water in the water bath should be at least level with the medium in the containers as this may lead to uneven cooling of the agar.

9.7.2.3 Allow sufficient time for the medium to cool to this temperature before continuing. As a rough guide containers should be 'hand' hot after they have been removed from the waterbath, dried and the contents mixed. However, media should not be held molten in the waterbath for more than 4 h.

9.7.2.4 Allow broths to cool to ambient temperature on the bench before addition of heat sensitive supplements.

9.8 ADDITION OF SUPPLEMENTS

9.8.1 Reconstitute commercially available supplements according to their kit inserts/literature held in the 'Kit Inserts — Current Versions' file. Aseptic handling of supplements is necessary and are to be rehydrated with sterile liquids. Use a 5 ml sterile syringe to dispense the supplement into the medium.

9.8.2 If supplements are prepared in-house, filter sterilize the supplement as follows:

- Aspirate the supplement liquid using a 5 ml sterile syringe
- Remove syringe needle carefully and fit a sterile 0.2 pm filter membrane to the syringe tip - Press syringe plunger to pass the supplement liquid through the filter membrane into the tempered medium

9.8.3 Mix the completed medium well using a roller mixer before dispensing.

9.9 POURING OF PLATES AND SOLIDIFICATION OF MEDIA

9.9.1 Hand Pouring of Plates

1.1Agar media must be tempered to 47 - 50 °c to avoid excessive condensation, heat inactivation of labile supplements and deformation of the plastic base of the Petri dish.

1.2Remove media container from waterbath and dry outer surface with a clean disposable towel to prevent water running down the outer surface and contaminating the media during the pouring process.

9.9.1 .3 Mix the tempered medium on a hot plate stirrer.

1.4Before pouring, flame the inner neck of the container to ensure aseptic techniques.

9.9.2 Automatic Pouring of Plates

2.1For pouring of media using the automatic media preparator, refer to the 'Operation and Maintenance of the Automatic Media Preparator (MPHL-AMPOI)' (EQP-PCD-MB-021).

9.9.3 Dispense an Agar Volume of:

- 18-20 ml per plate when using standard 90 mm Petri dishes
- 5-10 ml per plate when using 55 mm Petri dishes
- 45-50 ml per plate when using 140 mm Petri Dishes

9.9.3.1 Lightly flame agar medium if bubbles form on medium surface.

9.9.4 Solidification of Media

4.1After pouring the agar medium, leave the plates on the media room bench until the agar medium has set to a firm gel.

9.9.4.2 Remove sterilized bottled media from the autoclave when cool and then tighten their caps.

9.10 LABELLING OF MEDIA

9.10.1 Whenever practical, all media plates and bottles are to be individually labelled with the Laboratory Batch No., as follows:

Laboratory Batch No. consists of the medium abbreviation (2 to 4 letters) followed by the expiry date (if the label dispenser permits, the full date (the day, month and year) is to be printed i.e. [ddmmyy]; if the label dispenser permits less digits, then the [ddmm] are at least to be printed).

Example of Label: 'MAC120709'. This means that the Mac Conkey Agar No. 3 medium will expire on the 12th of July 2009.

Media abbreviations and shelf-lives are listed in the 'Microbiological Culture Media Production Information' (RS-MB-069).

9.10.2 In case of large batches of the same medium being sterilised separately on the same day, identify the separate batches by a letter at the end of the Laboratory Batch No. e.g. BPW120709A, BPW120709B, etc.

9.10.3 In case of media whose base is prepared on one day and to which supplements shall be added on the day of use, give the Laboratory Batch No. to that sub-batch on the day of reconstitution. Data is to be recorded in the 'Media Production Form' (RS-MB-211).

Example: if 5 bottles of the base of the Bolton Broth are prepared on the 17th March, and the supplements are added to the 1st 2 bottles on the 20th March, then the Laboratory Batch No. to these 2 reconstituted bottles is BOB250311 (5 days from the 20th March). The Laboratory Batch No. of the other 3 bottles shall be given according to the next date of reconstitution.

9.10.4 Attach labels containing the required Laboratory Batch No. to the set plates and media bottles using the label dispenser.

Alternatively, large batches of agar plates and media bottles may be identified by affixing a media label found in the 'Media and Reagent Labels' file onto the container or in front of the relevant stack of media on the shelf. This label clearly identifies the medium identity, the volumes, conditions of storage, the date of preparation and the media batch expiry date.

9.11 PACKING, QC AND STORAGE OF MEDIA

9.11.1 Packing of Agar Plates into Bags

After agar plates are set and labelled, stack Petri dishes to a height of not more than 10 plates.

Place shrink wrap sleeves over plates and use the hot air blower to seal the shrink wrap over these plates. (This helps retard the loss of moisture from the medium.)

9.11.2 QC

Perform QC checks as per, 'QC of Microbiological Culture Media' (MPQ-PCD-003).

9.11.3 STORAGE

9.11.3 STORAGE

9.11.3.1 Preferably, after preparation and while media is being QC checked, media is first stored in a fridge dedicated for quarantine of media or segregated as specified in the 'Microbiological Culture Media Production Information' (RS-MB-069). Then, if the media passes the QC checks, media can be positively released for use in their appropriate storage location as indicated in the same RS-MB-069.

9.1 1 .3.2 However, in view of limited storage availability, especially for refrigerated media, freshly prepared media may be placed with other previously prepared media while ensuring effective stock rotation by adopting the 'first-in, first-out' policy. QC shall be performed in parallel as per, 'QC of Microbiological Culture Media' (MPQ-PCD-003).

Note 8: Store prepared plates: medium side up.

9.1 1 PREPARATION OF REAGENTS

2.1 For preparation of reagents, follow the appropriate documented procedures held in the 'Media Preparation and QC Manual' and in the 'Reagents Production Information' (RS-MB-220).

2.2 Record each reagent prepared in the 'Reagents Production Form' (RS-MB-222).

9.1 2.3 Attach a label to the reagent bottle containing the following information:

- Reagent name (including %, m/m, Molarity, etc.)
- Volume prepared
- Storage conditions
- Date of Preparation
- Shelf life

COMMUNICATION OF TEST METHOD

Officers are to indicate that they have read and understood this version of the test method in the table below.

END

A.3.2 QC of Microbiological Culture Media

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10.5.3.3 Several test microorganisms can be streaked on the same plate as parallel lines without 'crossing' across line streaked; streaks should be distinguishable to allow observation of typical morphology.

10.5.3.4 Incubate at the recommended conditions as indicated in the 'Media Growth Checks' (RS-MB-206).

10.5.3.5 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1)

10.5.4 Specificity testing

Note 8: The specificity of the culture medium is given by essential indicative physiological characteristics to differentiate related organisms by the presence, absence and/or grade of expression of biochemical responses and colony sizes and morphology.

For specificity testing of plate media, inoculate using a suspension of the non-target microorganism containing at least 10^3 cfu on to the plate.

10.5.4.2 Inoculate by streaking each test microorganisms in a way to obtain discrete colonies.

10.5.4.3 Incubate at the appropriate conditions as indicated in the 'Media Growth Checks' (RS-MB-206).

10.5.4.4 The amount of growth on the plates after incubation is assessed as follows:

- 0 corresponds to no growth; - 1 corresponds to weak growth
- 2 corresponds to good growth

10.5.4.5 Assess the size and appearance of the colonies on or in the medium under test by comparison with the recovery on the non-selective culture medium (TSA or BA).

1 0.5.4.6 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1)

10.5.5 Testing of culture media used for membrane filtration

Note 9: To test the performance of a culture medium for use in membrane filtration, use working cultures and inocula that give a range of 80 cfu to 120 cfu per plate with a minimum number of 50 cfu per plate.

Filter the suspension medium dilution chosen to give a range of cfu/plate as per Note 7 above according to the membrane filtration procedure using the 0.45 pm or 0.2 pm sterile filters as per relevant procedure.
Example 1: When testing the TSC medium use the 0.2 pm sterile filter.
Example 2: For Legionella testing, use the 0.2 pm Supor 200 membrane filter

Place the membrane on the surface of the agar under test.

10.5.5.3 Incubate at the recommended conditions as indicated in the 'Media Growth Checks' (RS-MB-206).

Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1)

10.5.7 Validation of MacConkey plates supplemented with 1 mg/L cefotaxime

Proceed as per procedure 'Validation of selective MacConkey agar plates supplemented with 1 mg/L cefotaxime for monitoring of ESBL and AmpC producing E. coli in meat and animals' (MPQ-PCD-058)

10.5.8 Growth Checks of Media used for Legionella Testing

With reference to RS-MB-206:

GVPC:

Legionella pneumophila sgpl (WDCM 00107 or 00180) (Productivity Test)

- Reconstitute a bead inoculated with Legionella pneumophila sgpl in 1 ml MRD.
- Vortex and let bead settle.
- Inoculate an OLD and NEW GVPC (Note: CYE can be used as a Reference Medium instead of GVPC) with 100 gl of sample from the 1 ml MRD.
- Also transfer 100 pl of sample to 900 Vl MRD (1 : 10 dilution).
- Vortex and Inoculate an OLD and NEW GVPC from the 1:10 dilution. • Incubate at 36 ± 2 °c for up to 5 days.

Legionella anisa (WDCM 00106) (Productivity Test)

- Reconstitute a bead inoculated with Legionella anisa in 1 ml MRD.
- Vortex and let bead settle.
- Inoculate an OLD and NEW GVPC (Note: CYE can be used as a Reference Medium instead of GVPC) with 100 pl of sample from the 1 ml MRD. • Incubate at 36 ± 2 °c from 5 to 10 days.

Determine the Productivity Ratio for both cultures as per Section 10.5.1.8 above.
The Productivity Ratio (Pr) should be 20.5

E.faecalis (WDCM 00009 or 00087) and E.coli (WDCM 00012 or 00013) (Selectivity Test)

- Inoculate these Non-Target Organisms, E.faecalis and E.coli, on OLD and NEW GVPC using a suspension containing a count ranging from 10^4 - 10^6 in TSB broth.
- Using a 10 pl loop streak a line of each of the organisms on both OLD GVPC (Reference Medium) and NEW GVPC (Test Medium).
- Incubate at 36 ± 2 °c for 3 days.
- The amount of growth on the plates after incubation is assessed as follows:

- 0 corresponds to no growth
- 1 corresponds to weak growth.
- Record the observations and results in the medium's 'Media Production Form' (RS-MB-070)

10.5.8.2 CYE (BCYE with Cysteine)•. (Productivity Test)

Legionella pneumophi/a sgpl (WDCM 00107 or 00180)

- Reconstitute a bead inoculated with Legionella pneumophila sgpl in 1 mL MRD.
- Vortex and let bead settle..
- Inoculate an OLD and NEW CYE with 100 pl of sample from the 1 ml MRD.
- Transfer 100 pl of sample to 900 Vl MRD to do a 1:10 dilution if necessary.
- Vortex and Inoculate 100 pl on OLD and NEW CYE from the 1:10 dilution. • Incubate at 36 ± 2 °c for up to 5 days.

- Determine the Productivity Ratio for both cultures as per Section 10.5.1.8 above.
- The Productivity Ratio (Pr) should be 20.7.
- Record the observations and results in the medium's 'Media Production Form' (RS-MB-070).

WCY (BCYE without Cysteine): (Confirmation Test)

Legionella pneumophila sgpl (WDCM 00107 or 00180)

- Reconstitute a bead inoculated with Legionella pneumophila sgpl in 1 ml MRD.
- Vortex and let bead settle.
- Inoculate a WCY and CYE (reference medium) using a 10 pl loop. Streak medium. • Incubate at 36 ± 2 ° c for up to 5 days.
- Record the observations and results in the medium's 'Media Production Form' (RS-MB-070).

10.6 LIQUID MEDIA

10.6.1 Qualitative tube method for performance testing of selective liquid media

The volume used for testing should contain 100 cfu for productivity tests of pre-enrichment and enrichment media.

The volume of the inoculum shall be in the same ratio as used in the relevant International Standard, usually 10 % of the medium under test.

Select a number of tubes each containing 10 ml of medium or 10 ml portions from each batch to be tested.

10.6.2 Turbidity score assessment for liquid media - BB, BHI, BPW and TSB

.1 Inoculate one tube of test broth with an inoculum containing 100 cfu of target microorganism and mix.

1 0.6.2.2 Incubate the tubes under the conditions defined for each medium in the 'Media Growth Checks' (RS-MB-206).

1 0.6.2.3 Check for turbidity and record the observations and results in the medium's 'Media Production Form (RS-MB-070 or 21 1).

10.6.3 Inoculation of target organisms

1 0.6.3.1 Inoculate one tube of test broth with an inoculum containing 100 cfu of target microorganism and mix.

1 0.6.3.2 Incubate the tubes under the conditions defined for each medium in the 'Media Growth Checks' (RS-MB-206).

1 0.6.3.3 Remove one loopful (10 PI) from the tube containing the target organism and streak on a plate of a non-selective medium (e.g. TSA or BA).

1 0.6.3.4 Incubate the plates under the conditions as specified in the 'Media Growth Checks' (RS-MB-206).

1 0.6.3.5 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1).

10.6.4 Inoculation of non-target microorganisms

10.6.4.1 Inoculate one tube of test broth per microorganism with an inoculum containing at least 10^4 cfu and mix.

10.6.4.2 Incubate the tubes under the conditions defined for each medium in the 'Media Growth Checks' (FRS-MB-206).

10.6.4.3 Remove one loop (10 PI) from the culture of non-target microorganism and streak on a plate of a non-selective medium (e.g. TSA).

10.6.4.4 Incubate the plates under the conditions as specified in the 'Media Growth Checks' (RS-MB-206).

10.6.4.5 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 211)

10.6.5 Inoculation of target and non-target organisms in the same tube

Inoculate one tube of test broth with SIOO cells of target microorganism and the same tube with a higher number of non-target microorganisms (2 1000 cells for every tube) and mix.

10.6.5.2 Incubate the tubes under the conditions defined for each medium in the 'Media Growth Checks' (RS-MB-206).

10.6.5.3 Remove one loopful (10 PI) and streak on a plate of the specific medium for the target microorganism.

10.6.5.4 Incubate the plates under the conditions as specified in the 'Media Growth Checks' (RS-MB-206).

10.6.5.5 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 211)

Productivity of the liquid test broth is satisfactory if good growth (at least 10 cfu or a line of confluent growth) of the target microorganism is obtained on the specific medium for that organism. Selectivity of the liquid test broth is either satisfactory if no growth, or less than 10 cfu, or less than 100 cfu of non-target microorganisms occur on the non-selective agar plate, as specified in RS-MB-206.

10.6.6 Method for quantitative testing of diluents

Note 10: The method determines the ability of the diluents to support the survival of microorganisms without undue multiplication or reduction during the period of contact before plating on to agar or inoculation into liquid media.

Use suspension of an inoculum level of 10^3 to 10^4 cfu.

10.6.6.2 Inoculate a test portion (e.g. 9 ml) of the diluents with 1 ml of test microorganism suspension containing around 10^4 cfu/ml and mix.

10.6.6.3 Immediately remove 0.1 ml of inoculated diluents and spread over the surface of a non-selective agar such as TSA (to).

1 0.6.6.4 Hold the inoculated diluents at ambient temperature for 45 min as specified in ISO 6887.

10.6.6.5 Mix and then remove the same volume (0.1 ml) and plate again on the reference medium (ti).

10.6.6.6 Incubate the reference medium at an appropriate temperature and time as specified in the 'Media Growth Checks' (RS-MB-206).

10.6.6.7 After incubation count the colonies on the plates to and ti. The number of microorganisms, ti, after incubation of the diluents shall be within ± 30 % of the initial count (to).

10.6.6.8 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1)

Table summary for different innocula

Productivity	Quantitative	Solid media	1 0^2 cfu
		Li uid media	10^3 to 10^4 cfu
	Qualitative	Solid media	10^3 to 10^4 cfu
		Li uid media	cfu
Selectivity		Solid/liquid media	At least 10^4
S ecificit		Solid media	At least 10^3

10.7 No Growth Checks on Media

10.7.1 In exceptional cases (e.g. Water agar) no growth checks are carried out on media when it is shown that the medium's role is not of supporting growth of the desired organism(s).

10.8 ACCEPTABLE QC CHECKS RESULTS

10.8.1 If the results obtained for the above QC checks are acceptable and within expected tolerances mark the media as 'PASS' in the medium's 'Media Production Form' (RS-MB-070 or 21 1) accordingly and transfer the media to their appropriate storage location indicated in the 'Microbiological Culture Media Production Information' (RS-MB-069).

10.9 UNACCEPTABLE QC CHECKS RESULTS

10.9.1 If the results obtained are outside the expected tolerances and recoveries, or not as prescribed in the 'Microbiological Culture Media Production Information' (RS-MB-069), inform the Technical Officer who shall decide about the corrective action(s) to be taken. (Important: inform the Technical Officer immediately especially if the media is being QC checked in parallel to use for testing samples.

10.9.2 Record the evaluation of the impact of this anomaly and the corrective action taken in the 'Corrective Action' Form (RS-121).

10.9.3 If it is decided to dispose of the media, mark as 'FAIL' in the medium's 'Media Production Form' (RS-MB070 or 211) and forward the record sheet to the Technical Officer for confirmation (insert signature) near same.

11.0 REFERENCES

11.1 MSA EN ISO 6887 — 1:2000: Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions

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11.2 SM EN ISO 7218:2024. Microbiology of the food chain — General requirements and guidance for microbiological examinations.

11.3 MSA EN ISO 1 1133:2014 and its amendments: Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media

COMMUNICATION OF TEST METHOD

Officers are to indicate that they have read and understood this version of the test method in the table below.

END

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1 .0 INTRODUCTION

1 .1 The routine quality control (QC) of bacteriological culture media is essential in order to ensure good laboratory practice necessary to maintain the standards and performance of any bacteriological technique.

2.0 PURPOSE

2.1 This procedure ensures that all bacteriological culture media prepared in-house as per, 'Preparation of Microbiological Culture Media and Reagents' (MPQ-PCD-002) and also bacteriological culture media procured ready-for use are of a high standard of quality.

3.0 SCOPE

3.1 Bacteriological culture media prepared in-house and procured ready-for use.

4.0 DEFINITIONS

4.1	Target microorganism:	microorganism or group of microorganisms to be detected or enumerated.
4.2	Non-target microorganism:	microorganism that is suppressed by the medium and/or conditions of incubation or does not show expected characteristics of the target microorganism.
4.3	Productivity of culture medium:	level of recovery of a target microorganism from the culture medium under defined conditions.
4.4	Selectivity of culture medium:	degree of inhibition of a non-target microorganism on or in a selective culture medium under defined conditions.
4.5	Specificity of culture medium:	demonstration, under defined conditions, that non-target Microorganisms, if able to grow on the medium, do not show the same visual characteristics as target microorganisms.

5.0 RESPONSIBILITY

5.1	Chemical and Physical QC Checks:	Media Preparation Officers (M.P.O.'s)
5.2	Sterility QC Checks:	M.P.O.'s as per (MPQ-PRG-OOI)
5.3	Growth Checks:	Quality Assurance Officers (Q.A.O.'s) and Officers indicated in the 'Authorisation of PHL Personnel' (RS-IOI)
5.4	Recording Media as 'PASS/FAIL':	Q.A.O.'s and Officers indicated in the 'Authorisation of PHL Personnel' (RS-IOI)

6.0 FREQUENCY

6.1 After media production and procured ready-to-use media

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7.0 ASSOCIATED DOCUMENTS

7.1 'MPHL Food Test Methods QC Schedule' (FOD-PRG-MB-002)
7.2 'MPHL Water Test Methods QC Schedule' (WAT-PRG-MB-002)
7.3 'MPHL Culture Test Methods QC Schedule' (GPR-PRG-MB-006)
7.4 'Miscellaneous Duties for Media Preparation Officers' (MPQ-PRG-OOI)
7.5 'Preparation of Microbiological Cufture Media and Reagents' (MPQ-PCD-002)
7.6 'Maintenance of Reference and Working Cultures' (CPM-PCD-MB-009)
7.7 'Validation of selective MacConkey agar plates supplemented with 1 mg/L cefotaxime for monitoring of ESBL and Ampc producing E. coli in meat and animals' (MPQ-PCD-058)
7.8 'Microbiological Culture Media Production Information' (RS-MB„069)
7.9 'Media Production Form' (RS-MB-070)
7.10 'Authorisation of PHL Personnel' (RS-IOI)
7.1 1 'Corrective Action' Form (RS-121)
7.12 'Method Performance — Legionella' (RS-MB-201)
7.13 'Media Growth Checks' (RS-MB-206)
7.14 'Media Production Form - BOB, ITC, PB, RPF, TSC' (RS-MB-211)
7.15 'Kits & Reagents — Instructions for Use' file
7.16 Reference Cultures (E.g. Lenticules) Quality Certificates

8.0 SAFETY CONSIDERATIONS

8.1 Normal microbiology laboratory precautions apply.

9.0 EQUIPMENT

9.1 Usual laboratory equipment and in addition:

9.1 .1 pH meter
9.1 .2 Waterbath at 47 - 50 °c
9.1 .3 Incubators at temperatures listed in RS-MB-069 and RS-MB-206

10.0 PROCEDURE

10.1 CULTURE MEDIA CHARACTERISTICS

Note 1: Quality assessment by visual inspection shall ensure that each culture medium conforms to stated recommendations e.g. volume and/or thickness; appearance, colour and homogeneity; gel consistency; moisture content.

10.1 .1 Refer to 'Microbiological Culture Media Production Information' (RS-MB-069) for culture media characteristics such as colour, consistency, etc.

10.1 .2 Carry out visual inspection on all batches of media prepared in-house.

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10.1 .3 Record the observations in the medium's 'Media Production Form' (RS-MB-070 or 21 1) accordingly.

10.2 POST STERILISATION pH CHECKS

10.2.1 Refer to media target pH's in the 'Microbiological Culture Media Production Information' (RS-MB-069).

10.2.2 Operate the calibrated pH meter as per its operation and maintenance procedure, currently in use in the Media Preparation 1.

10.2.3 Perform pH checks on all batches of media prepared in-house.

10.2.4 Check the pH of the final cooled product, e.g. on a bottle of solid or liquid media. In the case of solid media poured in plates, carry out the pH check on a sample of the medium poured in a sterile container from a large volume.

- Carry out the pH check at ambient temperature.
- Leave the pH probe in the medium for some time until the reading has stabilized.
- Take an average of the pH measurement when reading the final pH.
- pH of the medium should fall within the specified tolerances.

10.2.5 Record the observations in the medium's 'Media Production Form' (RS-MB-070 or 211) accordingly.

10.2.6 Media used for pH checks must be discarded as it will be contaminated.

10.3 STERILITY CHECKS

10.3.2 Perform sterility checks on all batches of media prepared in-house.

10.3.3 For media batches of < 1 Litre: carry out sterility checks on 1 plate, tube or bottle.
For media batches > 1 Litre: Carry out sterility checks on at least 1 plate, tube or bottle or 1% of plates, tubes or bottles from the beginning, middle and end of a pouring or dispensing process.

10.3.3. Label the control plates or bottles with the laboratory batch no. and the sterility check date.

10.3.4 Incubate the control plates or bottles at the temperatures indicated in the 'Microbiological Culture Media Production information' (RS-MB-069).

10.3.5 After 24 h, observe the media incubated. Check for growth on solid media and turbidity in broths. Reincubate for a further 48 h.

Note 2: VRG: In view that this medium has a short shelf life of 4 hours, the sterility check is performed for 24 h

10.3.6 Re-check the media for sterility after completion of 72 h of incubation.

10.3.7 Record the sterility checks observations in the medium's 'Media Production Form' (RS-MB-070 or 21 1) accordingly.

10.3.8 If the control medium is found to contain growth, incubate a further 3 control media plates or bottles as per Section 10.3.4 - 10.3.6.

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10.4 POST STERILISATION VOLUME CHECKS

10.4.1 Perform volume checks gravimetrically on representative bottles of media whose volume is considered critical e.g. diluents.

10.4.2 The volume delivered by a dispenser for liquid or molten culture media shall achieve an accuracy of ±5 % of the designated volume. The accuracy required for dispensing measured volumes of dilution fluid and other critical applications shall be ±2 % of the designated volume. The general volumes of media dispensed together with the related tolerance limits are listed in 'Microbiological Culture Media Production Information' (RS-MB-069)

1 0.4.3 Record volumes in the medium's 'Media Production Form' (RS-MB-070 or 211) accordingly).

10.5 GROWTH CHECKS

10.5 SOLID MEDIA

1 0.5.1 Productivity - Quantitative method

For the quantitative enumeration test, a level of around 10^2 cfu is necessary to achieve sufficient precision - 100 ± 20 cfu per plate. Thereby, innocula that will result in a range of 80 cfu to 120 cfu per plate with a minimum number of 50 cfu per plate should be used.

Note 3: The pour plate method shall be used for culture media normally used for enumeration in this way.

Note 4: Inoculate the reference medium (TSA or BA) from a previously accepted batch using the same procedure.
Note 5: Ensure that the surfaces of the plates are adequately dried.

Note 6: VRG, RPF, TSC, Chrom ID EHEC Agar: In view that these media have a short shelf life (ranging from 4 hours to 2 weeks accordingly), QC is to be initiated immediately. Plates shall be inoculated with microorganisms as indicated on the 'General Purpose Form' titled 'QC of the following Culture Media used for Food and Environmental Samples Testing' which is held in the FAL by the Officers using this media for such testing. These officers shall inform the Q.A.O.'s of the incubated plates in order to proceed from Section 10.5.1 .4 below.

Inoculate by spreading the inoculum on the media.

Incubate at the appropriate conditions as indicated in the 'Media Growth Checks' (RS-MB-206).

Count the colonies present on each plate.

Assess the size and appearance of the colonies on or in the medium under test by comparison with the recovery on the non-selective reference culture medium (TSA or BA).

Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 211)

To interpret the result, calculate the productivity ratio, PR and where appropriate the selectivity factor, SF (as per Section 10.5.3.5 below).

The productivity ratio, PR is determined using the following formula:

P_R =No of colonies in culture medium under test
No o colonies in reference medium T A or BA)

The PR shall be 2 0.5 for comparison of a selective medium with the non-selective reference medium. The PR shall be 0.70 for comparison of a non-selective medium with a non-selective reference medium and in special cases where comparison is made with the previous batch.

10.5.2 Productivity - Qualitative method

The volume of suspension used for testing should contain 10^3 to 10^4 cfu for qualitative tests of plate media.

10.5.2.2 Inoculate by streaking each test microorganisms in a way to obtain discrete colonies.

10.5.2.3 Incubate at the appropriate conditions as indicated in the 'Media Growth Checks' (RS-MB-206).

10.5.2.4 The amount of growth on the plates after incubation is assessed as follows:

- 0 corresponds to no growth;
- 1 corresponds to weak growth
- 2 corresponds to good growth

Assess the size and appearance of the colonies on or in the medium under test by comparison with the recovery on the non-selective reference culture medium (TSA or BA).

10.5.2.6 Record the observations and results in the medium's 'Media Production Form' (RS-MB-070 or 21 1)

Note 7: Target microorganisms shall score 2 and have typical appearance, size and colony morphology.

10.5.3 Selectivity testing

For selectivity testing of culture media, inoculate using a suspension of the non-target microorganism containing at least 10^4 cfu on to the plate or into the tube of medium.

10.5.3.2 From the inoculum streak each test microorganism as a single straight line using a 1 pl loop on the surface of the test medium.

A.3.3 Direct Enumeration of ß-glucuronidase positive *E. coli* at 44 º c.

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1.0 INTRODUCTION

1 .1 The presence of Escherichia coli (E. coli) in food is regarded as an indication of contamination with organisms of faecal origin. This implies that such contaminated food may also harbour enteric pathogens.

1 .2 Microbiological guidelines for E. coli counts in certain ready-to-eat foods are published by several food safety organisations. Commercial guidelines for ready-to-eat food generally set the target value at 10 cfu/g or ml for E. coli. Counts of 100 cfu/g or greater are considered as unsatisfactory for ready to eat food.

1 .3 This test method is a pour plating method based on ISO 16649 - 2:2003.

2.0 SCOPE

2.1 The procedure described is applicable to the microbiological examination of products intended for human consumption or feeding of animals for E. coli strains which are capable of growth at 44 °c and that are ß-glucuronidase positive.

3.0 PRINCIPLE

3.1 This test method employs a colony count technique at 44 °c on a solid chromatogenic medium. 5bromo-4-chloro-3-indolyl ß-D-glucuronide is the substrate used for the detection of ß-glucuronidase activity. E. coli strains which do not grow at 44 °C, and in particular those that are ß-glucuronidase negative, such as E. coli 0157, will not be detected.

4.0 DEFINITIONS

4.1 For the purpose of this test method E. coli are bacteria, which under the test conditions at 44 °c, form typical blue colonies on tryptone—bile—glucuronide medium (TBX).

5.0 RESPONSIBILITY

5.1 Officers trained to perform the procedure.

6.0 FREQUENCY

6.1 As required.

7.0 ASSOCIATED DOCUMENTS

7.1 'MPHL Food Test Methods QC Schedule' (FOD-PRG-MB-002)
7.2 'Quality Assurance' (CPM-PCD-008)
7.3 'Measurement Uncertainty' (GPR-PCD-014)
7.4 Preparation of Samples and Dilutions' (FOD-PCD-MB-002)
7.5 'Proficiency Testing Evaluation Form' (RS-109)
7.6 'Food & Hygiene Testing' (RS-MB-148)

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8.0 SAFETY

8.1 Normal microbiology laboratory precautions apply.

8.2 In addition, care must be taken of the boiling waterbath when melting agars or boiling confirmatory test media prior to inoculation. Use heat and water resistant gloves when removing containers and avoid leaning over the bath.

9.0 APPARATUS

9.1 Usual laboratory equipment and in addition:

9.1 .1 Incubators: 37 ± 1 °c and 44± 1 °c or cyclic incubator
9.1 .2 Water bath: 46 ± 1 °c
9.1 .3 Colony Counter (optional)
9.1 .4 Petri dishes (sterile) of approximately 90 mm diameter
9.1 .5 Pipettes (sterile; total delivery) Iml volume

1 0.0 CULTURE MEDIA AND REAGENTS

1 0.1 Commercial dehydrated media may be used. The manufacturer's instructions must be carefully followed.

10.1.1 Maximum recovery diluent (MRD) pH
7.0± 0.2 at 25 °c
10.1.2 Buffered peptone water (BPW) pH
7-2 7.0± 0.2 at 25 °c
10.1 .3 Tryptone—bile—glucuronide medium (TBX) pH
7.2 ± 0.2 at 25 °c

1 1 .0 PROCEDURE

1 1 .1 Sample Preparation and Dilutions

1 1 .1 .1 Prepare a 10-1 homogenate in MRD or BPW, and further decimal dilutions as required in MRD as per procedure, 'Preparation of Samples and Dilutions' (FOD-PCD-MB-002).

1 1 .2 Inoculation and Incubation

1 1 .2.1 Transfer 1 ml of the sample if the product is liquid, or 1 ml of the initial suspension (10-1) in the case of other products, to a Petri dish. Repeat the procedure for each dilution.

1 1 .2.2 Pour approx. 15 ml of the T BX tempered to 46 ± 1 °c into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment the medium is poured into the dishes should not exceed 45 min.

1 1 .2.3 Carefully mix the inoculum with the medium and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface.

1 1 .2.4 After complete solidification, invert the prepared dishes and incubate them at 44 ± 1 °c for 18 to 24 h. Alternatively incubate at 37 ± 1 °c for 4 h and then at 44 ± 1 °c for a further 18 to 24 h. This is especially

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recommended if the presence of stressed cells is suspected. In any case the incubation temperature must not exceed 45 ^{c}c.

11.3 Counting of Colonies

1 1 .3.1 Examine the plates under subdued light for blue colonies (ß-glucuronidase positive) as soon as they are removed from the incubator. If this is not possible then store the plates at 5 ± 3 °c for not more than 24 h.

11.3.2 For enumeration, preferably select dishes containing not more than 300 colonies, with 10 to 150 typical colonies at 2 consecutive dilutions and count these suspect colonies. If less than one quarter of the dish is over grown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. If more than one quarter is overgrown by spreading colonies, discard the count.

12.0 RECORDING THE OBSERVATIONS

12.1 Record the colony counts obtained for each plate on the 'Food & Hygiene Testing' (RS-MB-148).

13.0 CALCULATION OF RESULTS

13.1 Preferably, colony counts are to be calculated using 2 consecutive dilutions giving 10 to 150 typical colonies on each plate. Calculate E. coli count per g or ml as follows:

$$\text{E. coli count} = \frac{\Sigma c}{v(n1 + 0.1n2)\, d}$$

where:

Σcis the sum of colony counts on all the selected plates ni is the number of plates counted at the first dilution rt2 is the number of plates counted at the second dilution d is the dilution from which the first count was obtained. v is the volume of inoculum in ml.

13.2 Round the result to two significant figures.

14.0 REPORTING OF RESULTS

14.1If no typical growth is obtained from 1 ml of the test sample (liquid), report as:
Less than 1 cfu/ml

14.2 If no typical growth is obtained from 1 ml of the 10-1 dilution, report as: Less than 10 cfu/g or ml

14.3 If the plates of the test sample (neat liquid or suspension) or of the first dilution contain less than 10 typical colonies, but at least 4, calculate the result as per Section 13.1 and report the estimated count 'a', rounded to two significant figures. a cfulg or ml (estimate)

14.4 If the total is 3 to 1, report the result as:
Present but less than (4 x d) lg or ml

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14.5 If the total number of typical and atypical colonies of the first selected dilution (dl) is >300, and if no typical growth is observed from the subsequent dilution (d2), report the estimated count as follows:
Less than 11 d2 and more than 1/ dl (1/ E.coli <1/ d2) lg or ml

14.6 If typical organisms are detected with counts between 10 and 99 per gram or ml, report in the form of: a cfulg or ml where: a is a number between 10 and 99.

14.7 If the test organisms are detected at counts of 100 or higher per gram or ml, report in the form of: ax 10^b cfu/g or ml

where: a is never less than 1 .0 or greater than 9.9 b
represents the appropriate power of ten.

14.8 If the number of typical colonies on each plate of the highest dilution is > 150, and in the presence of background flora, report in the form of:
>150/d cfu/g or ml
where: d is the highest dilution

Round up the colony count if the last figure is 5 or more and down if the last figure is 4 or less.

15.0 MEASUREMENT UNCERTAINTY

15.1 Refer to 'Measurement Uncertainty' (GPR-PCD-OI 4).

16.0 QUALITY CONTROL

16.1 Refer to the 'Quality Assurance' procedure (CPM-PCD-008) and to the 'MPHL Food Test Methods QC Schedule' (FOD-PRG-MB-002).

16.2 Internal PT: Spike 1 sample homogenate with the reference cultures listed in the record sheet RS-MB148, prepare the necessary dilutions and continue as per Section 1 1 .2. Count the colonies, calculate and report the results obtained as per Sections 1 1 .3, 12 to 14 accordingly in the 'Proficiency Testing Evaluation Form' (RS-109).

16.3 Routine QC: Use working cultures derived from the reference cultures listed in the record sheet RS-MB148, which had been previously prepared by the Quality Assurance Officers for use and record the results obtained in the same record sheet.

17.0 REFERENCES

17.1 MSA ISO 16649 - 2:2003: Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of ß-glucuronidase — positive Eschericia coli — Part 2: Colony count technique at 44 °c using 5-bromo-4-ch/oro-3-indolyl ß-D-glucuronide.

17.2 ISO 7218:2007 and its amendment: Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations

17.3 Health Protection Agency (Nov 2009). Guidelines for Assessing the Microbiological Safety of Ready-toEat Foods placed on the Market. Communicable Disease and Public Health.

Microbiology - Public Health Laboratory	
PROCEDURE — Direct Enumeration of ß-glucuronidase positive E. coli at 44 °c.	Page 5 of 5
	Copy No. 1

Flow Diagram

Prepare a 10-1 dilution of sample

U

Homogenise by stomaching

Prepare further dilutions if required in MRD

Starting with the highest dilution inoculate 1 ml of each dilution into an empty Petri dish and add approximately 15 ml of tempered T BX agar.
Mix by gentle rotation and allow to set.

Incubate at 44 °c for 18 - 24 h
(Or at 37 ± 1 °c for 4 h and 44 °c for 18 - 24 h)

Count typical colonies blue

Calculate E. coli count

COMMUNICATION OF TEST METHOD

Officers are to indicate that they have read and understood this version of the test method in the table below.

END

A.3.4 Preparing strains for MicrobesNG in inactivation buffer

Version 20220425

Preparing strains for MicrobesNG in inactivation buffer

To maximise the chance of successful DNA extraction from your strain it is important that you prepare your strain sample following the steps below. **We do not grow your samples for DNA extraction**. Instead, we extract it directly from an aliquot taken from the tube you send us. It is important that enough cells have been collected and added to the tube as per these instructions, otherwise the DNA extraction will likely fail!

These instructions are to prepare cells that will be submitted to MicrobesNG in DNA/RNA Shield (herein DRS) from Zymo Research. This buffer effectively inactivates pathogens, including tough-to-lyse microbes or viruses, without the need for additional steps (i.e. heat-treatment, homogenisation, or alcohol sterilisation).

MicrobesNG aims to provide customers with strain submission tubes containing DRS inactivation buffer. In the event that we are not able to provide these tubes you can also source the DRS buffer and use 2mL screw cap microfuge tubes to prepare your strains following the instructions below. (IMPORTANT: only use screw cap tubes with 11mm outer diameter, NOT storage tubes with 13mm outer diameter, these do not fit our standard centrifuge rotors!)

In the UK DNA/RNA Shield can be obtained from Cambridge Biosciences (https://www.bioscience.co.uk/cpl/dna-rna-shield-reagent).

Outside the UK you can find your local distributor from the manufacturer's website (https://www.zymoresearch.com/pages/our-distributors).

IMPORTANT:

For Health and Safety reasons we advise that you read the manufacturer's Safety Data Sheet for DRS before handling the DRS tubes. A copy of the SDS can be obtained from the manufacturer's website:
https://files.zymoresearch.com/sds/dna_rna_shield_ce_eu.pdf (last accessed 5th May 2021)

Preparing strains for MicrobesNG in inactivation buffer Page 1 of 8

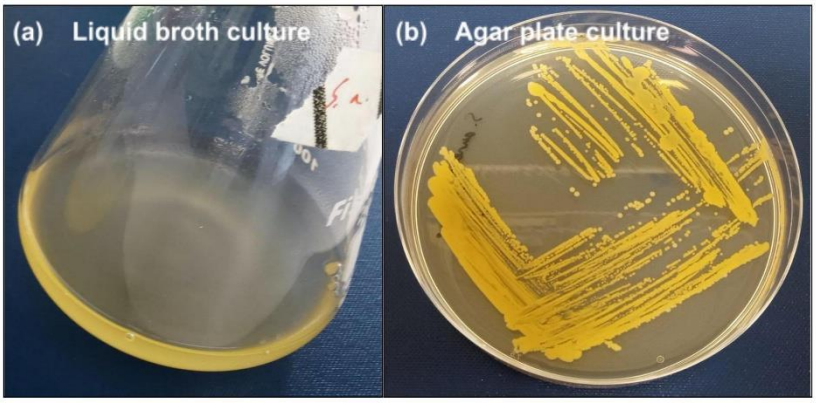
Version 20220425

MicrobesNG STRAIN PREPARATION PROTOCOL

(To see visual summary of the process see **Appendix A**)

Growing your cells

1. Take a single colony of the strain to be sequenced, mix in 200 µl of sterile buffer (for example 1xPBS). Cells can then be grown in (a) **liquid broth** (PICTURE 1a) or (b) **agar plate** (PICTURE 1b) .
    a. If you grow your cells in **liquid broth** we recommend that you use 100 µL to inoculate the liquid broth and you streak out the remaining 100 µL on a suitable agar plate to determine that the culture is pure.
    b. If you grow your cells in **agar plates** we recommend that you try to grow *at least one plate* with a lawn of bacteria in ½-⅔ of the plate and then streak out on the rest of the plate to determine that the culture is pure. Depending on the species you may need to grow more than one plate per strain.

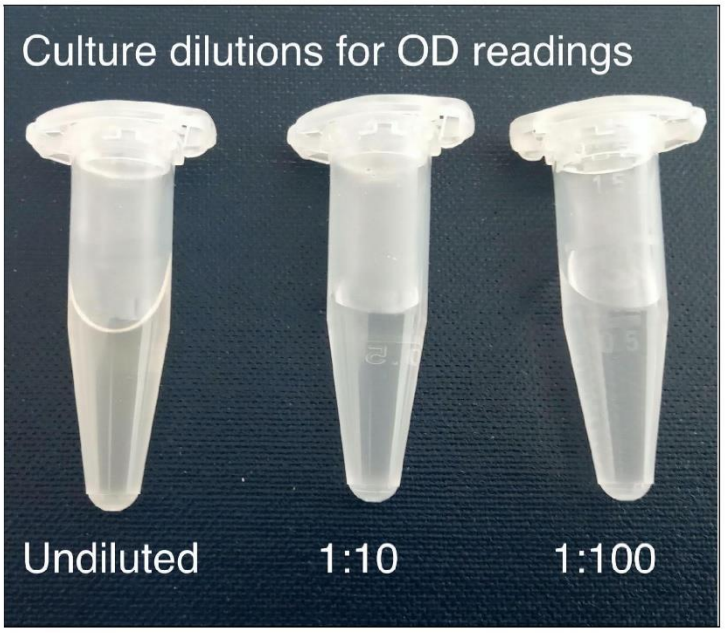


PICTURE 1. EXAMPLES OF CELL CULTURES. These images show growth examples of a *Staphylococcus aureus* strain. **(a)** Liquid broth culture at log-phase after 6 hours of growth; **(b)** Agar plate culture after overnight growth.

Collecting your cells

2. Measure the OD600nm of your liquid culture or your agar plate cells harvested and resuspended in PBS.
    a. **Liquid broth**: measure OD600nm to verify that cells are in the exponential growth phase. This is usually between 0.5 and 1.0 depending on the organism you are culturing.
    b. **Agar plate**: using a large sterile loop take **all** bacterial culture off the plate and mix into a 5mL or 10mL PBS. Measure the OD600nm of the resuspended cells.

    OD measures should only be considered as valid when they are below 1.0, so you may need to prepare a serial dilution of your culture when measuring the OD until one of the dilutions has an OD below 1.0 (PICTURE 2), and then calculate the OD of the stock culture by multiplying the measured value by the dilution factor. Cells should be harvested during the exponential growth phase.



PICTURE 2. SERIAL DILUTION TO MEASURE OD600nm. This image shows a serial dilution of *Staphylococcus aureus* liquid culture that has been prepared to measure OD600nm. Apart from the undiluted culture, two dilutions from the stock culture have been prepared (1:10 and 1:100). If the OD600nm of the undiluted culture is higher than 1.0 then the OD600nm reading of the first dilution that is below or equal to 1.0 will be used to estimate the OD600nm of the stock culture (multiplying the measured value of the dilution by the dilution factor).

3. Harvesting cells. We require **the equivalent of 8 - 12 OD600nm or 4 - 6 x 10^9 cells**[1] (e.g. 8 - 12 mL culture at 1.0 OD600nm)

   Harvest an equivalent OD600nm of 8 - 12, or 4 - 6 x 10^9 cells. If cells are grown in bigger volume containers (e.g. 50mL Falcon tubes) you can do the first centrifugation in the container, resuspend them in 1mL PBS and transfer them to the microfuge tube for the second centrifugation.

KEEP THE AMOUNT OF CELLS HARVESTED WITHIN THIS RANGE:

MINIMUM: equivalent of **8 OD600nm** or **4 x 10^9 cells**
MAXIMUM: equivalent of **12 OD600nm** or **6 x 10^9 cells**

a. If you have harvested more cells than the upper limit **remove the excess**.

b. If you have not reached the lower limit **you need to grow more cells until you reach this value**. Once you have the right number of cells centrifuge to get a pellet.

c. We have optimised our DNA extraction for strain samples within this range. **Samples outside this range of cells will likely fail DNA extraction** and it is possible we will need to request you to send a DNA (not strain!) sample, which will delay your project.

What if you cannot measure OD600nm for your strain?

We strongly recommend that cell concentrations are estimated using OD600nm measurements. However some strains (e.g. Actinobacteria) grow forming cell aggregates that do not produce homogeneous cell suspensions, thus OD600nm measurements would not be valid to estimate cell concentrations.

[1] ***X equivalent OD600nm*** refers to the **amount of cells** that, if they were resuspended in **1mL** of liquid culture, would produce an OD600nm value of **X**.
For example, if you need an *equivalent OD600nm* of **10**, this means that you need the number of cells that would produce an OD600nm of 10 if they were suspended in 1mL of liquid. If you grow your cells in a plate, collect in 10mL of PBS and the OD600nm of the resuspended cells is **2.5** (you would have measured 0.25 OD600nm of a 1:10 dilution of the culture for a valid OD600nm reading), you will need to harvest 10 (*target* equivalent OD)/2.5 (*observed* OD) = 4 mL of culture. If you then pellet these 4mL, remove the liquid and resuspend the cells in 1 mL of liquid, the OD600nm of this new suspension would be OD600nm = 10.
REMEMBER: **equivalent OD600nm** relates to **amount of cells**, and it is used as a standardised way of knowing how much culture you need to pellet to obtain a desired number of cells.

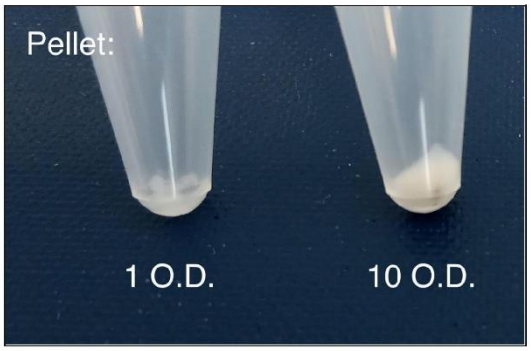
When OD600nm cannot be used as a proxy for cell concentration, or if you are absolutely unable to measure OD600nm, you can use the table below as a guideline for pellet size to prepare:

Pellet type	Pellet amount to harvest
Wet-compact, when most/all supernatant is removed the pellet is not disturbed (e.g. *E. coli* pellets)	12-18 mg
Wet-loose pellet, you need to leave some supernatant with the cells because it cannot be removed without disturbing the pellet, cells can be resuspended easily (e.g. *Streptococcus* sp. pellets)	30-50 mg (or 30-50 uL of wet pellet)

IMPORTANT! MAXIMUM CELL PELLET SIZE is 50 µL *or* 50 mg

Do NOT load more than these values in the DRS tube, otherwise there will be too many cells to be protected from degradation by the provided DRS buffer.

4. The cells should be concentrated by centrifugation in a 1.5mL or 2mL microfuge tube, washed with 1mL PBS and pelleted again (PICTURE 3).



PICTURE 3. CELL PELLETS. This picture shows two examples of harvested, PBS washed, pellets of *Staphylococcus aureus* cells. The tube on the right shows the cell pellet size for an equivalent OD600nm of 10, this is the amount of cells we would require you to resuspend in DRS buffer and send to us. The volume of the pellet is lower than 50 µL (here it is around 10-15 µL) Just for comparison, the pellet on the left is for an equivalent OD600nm of 1 - **this will NOT be enough for submission to MicrobesNG!**

5. Resuspend the final pellet in **0.5 mL** of **1x DNA/RNA Shield buffer** in the 2 mL **screw cap tube** provided.

6. (If your DRS tubes do not have a barcode label) **Label the strain tube(s)** with MicrobesNG barcode **number** (MicrobesNG's sample ID). The barcode number can be found in your project portal. Print your own label for the tube with the appropriate barcode number (please make sure the labels are printed and **NOT written by hand**), and attach the label lengthwise down the DRS tube with transparent adhesive tape - i.e. Sellotape.

7. **IMPORTANT: Fill in the sample information in your project portal at this point. We will not be able to accept your samples if you have not entered the sample information in your project portal.**

Shipping your strains

8. Strain samples in DNA/RNA later can be sent at room temperature for shipments up to 5-7 days, although shipping the samples at 4oC (blue ice) would be recommended if longer shipping times are expected.

9. **IMPORTANT:** To prevent accidental damage and leakage of the tubes during shipment, we strongly recommend that you put the tubes in a **rigid storage box**[2] (if we sent you the empty tubes in a box please use it to ship your samples to us). We would really appreciate if you could load the samples row by row in barcode order as this greatly reduces the handling time when receiving your samples.

   For shipments of **more than 10 strain tubes**, please do NOT send loose tubes in a bag, put them in a tube box instead

10. Samples (ideally in a tube storage box) should be shipped inside a rigid package (e.g. polystyrene or cardboard package) that will further protect them from breaking accidentally during shipment. We strongly recommend that you follow **packing instruction P650** (you can find this instruction in your project portal or in https://microbesng.com/microbesng-faq/).

[2] We have received cracked tubes damaged during transport shipped loose in envelopes. We have not observed this problem when tubes are shipped inside storage boxes (cardboard or plastic) shipped in padded packages (e.g. polystyrene box).

11. Attach the return label that you have received outside of your package. This will help our Laboratory Team with the identification of your project.

12. **VERY IMPORTANT: make sure that you have entered the sample information in your project portal before shipping your samples!**

    REMEMBER: We will NOT be able to accept your samples if you have not entered the sample information in your project portal.

13. Your samples are now ready to be shipped. Further information in shipping your samples to MicrobesNG can be found in https://microbesng.com/microbesng-faq/

Version 20220425
APPENDIX A
Stop culture at mid Log phase
O.D.600nm Measurement:
- Blank solution (PBS or broth media)
- 1 mL of culture (no dilution)
- 0.9 mL blank solution + 0.1 mL of culture (1:10 dilution)
- 0.95 mL blank solution + 0.05 mL of culture (1:20 dilution)
...
Use dilution with O.D. between 0.2-1.0 (max 2 but not ideal) to calculate the OD of your culture :
O.D. x Dilution factor
Centrifuge the volume required :
10 (8 to 12) equivalent O.D. (see Example below)
Discard supernatant and resuspend cells in RNA/DNA shield.
Direct culture or sub-culture
Inoculate 1 colony in broth
Streak cells on plate to confirm absence of obvious contamination
Resuspend colonies
5-10 mL PBS
Example: A 10 mL culture with an O.D.600nm of 0.25 after a 1 in 10 dilution has an OD of 2.5 (= 0.25 OD/mL x 10 [dilution factor]). This means that 1 mL of this culture contains the same number of cells than 2.5 mL of a 1.0 OD culture, which is to say that our culture has an equivalent OD of 2.5 per mL of culture). We need to harvest 10 (8-12) equivalent OD, that is the number of cells that would give an OD of 10.0 in 1mL of culture = 10 equivalent OD [the number of cells we want to harvest to give 10.0 OD in 1 mL]) / 2.5 OD/mL [i.e. our culture's equivalent OD per mL]= 4 mL (acceptable range 3.2-4.8 mL for equivalentOD 8-12) of culture to be pelleted, resuspended in DNA/RNA Shield and sent to us.
Preparing strains for MicrobesNG in inactivation buffer
Page 8 of 8

Appendix B: Additional Results

B.1 Rt PCR CTX-M Results



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B.1.1 Quantitation Report

Experiment Information

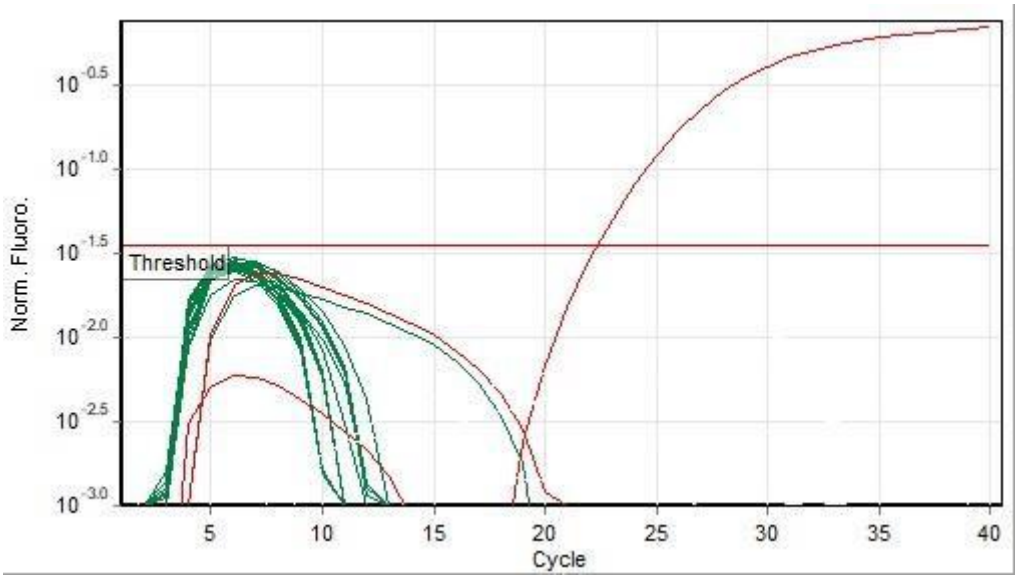
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Operator	AB
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Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.

Quantitation Information

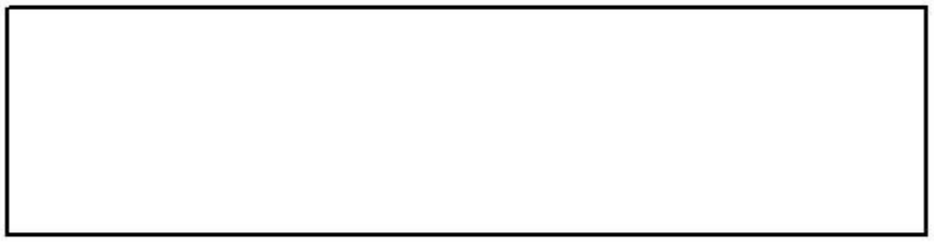
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Start normalising from cycle	1

Noise Slope Correction	Yes
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Yellow



Standard Curve



No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
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16		16	Unknown				

17		17	Unknown				
18		18	Unknown				
19		19	Unknown				
20		20	Unknown				
21		NTC	NTC				
22		PC	Positive Control	22.33			

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.2 Quantitation Report

Experiment Information

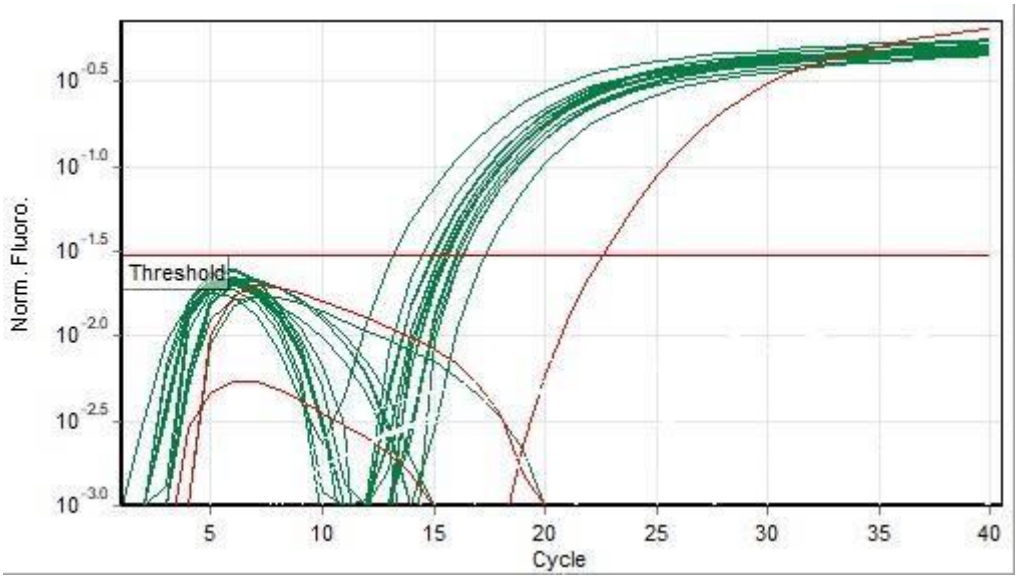
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Operator	AB
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Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.

Gain Orange	5.

Quantitation Information

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Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve

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22		PC	Positive Control	22.55			

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.3 Quantitation Report

Experiment Information

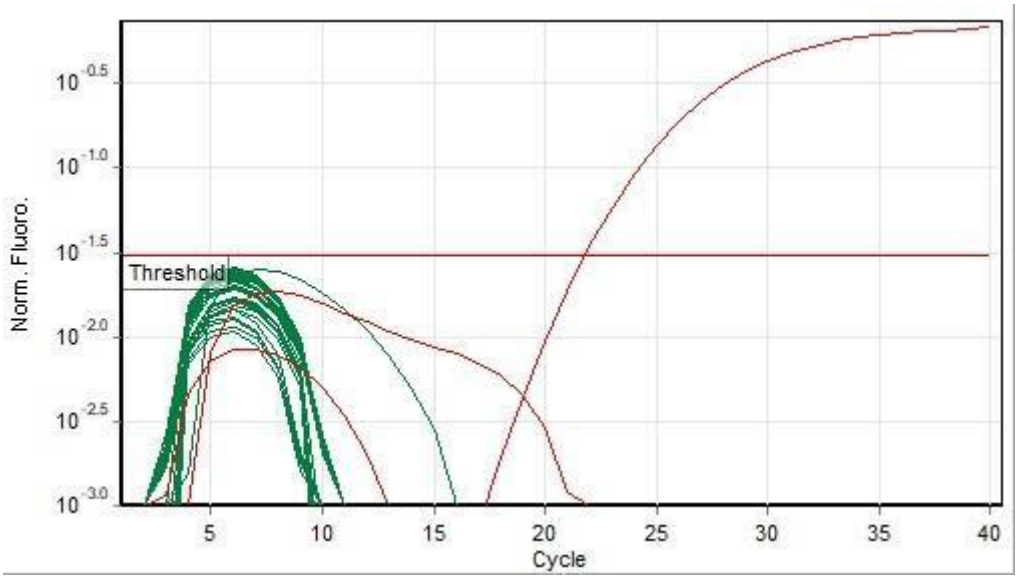
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Quantitation Information

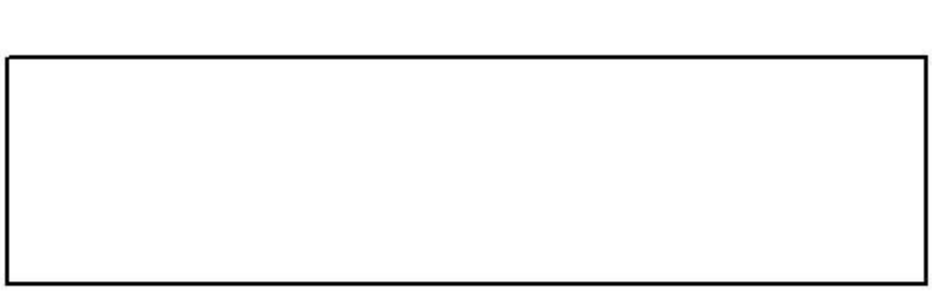
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Reaction Efficiency Threshold	Disabled
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Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Yellow



Standard Curve



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34		PC	Positive Control	21.75			

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.4 Quantitation Report

Experiment Information

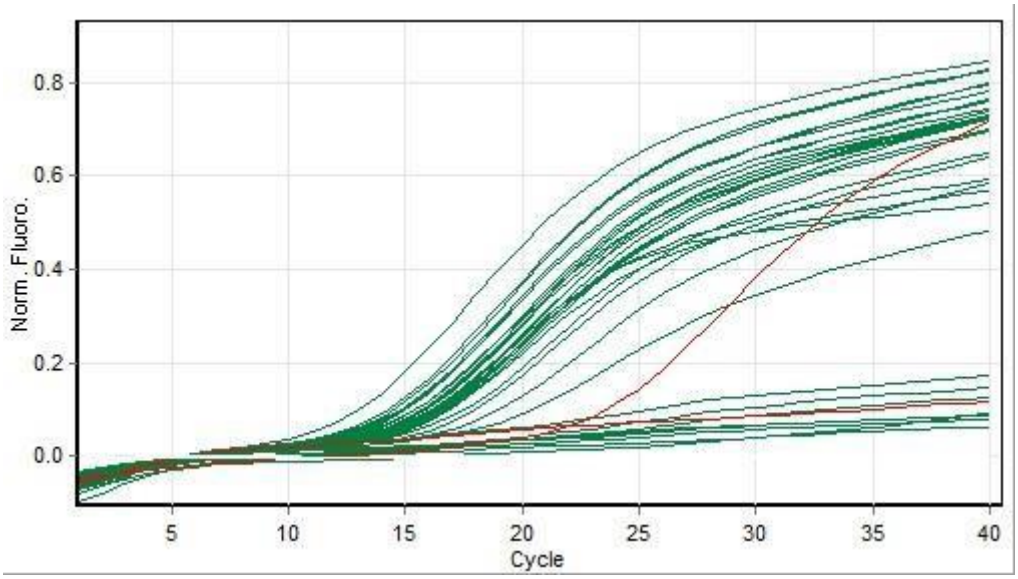
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Gain Yellow	5.
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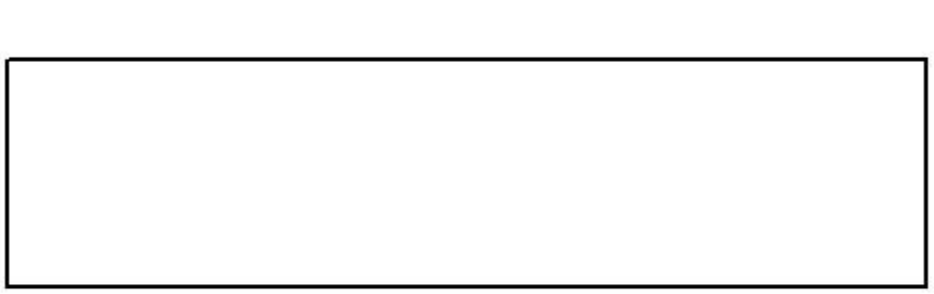
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Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve



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33		NTC	NTC				
34		PC	Positive Control				

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.5 Quantitation Report

Experiment Information

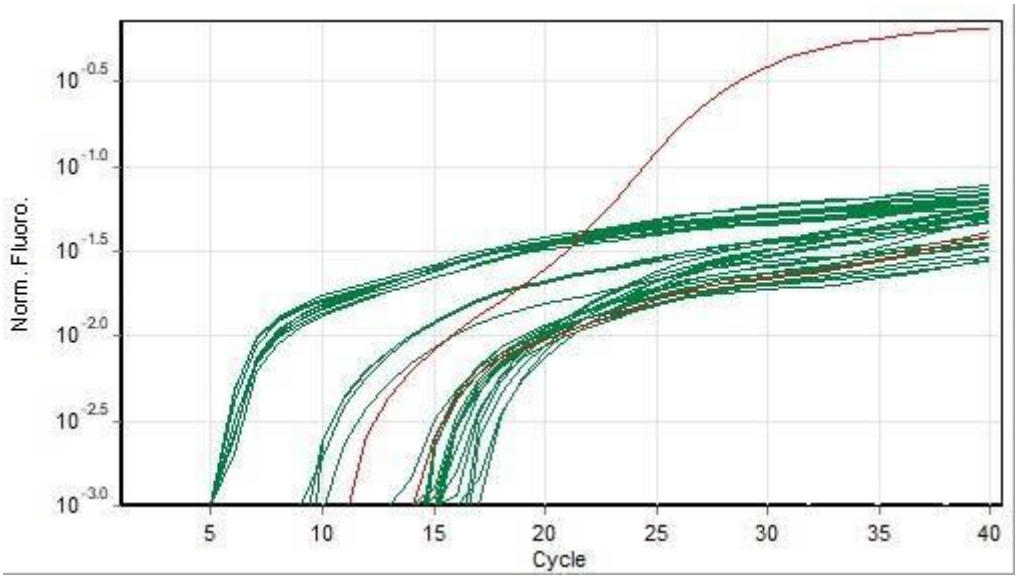
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Gain Yellow	5.

Gain Orange	5.

Quantitation Information

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Reaction Efficiency Threshold	Disabled
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Digital Filter	Light
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Imported Analysis Settings	

Quantitation data for Cycling A.Yellow



Standard Curve

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No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		9	Unknown				
10		10	Unknown				
11		11	Unknown				
12		12	Unknown				
13		13	Unknown				
14		14	Unknown				
15		15	Unknown				
16		16	Unknown				
17		17	Unknown				
18		18	Unknown				
19		19	Unknown				
20		20	Unknown				
21		21	Unknown				
22		22	Unknown				
23		23	Unknown				
24		24	Unknown				
25		25	Unknown				
26		26	Unknown				
27		27	Unknown				
28		28	Unknown				
29		29	Unknown				

30		30	Unknown				
31		NTC	NTC				
32		PC	Positive Control				

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.6 Quantitation Report

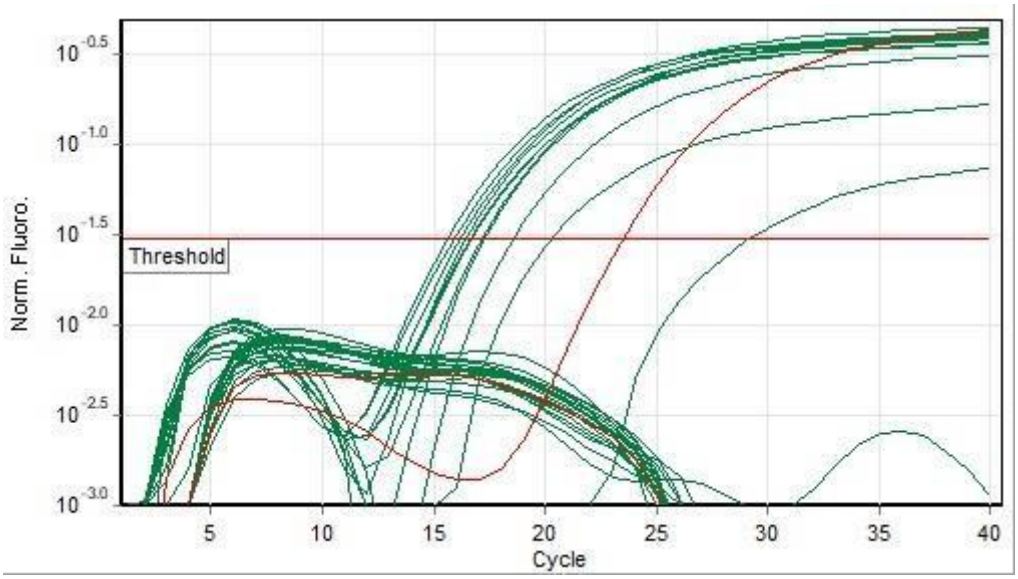
Experiment Information

Run Name	Anton Run 2023-04-11 (1)
Run Start	11/04/2023 16:36:28
Run Finish	11/04/2023 18:25:05
Operator	AB
Notes	
Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.

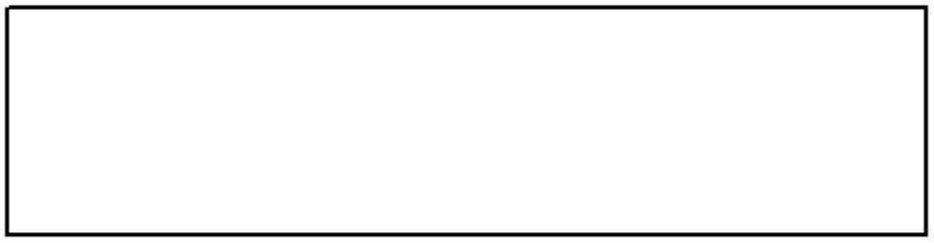
Quantitation Information

Threshold	0.030
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	Yes
No Template Control Threshold	% 5
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve



No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		1	Unknown				
2		2	Unknown				
3		3	Unknown				
4		4	Unknown				
5		4	Unknown	29.21			
6		6	Unknown				
7		7	Unknown				
8		8	Unknown				

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		9	Unknown				
10		10	Unknown				
11		11	Unknown				
12		12	Unknown				
13		13	Unknown				
14		14	Unknown				
15		15	Unknown				

16		16	Unknown	18.56			
17		17	Unknown				
18		18	Unknown				
19		19	Unknown				
20		20	Unknown	20.30			
21		21	Unknown	16.69			
22		22	Unknown	16.59			
23		23	Unknown	17.25			
24		24	Unknown	17.13			
25		25	Unknown				
26		26	Unknown				
27		27	Unknown	15.56			
28		28	Unknown	15.87			
29		29	Unknown	16.40			
30		30	Unknown	16.17			
31		NTC	NTC				
32		PC	Positive Control	23.45			

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.7 Quantitation Report

Experiment Information

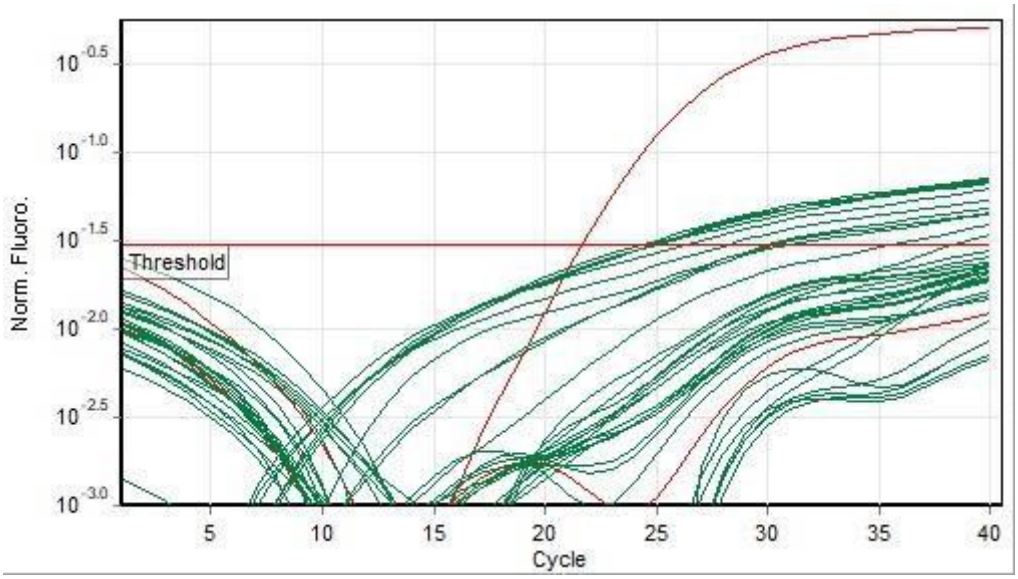
Run Name	ANTON RUN 2 2022-11-08 (1)
Run Start	08/11/2022 11:08:13
Run Finish	08/11/2022 12:57:02
Operator	JS
Notes	
Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.

Quantitation Information

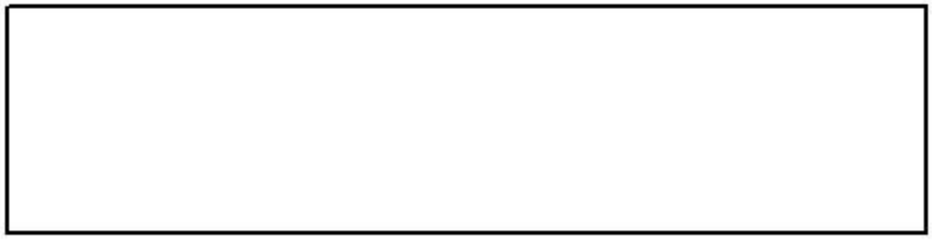
Threshold	0.030
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	6
Noise Slope Correction	Yes
No Template Control Threshold	% 5
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation

Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Yellow



Standard Curve



No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		PC	Positive Control	21.68			

2		NTC	NTC				
3		11	Unknown				
4		12	Unknown		NEG (NTC)		
5		13	Unknown	35.78			
6		14	Unknown				
7		15	Unknown				
8		16	Unknown	24.33			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		17	Unknown	30.16			
10		18	Unknown	24.99			
11		19	Unknown				
12		20	Unknown	38.49			
13		21	Unknown				
14		22	Unknown				
15		23	Unknown				
16		24	Unknown	25.03			
17		25	Unknown				
18		26	Unknown				
19		27	Unknown				

20		28	Unknown	30.94			
21		29	Unknown				
22		30	Unknown	26.40			
23		31	Unknown				
24		32	Unknown				
25		33	Unknown		NEG (NTC)		
26		34	Unknown				
27		35	Unknown				
28		36	Unknown				
29		37	Unknown				
30		38	Unknown	24.81			
31		39	Unknown	28.26			
32		40	Unknown				
33		41	Unknown	30.47			
34		42	Unknown				
35		43	Unknown				
36		3	Unknown				

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.8 Quantitation Report

Experiment Information

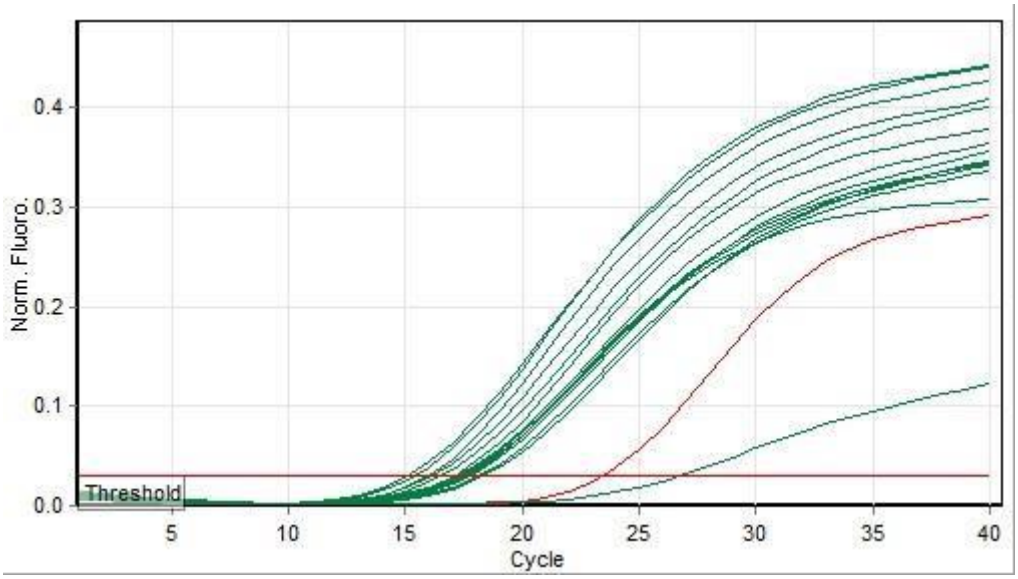
Run Name	ANTON RUN 2 2022-11-08 (1)
Run Start	08/11/2022 11:08:13
Run Finish	08/11/2022 12:57:02
Operator	JS
Notes	
Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.

Quantitation Information

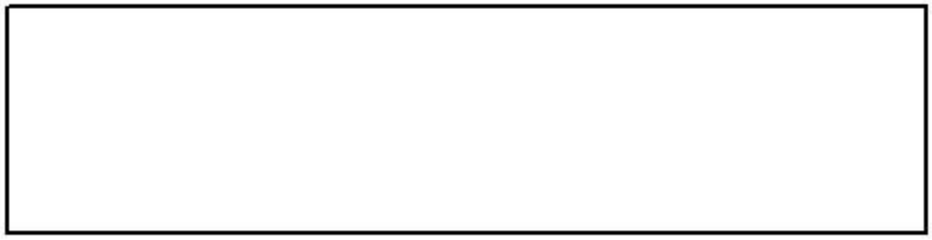
Threshold	0.030
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	6
Noise Slope Correction	Yes
No Template Control Threshold	% 10
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation

Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve



No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		PC	Positive Control	23.51			
2		NTC	NTC		NEG (NTC)		

3		11	Unknown	17.77			
4		12	Unknown	26.76			
5		13	Unknown	17.11			
6		14	Unknown		NEG (NTC)		
7		15	Unknown		NEG (NTC)		
8		16	Unknown	15.11			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		17	Unknown	17.36			
10		18	Unknown	18.20			
11		19	Unknown		NEG (NTC)		
12		20	Unknown		NEG (NTC)		
13		21	Unknown	18.42			
14		22	Unknown		NEG (NTC)		
15		23	Unknown		NEG (NTC)		
16		24	Unknown	17.64			
17		25	Unknown	15.94			
18		26	Unknown		NEG (NTC)		
19		27	Unknown		NEG (NTC)		
20		28	Unknown	17.18			

21		29	Unknown		NEG (NTC)		
22		30	Unknown		NEG (NTC)		
23		31	Unknown		NEG (NTC)		
24		32	Unknown		NEG (NTC)		
25		33	Unknown		NEG (NTC)		
26		34	Unknown	17.48			
27		35	Unknown		NEG (NTC)		
28		36	Unknown	15.40			
29		37	Unknown		NEG (NTC)		
30		38	Unknown	16.17			
31		39	Unknown		NEG (NTC)		
32		40	Unknown		NEG (NTC)		
33		41	Unknown		NEG (NTC)		
34		42	Unknown		NEG (NTC)		
35		43	Unknown		NEG (NTC)		
36		3	Unknown	16.55			

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.9 Quantitation Report

Experiment Information

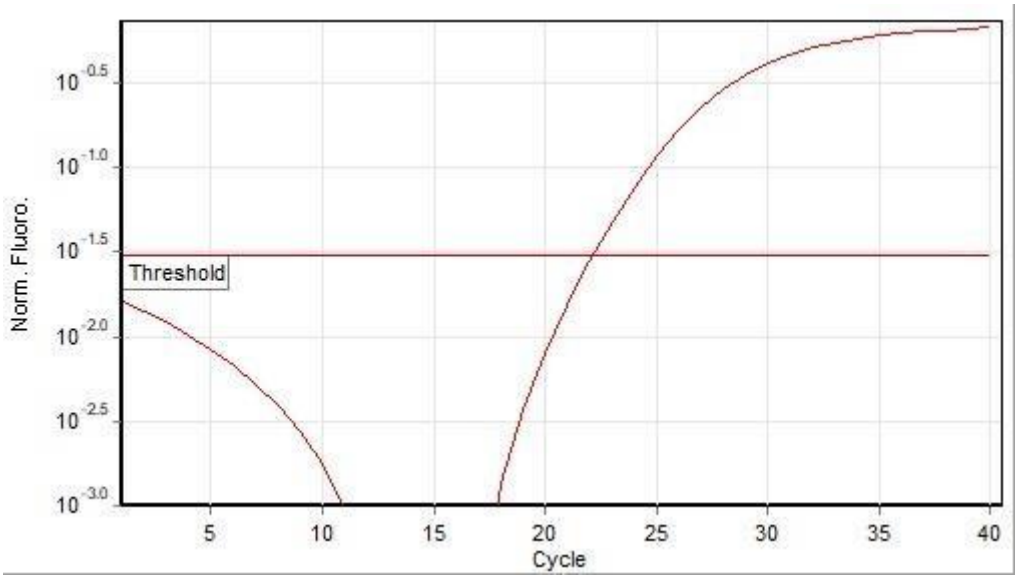
Run Name	Anton run 1 (1)
Run Start	01/11/2022 17:07:43
Run Finish	01/11/2022 18:53:29
Operator	JS
Notes	
Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.

Quantitation Information

Threshold	0.030
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	6
Noise Slope Correction	Yes
No Template Control Threshold	% 5
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1

Imported Analysis Settings	

Quantitation data for Cycling A.Yellow



Standard Curve

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		PC	Positive Control	22.12			
2		NTC	NTC		NEG (NTC)		
3		1	Unknown		NEG (NTC)		
4		2	Unknown		NEG (NTC)		

5		3	Unknown		NEG (NTC)		
6		4	Unknown		NEG (NTC)		
7		5	Unknown		NEG (NTC)		
8		6	Unknown		NEG (NTC)		

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		7	Unknown		NEG (NTC)		
10		8	Unknown		NEG (NTC)		
11		9	Unknown		NEG (NTC)		
12		10	Unknown		NEG (NTC)		

NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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B.1.10 Quantitation Report

Experiment Information

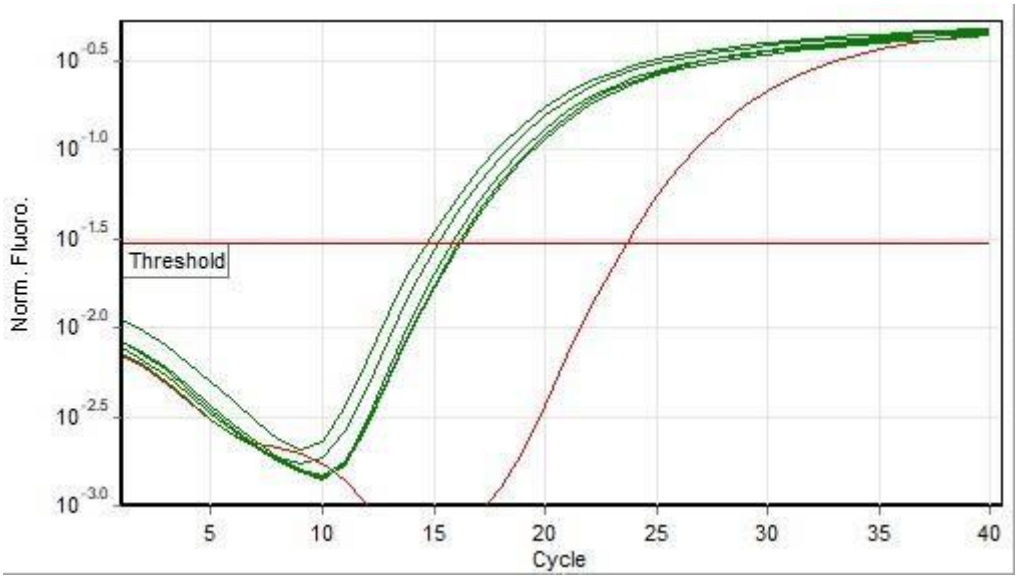
Run Name	Anton run 1 (1)
Run Start	01/11/2022 17:07:43
Run Finish	01/11/2022 18:53:29
Operator	JS
Notes	
Run On Software Version	Rotor-Gene 2.2.3.11
Run Signature	The Run Signature is valid.
Gain Green	5.

Gain Yellow	5.
Gain Orange	5.

Quantitation Information

Threshold	0.030
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	6
Noise Slope Correction	Yes
No Template Control Threshold	% 5
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

Quantitation data for Cycling A.Green



Standard Curve



No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		PC	Positive Control	23.61			
2		NTC	NTC		NEG (NTC)		
3		1	Unknown		NEG (NTC)		
4		2	Unknown		NEG (NTC)		
5		3	Unknown	16.16			
6		4	Unknown	16.07			
7		5	Unknown		NEG (NTC)		
8		6	Unknown	15.79			

(Continued on next page)...

No.	Colour	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
9		7	Unknown	15.19			
10		8	Unknown		NEG (NTC)		
11		9	Unknown	14.69			
12		10	Unknown		NEG (NTC)		

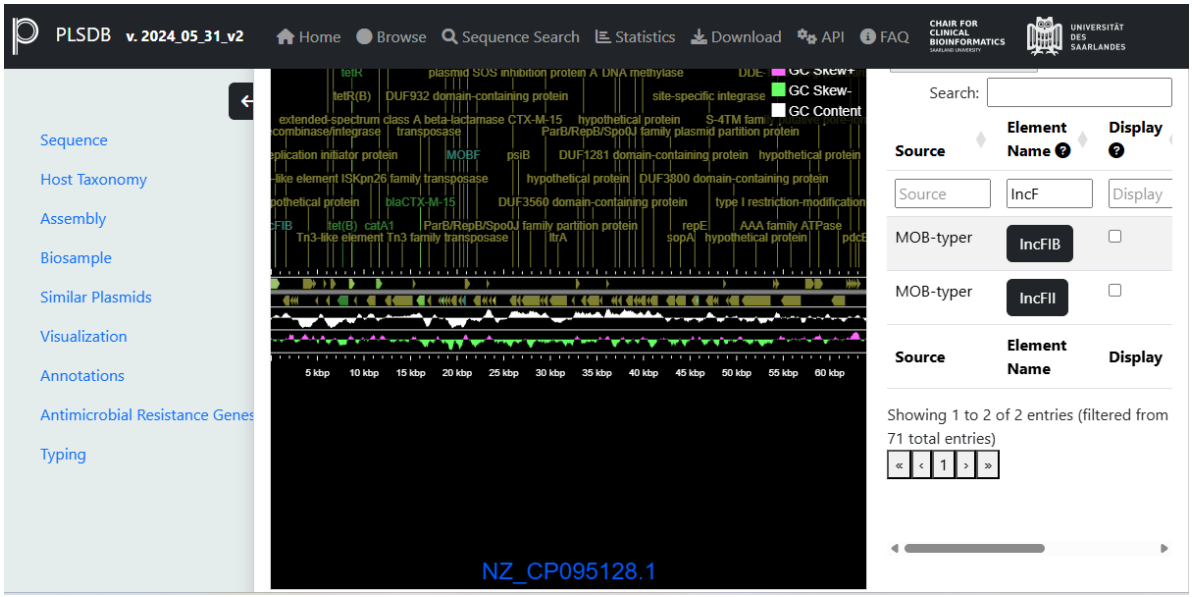
NEG (NTC) - Sample cancelled due to NTC Threshold. NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold. **Legend:**

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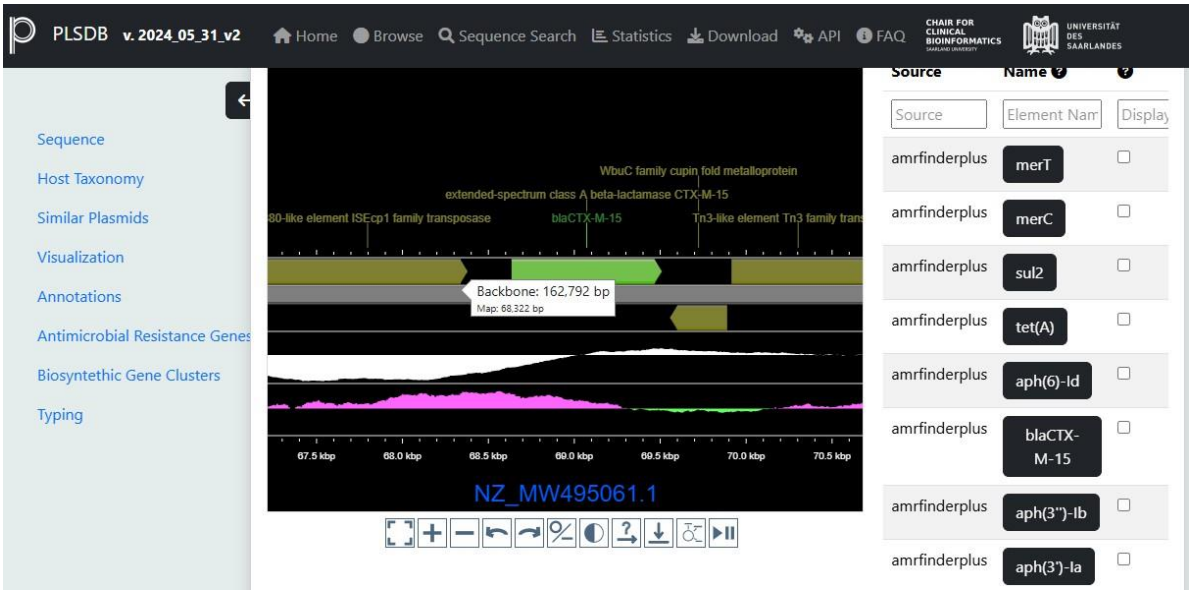
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B.2 Plasmids

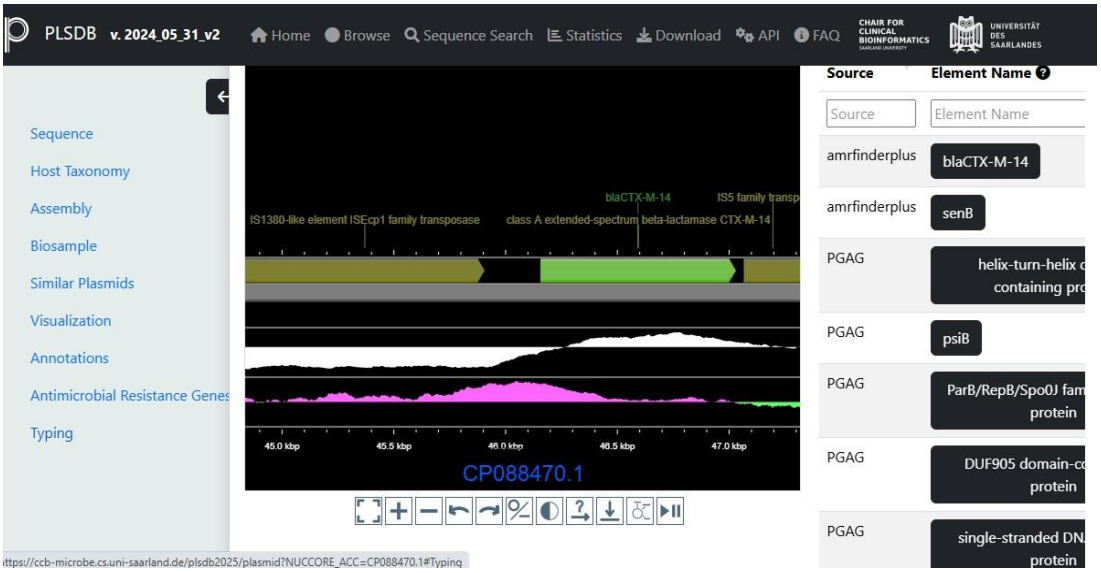
B.2.1 Accession number NZ_CP095128.1



B.2.2 Accession number NZ_MW495061.1



B.2.3 Accession number CP088470.1



B.2.4 Accession number NZ_MW495061.1

