

**A STUDY OF THE PRACTICAL CHALLENGES
ASSOCIATED WITH DEVELOPING
A NEW TEST FOR BOVINE TUBERCULOSIS**

By

ANDREA PIROVANO

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Supervisors

DR. CATHERINE REES

DR. BENJAMIN SWIFT

ABSTRACT

Bovine tuberculosis, a chronic disease caused by *Mycobacterium bovis*, is a major economic problem affecting farmers both in the UK and worldwide. In order to prevent and control bovine tuberculosis it is very important to be able to promptly and definitively diagnose infected animals. However, the current diagnostic tools show some limitations which can contribute to cause economic losses and to the transmission of the disease to other animals in a herd, to wildlife and potentially to humans. Therefore, there is an urgent need to have available new diagnostic methods that allow the definitive detection of bovine tuberculosis. In this work, the practical issues associated with the cost-effective production of two diagnostic kits that employ mycobacteriophage (Actiphage™ and Actiphage™ Rapid) were investigated. This technology exploits the ability of bacteriophage to infect live mycobacterial species in a strain specific manner. This technique was originally developed as a tool for the diagnosis of human tuberculosis (*FastPlaqueTB*™, BIOTEC) and has now been refined to offer transformational tools with significant applications in veterinary, agriculture and - potentially – the human sector. These kits are manufactured and distributed by PBD Biotech Ltd. (Thurston, Suffolk, UK) and allow the detection of the presence of *M. bovis* and *M. avium* subsp. *paratuberculosis* in diverse sample types, such as blood and milk. The assays can therefore be used for infection control and as a part of a quality assurance programme to confirm the absence of these organisms in non-pasteurized dairy products. Furthermore, the methods can be adapted to detect other types of mycobacteria and applied to detect infection not only in cattle but also in other animal hosts including sheep, goats, deer, horses, alpacas, llamas as well as companion animals and exotic species.

In order to develop the simplest method that gives a reproducible titre of at least 10^{10} pfu ml⁻¹, three different ways of manufacturing the freeze-dried bacteriophage supplied with the kits were explored. With all of the three methods, there was no difference in the titre of the phage culture before and after freeze drying. This suggests that the process does not have a negative impact on the viability of the phage. With the first two methods employed, the titre obtained was normally 10^9 pfu ml⁻¹. Only one time, using method 2, the titre obtained was 10^{10} pfu ml⁻¹. This titre seems to have been routinely

achieved modifying method 2 in order to ensure an optimal elution of the bacteriophage from the agar to the medium. Shelf life studies on the freeze dried phage are being performed and, so far, they revealed an average stability of about 3 months if stored at +5 / +8°C. The practical issues related to manufacturing of the virucide tablets supplied with the Actiphage™ Core kit were also explored and a softer formula led to jamming of the tablet press and broken tablets but also produced a tablet that was easier to dissolve. Once the production issues were solved by adjusting the machine settings, a procedure for checking the weights of the tablets was developed. Finally, work was carried out to identify the best source of the other kits components and to design and test packaging materials, and finally an evaluation of the cost of competitor tests was performed to better understand the market price that could be charged for Actiphage™ tests.

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LIST OF ABBREVIATIONS

Ab	Antibodies
AFB	Acid fast bacteria
BCG	Bacillus Calmette Guerin
bTB	Bovine tuberculosis
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EN	Electronic nose
FAS	Ferrous Ammonium Sulphate
γ-IFN	Gamma-Interferon
GB	Great Britain
IGRA	Interferon-gamma release assay
LF	Lateral Flow
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
MTC	<i>Mycobacterium tuberculosis</i> complex
NSR	Non-Specific Reactors
NVL	Non-Visible Lesions
OD	Optical Density
PCR	Polymerase Chain Reactions
RNA	Ribonucleic Acid
ssRNA	single stranded Ribonucleic Acid
PBMCs	Peripheral Blood Mononuclear Cells
PPD	Purified Protein Derivative
Se	Sensitivity
Sp	Specificity
spp.	Species
SICCT	Single Intradermal Comparative Cervical Test
TB	Tuberculosis
VOCs	Volatile organic compounds

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 BOVINE TUBERCULOSIS

Bovine tuberculosis (bTB) is a zoonotic infectious disease of international importance caused by *Mycobacterium bovis*. It affects primarily cattle, but can infect humans as well as a variety of other domesticated and wild animals (Crawshaw, 2013). *Mycobacterium bovis*, with *M. tuberculosis*, *M. caprae*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. cannettii* and *M. bovis* BCG, form part of the *Mycobacterium tuberculosis* complex group of pathogens.

The history of bTB control and eradication is long; in Britain tuberculosis was responsible for over 800,000 human deaths between 1850 and 1950, and many of these cases were due to *M. bovis* infections transmitted via raw milk (Waddington, 2002). Despite compulsory eradication schemes being in place since the 1950s, the UK has struggled to eradicate the disease; in fact, the incidence of bTB outbreaks has increased and control measures continue to be a significant economic burden for this UK agriculture sector (Szmaragd *et al.*, 2013). Costs associated with the inability to control this disease include reduced productivity in affected animals, testing programs, culling of affected animals, and restrictions on movement and trade. Outside the UK bTB is listed in the top 14 principal diseases that affect livestock production in Africa and South Asia, and the direct impact of this disease in these countries is estimated to be US\$300 million annually (Vordermeier *et al.*, 2016) while globally the disease costs US\$3 billion annually (Waters *et al.*, 2012).

Although in developed countries bTB only rarely poses a threat to human health, it causes a serious food security challenge to the agricultural industry in terms of dairy and meat production (Marsden and Morley, 2014; Tomlinson, 2013). Cattle which are identified as infected - or are thought to have a high chance of being TB infected - are slaughtered and thus the farmer loses the value of the animal and its output, although in GB the Government pays farmers compensation for slaughtered animals based on their market value and so a large part of the economic cost is borne by the taxpayer (DEFRA 2014b; Krebs *et al.*, 1997). In addition, when an animal in a herd tests positive for the disease, the entire herd is put under movement restrictions until all the remaining animals have given repeated test negative results (DEFRA 2014b; Krebs *et al.*, 1997). Until that point cattle cannot be moved to market, nor can animals be brought

into the herd to replace slaughtered animals (DEFRA 2014b; Krebs *et al.*, 1997). Within the past 10 years over £500 million on testing, compensation and research with further costs being borne by the agricultural industry (Godfray *et al.*, 2013).

bTB-infected cattle may facilitate the spread of infection to other domestic and wild animals (Caron *et al.*, 2013; McCallan *et al.*, 2014). In order to control and eradicate TB from cattle, all cattle are regularly tested via a skin test – the primary ante-mortem test for bTB in GB - at a frequency that is determined by the prevalence of bTB within the cattle population. In addition, all cattle moved to another farm are also tested prior to movement (Krebs *et al.*, 1997) and can lead to the identification of infected animals and outbreaks of disease (AHVLA 2013).

Spatio-temporal disease analysis (Lin *et al.*, 2015; Moustakas and Evans, 2015; Ruiz-Medina *et al.*, 2014) that describe time trends and geographic disparities of disease dynamics provide useful insights into the overall efficiency of control efforts and eradication of the disease (Booth *et al.*, 2005) as well allowing the relative efficiency of different management measures towards eradication to be monitored (Yu *et al.*, 2013). According to EU Directives 64/432/EEC and 97/12/EC the minimum testing frequency for cattle depends on the percentage of infected cattle herds. Annual testing is required unless the percentage of infected herds in a region is 1% or less. When the percentage of infected animals is 0.2% or less than 0.1% testing may be conducted every 3 or 4 years, respectively. Most places in GB tested cattle every 4 years during the 1990s (DEFRA 2014b; Krebs *et al.*, 1997). This frequency decided on economic grounds since increasing the frequency would increase the annual cost of testing (Krebs *et al.* 1997). In the case of bTB, monthly statistics are recorded as part of the legal requirements for EU Member States (DEFRA 2014b) and these data are publicly available. Scotland has had a risk based surveillance testing policy (Dorjee *et al.*, 2013) under which high risk herds are tested every 4 years and since September 2009 has been designated as bTB free while the rest of GB was not granted this status due to detection of a significant number of outbreaks of disease (Abernethy *et al.*, 2013). Wales has had an annual or even more frequent testing policy of all herds since January 2010 while in England the

number of herds tested annually (instead of every 4 years) has increased since 2010 and expanded further in the East and North regions in 2011.

Infection of cattle with *M. bovis* is usually chronic and can remain subclinical for a long period. Importantly, infected cattle can become infectious long before they exhibit any obvious clinical sign or lesions typical of bTB detectable even with the most careful veterinary examination.

The majority of currently employed bTB eradication programmes rely on two main diagnostic tools: tuberculin intradermal tests and interferon-gamma release assay (IGRA). These tests detect immune responses of infected animals to the presence of the bacteria and will be discussed in more detail in the next sections. However immune responses may be influenced by previous exposure to other mycobacterial agents, as a high level of molecular similarity exists among mycobacterial species (Lamont *et al.*, 2014).

Hence the current testing limitation and the significant economic losses that occur, as well as the risk of transmission among livestock, human and wildlife, emphasizes the desperate need for rapid and inexpensive diagnostic methods able to detect and distinguish *M. bovis* from other pathogenic and environmental mycobacteria (Vordermeier *et al.*, 2009). Development of more specific diagnostic tests, relying on the identification of the pathogen or its antigens, have proven to be challenging. Due to the slow growth of members of the *M. tuberculosis* complex (MTBC), culture is not suitable as a diagnostic test. Molecular-based methods (which are independent of viable mycobacteria) have not been promising in providing faster results when testing a variety of samples, including blood, due to low sensitivity even with high volume extraction (Pavlinac *et al.*, 2016).

1.2 Ante-mortem tests for bTB

1.2.1 Intradermal tuberculin (“skin”) tests

The skin tests are the international standard for ante mortem diagnosis of bTB in cattle herds and individual animals. They are based on obtaining a delayed-type hypersensitivity response to the intradermal injection of tuberculin, a crude protein extract from supernatants of mycobacterial cultures. A number of reports have provided written detailed historical accounts of the events that led to the development of tuberculin, first as a possible therapeutic vaccine for human TB and later as a diagnostic tool in human and veterinary medicine (Snider, 1982; Monaghan *et al.*, 1994; Kaufmann and Shaible, 2005). Briefly, the glycerol extract of pure liquid culture of tubercle bacilli, originally discovered by Robert Koch in 1890, has been refined over the years and now a purified protein derivative (PPD) tuberculin is used worldwide (Snider, 1982; Monaghan *et al.*, 1994; Cousins *et al.*, 1998; Anon, 2004 a,b). This is the complex water-soluble fraction of heat-treated products of growth and lysis of *M. bovis* (Anon, 2002). The bovine tuberculin currently in use in the UK and many other countries is a preparation of *M. bovis* AN5, a field strain isolated in England in 1948 and selected for its optimal growth *in vitro* (Inwald *et al.*, 2003). The organism is cultured in liquid synthetic medium, heat treated in free-flowing steam, filtered, concentrated by precipitation, washed and then re-dissolved into a sterile preparation free from intact mycobacteria (Francis *et al.*, 1978; Monaghan *et al.*, 1994; Anon, 2004b). Similarly, avian and human tuberculins, respectively, are obtained from cultures of specific strains of the avian (*M. avium* subsp. *avium*) and human (*M. tuberculosis*) TB bacilli. When these PPDs are injected into the skin of an animal that is not sensitised to tuberculin antigens, no significant local inflammatory response occurs. However, if tuberculin is injected into an animal whose immune system has been sensitised by infection with *M. bovis*, or by exposure to cross-reacting antigens, an inflammatory response is triggered resulting in swelling at the injection site that reaches its greatest intensity 48-72h after the injection but regresses rapidly afterwards (Lepper *et al.*, 1977; Francis *et al.*, 1978; Pollock *et al.*, 2003). This delayed-type hypersensitivity reaction to the intradermal injection of PPD tuberculin is mediated by a population of sensitised T-cells (Thorns and Morris, 1983)

but takes a number of weeks after infection to develop (Francis, 1947; 1958). The simplest format of the skin test is performed by injecting a small volume of bovine tuberculin into the skin. Approximately 72 h after the injection, the site of injection is examined for any evidence of inflammation. This is known as the single intradermal test (SIT) and it can be applied in the skin of the mid-cervical region (cervical SIT) or in the base of the tail (caudal fold SIT) (Francis *et al.*, 1978; Monaghan *et al.*, 1994; Anon, 2004c). When a cervical SIT is used, in addition to visual observation and palpation of the injection site, the skin fold thickness is measured with callipers to the nearest millimetre immediately before the injection of tuberculin and 72 h later ($\pm 4 - 6$ h) (Anon, 2004b). The principle of the SIT for cattle is, therefore, similar to that of the intracutaneous test developed in 1908 by the French physician Charles Mantoux for the diagnosis of *M. tuberculosis* infections in humans (Snider, 1982).

Reactivity to bovine tuberculin is usually greatest in cattle infected with *M. bovis* or *M. tuberculosis*. In comparative skin tests, animals infected with mycobacteria other than the MTB complex generally develop reactions to avian tuberculin much greater than those to bovine tuberculin (Pearson *et al.*, 1977; Ketterer *et al.*, 1981; Cooney *et al.*, 1997; Köler *et al.*, 2001; Hope *et al.*, 2005). This difference provides the basis for the SICCT test used in the UK and Ireland and overcomes most of the problems of non-specific reactions associated with the SIT. The single intradermal comparative cervical tuberculin (SICCT) test is more time consuming, as it requires simultaneous injections of both bovine and avian tuberculins side-by-side into the skin of the neck. The interpretation of the SICCT test is based on the observation that *M. bovis*-infected cattle tend to show a greater response to bovine tuberculin than to avian tuberculin, whereas infections with other mycobacteria promote the reverse response (Karlson, 1962; Lesslie *et al.*, 1975; Francis *et al.*, 1978; Kazda and Cook, 1988; Pollock *et al.*, 2003). This test therefore allows better discrimination than the SIT between animals infected with *M. bovis* and those sensitised to tuberculin after exposure to organisms of the *M. avium* complex or to environmental non-pathogenic mycobacteria (Francis *et al.*, 1978; Monaghan *et al.*, 1994). Which type of skin test to select generally depends on the prevalence of exposure to other sensitising environmental mycobacteria (Monaghan *et al.*, 1994). The non-comparative caudal fold SIT is the primary ante mortem test for

routine screening of individual cattle in USA, Canada and New Zealand (Tweedle and Livingstone, 1994; Anon, 2004c, 2004b) and was the test used to successfully eradicate bTB from Australia (Cousins *et al.*, 1998). The cervical SIT is the primary bTB screening test for both individual cattle and herds in continental Europe (Francis *et al.*, 1978; Monaghan *et al.*, 1994; Caffrey, 1994; Anon, 2004b) and the SICCT test is then mainly used as an ancillary serial test for reactors or inconclusive reactors to the SIT (Tweedle and Livingstone, 1994; Anon 2004c), although the γ -IFN test (section 1.2.2) is gradually replacing the SICCT test as the ancillary test of choice (Anon, 2004c, 2005b, Ryan *et al.*, 2000; Buddle *et al.*, 2001). By contrast in the UK and Ireland, studies evaluating the performance of the SIT disclosed a high incidence (6 – 12%) of cattle giving a false positive (non-specific) reaction to the “mammalian” tuberculin that was being used which was derived from three different strains of *M. tuberculosis* (Anon, 1965; Leslie *et al.*, 1975; Monaghan *et al.*, 1994). A double intradermal comparative test, with injection of avian and bovine tuberculins in the neck 48 h after a first injection, was adopted in GB over the period 1940-1943 (Kerr *et al.*, 1946; Anon, 1965). In 1947 this double intradermal comparative test was abandoned in favour of a single intradermal comparative tuberculin (SICCT) test after experimental work had shown that the second tuberculin injections were unnecessary (Anon, 1965). The Republic of Ireland and Northern Ireland also adopted the SICCT test in 1954 and 1958, respectively (Monaghan *et al.*, 1994). This is the skin test still in use in the UK and Ireland, although the original SICCT test protocol was slightly modified in 1975, when the more potent and specific Weybridge bovine PPD tuberculin replaced the old “mammalian” tuberculin (Lesslie *et al.*, 1975; Monaghan *et al.*, 1994; Inwald *et al.*, 2003).

In the UK and Ireland, the SICCT test is used for both routine screening and *ad hoc* testing of reactors and at-risk herds. In the Republic of Ireland and Northern Ireland, all cattle herds are routinely tested at 12month intervals, whereas in GB routine SICCT testing takes place every 12-48 months according to the proportion of herds in a specific area sustaining confirmed bTB breakdowns (defined as Low or High Risk areas; Anon, 2004b, 2005c; State Veterinary Service, 2005). The current rules of test interpretation and tuberculin dosage are essentially those recommended by Lesslie *et al.* in 1975, based on extensive field trials of the SICCT test using Weybridge bovine PPD (Fig. 1.1).

In herds in which *M. bovis* infection has been established by post mortem inspection of reactors and/or culture of tissue samples, the result of the initial (disclosing) and subsequent (short-interval) SICCT tests are assessed under the so-called “severe” interpretation. This involves lowering the cut-off point for an animal to be declared a reactor or inconclusive reactor (Leslie *et al.*, 1975; State Veterinary Service, 2005). Although this enhances the sensitivity of the SICCT test over its standard interpretation, severe interpretation is not used in routine screening tests because it reduces the specificity of the test. Test specificity (Sp) is the proportion of uninfected animals that are correctly identified as negative by a diagnostic test (Martin *et al.*, 1987); i.e., it would cause bTB to be unnecessarily diagnosed in too many animals or herds.

The sensitivity (Se) of a diagnostic test is defined as the proportion of infected animals detected as positive in the diagnostic assay. One problem for the SICCT test is that Se is known to vary due to a number of biological factors. For instance, due to the delayed hypersensitivity reaction, newly infected cattle generally do not react to intradermal injection of tuberculin with hypersensitivity to tuberculin usually developing in cattle 1-9 weeks after infection with *M. bovis*, and a full response is likely to develop in most animals 3-6 weeks post infection (Francis, 1947; 1958; Kleeberg, 1960). This situation is complicated because a state of anergy may develop in cattle with advanced or generalised TB and also (temporarily) in animals subjected to stress (Lepper *et al.*, 1977; Pollock and Neill, 2002). For instance, tuberculous cows that have calved within the preceding four to six weeks sometimes fail to react to the tuberculin test (reviewed by Monaghan *et al.*, 1994). In addition, the administration of glucocorticoids, used to treat severe respiratory diseases or ketosis in dairy cattle, can also lead to a significant reduction in the size of the bovine tuberculin reaction in infected cattle (Doherty *et al.*, 1995a) or co-infection with viruses that depress the function of lymphocytes and macrophages (such as the bovine viral diarrhoea virus) may also have a transient effect on the Se of the SICCT test (Charleston *et al.*, 2001).

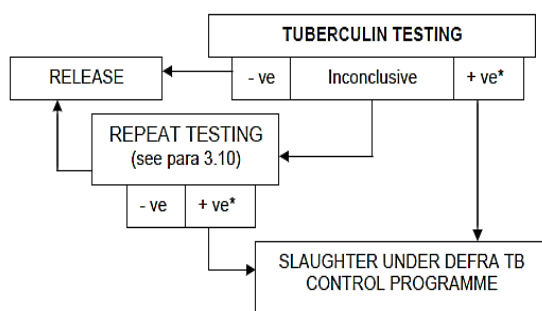


Figure 1.1 Skin test Interpretation

Standard Interpretation (England and Scotland)

Reaction	Result at Standard Interpretation
1. Animals showing a negative bovine reaction and a positive or negative avian reaction. 2. Animals showing a positive bovine reaction equal to or less than a positive avian reaction.	Pass (retain)
1. Animals showing a positive bovine reaction not more than 4mm greater than a positive avian reaction. 2. Animals showing a positive bovine reaction and a negative avian reaction where the difference is 4mm or less.	Inconclusive (retest)
1. Animals showing a positive bovine reaction more than 4mm greater than a negative or positive avian reaction.	Fail (remove)

Severe Interpretation (England and Scotland)

Reaction	Result at Severe Interpretation – England and Scotland
1. Animals showing a negative bovine reaction and a positive or negative avian reaction. 2. Animals showing a positive bovine reaction equal to or less than a positive avian reaction.	Pass (retain)
1. Animals showing a positive bovine reaction of not more than 2mm greater than a positive avian reaction.	Inconclusive (retest)
1. Animals showing a positive bovine reaction and negative avian reaction. 2. Animals showing a positive bovine reaction more than 2mm greater than a positive avian reaction.	Fail (remove)

Standard to Severe Interpretation

When carrying out a test using standard to severe interpretation, read the test at standard initially:

- if any IRs or reactors are detected, re-interpret the whole test using severe interpretation
- if no IRs or reactors are identified, carry out the whole test at standard interpretation.

A second issue for the SICCT is that after the intradermal injection of tuberculin in infected cattle, skin reactivity to a second injection is depressed for some time and this desensitisation can result in failure to identify infected cattle as reactors (Kerr *et al.*, 1946; Radunz and Lepper, 1985; Hoyle, 1990; Monaghan *et al.*, 1994; Doherty *et al.*, 1995b). The mechanisms responsible for desensitisation are poorly understood, but skin hypersensitivity to tuberculin is generally recovered by the 60th day after a tuberculin injection (Radunz and Lepper, 1985; Thom *et al.*, 2005). However, frequent tuberculin testing over long periods of time in chronically infected herds does not appear to have a significant desensitising effect on cattle (Costello *et al.*, 1997; Thom *et al.*, 2004; Thom *et al.*, 2005). To overcome this issue, skin testing protocols stipulate an interval of at least 42 days between two consecutive tests (Anon, 2004 b,c, 2005b; State Veterinary Service, 2005), which explains the 60-days interval applied in GB for repeat skin testing of infected herds, inconclusive reactors, etc. This time interval has been arrived at as a sensible compromise to deal with phenomena such as desensitisation and slow development of hypersensitivity to tuberculin.

A final issue that affects the Se of the SICCT is that non-specific hypersensitivity reactions to the skin (and other TB) tests in cattle have been described after natural, experimental or vaccinal exposure to various species of bacteria that share proteins with bovine PPD tuberculin. Hence when using the standard interpretation of the SICCT, the recognition of some *M. bovis* infected cattle can be temporarily masked by prior exposure to mycobacteria of the *M. avium-intracellulare* complex, and in this case the skin reaction to avian tuberculin in those animals exceeds that to the bovine tuberculin even though a strong reaction is given to the bovine PPD (Lesslie *et al.*, 1975; Hope *et al.*, 2005). This has been described in calves experimentally challenged with *M. avium* and *M. bovis* and this problem interferes with the performance of not only the SICCT but also with the standard γ -interferon test (section 1.2.2; Amadori *et al.*, 2002a; Hope *et al.*, 2005). In regions where infection with bacteria of the *M. avium-intracellulare* complex (which includes the causal organism of Johne's disease, *M. avium subsp. paratuberculosis*) is common, this has implications for the Se of the SICCT used in surveillance programs. Although this could be addressed by ignoring the reaction to avian PPD, as discussed above, this approach would have consequences in terms of

reducing the test specificity. The ideal screening test would have 100% Se and Sp, but no biological test for any disease is perfect. Furthermore, in quantitative diagnostic tests a cut-off point for a positive result has to be defined on the continuous scale of possible test readings and therefore as illustrated above, in practice compromises have to be reached for useful test interpretation. In the long term, a solution to the problem of non-specific sensitisation to the skin tests would be the replacement of PPD tuberculin with an *M. bovis*-specific purified antigen as the test reagent (Amadori *et al.*, 2002a; Pollock *et al.*, 2003).

In the UK, Ireland and other countries, some 50-80% of all cattle reacting to the skin test show no signs of disease at slaughter (no visible lesions and failure to isolate *M. bovis* on culture; Byrne, 1992; Tweedle and Livingstone, 1994; Goodchild and Clifton-Hadley, 2001; Anon, 2005a, Goodchild *et al.*, unpublished observations cited in de la Rua-Domenec *et al.*, 2005). This gives an impression of a test specificity far lower than that reported in field evaluations and therefore undermines confidence in the skin tests and other ante mortem diagnostic methods for bTB, such as the γ -IFN. This perception is based on the assumption often used in the study of other bacterial diseases that post-mortem examinations and bacteriological culture are the “gold standards” that should be used to assess the accuracy of immunodiagnostic bTB tests. It is known that post-mortem examinations and standard bacteriological tests for *M. bovis* are far less sensitive than the immunological tests which can detect subclinical infection (Corner, 1994; Monaghan *et al.*, 1994; Goodchild and Clifton-Hadley, 2001; Pollock and Neill, 2002; Vordermeier *et al.*, 2004). NVL reactors can occur due to a variety of reasons such as animals in the early stage of infection with *M. bovis*, when TB granulomas are too small or infrequent to be seen during post mortem examination. However, these may also represent true “false positive” non-infected animals exposed to antigens of environmental mycobacteria that cross-react with bovine tuberculin, and the fact that this possibility is widely discussed within the veterinary and farming communities, contributes to the lack of confidence in the accuracy of the skin test. Despite these problems, in regions where bTB is endemic or herds in which the presence of the disease has already been confirmed by post mortem or laboratory examination, tuberculin reactors are highly predictive of *M. bovis* infection and, to a point, it is insignificant

whether they are NVL or not. This was underlined by the findings of Swift *et al.* (2016) who showed that both VL and NVL SICCT-positive cattle contained detectable levels of MTBC bacteria in their blood, and so post-mortem identification of lesions is not a reliable confirmatory test for bTB infection. By contrast, as the prevalence of bTB falls, the predictive value of a positive skin test also declines and a larger proportion of positive test results will arise from non-specific sensitisation of cattle to *M. bovis* antigens (NSR). In such cases, the application of ancillary tests for serial re-testing may help clarify the status of tuberculin reactors (Buddle *et al.*, 2001).

1.2.2 The γ -interferon (γ -IFN) test

γ -IFN is a cytokine predominantly released by T lymphocytes after antigenic stimulation. γ -IFN has an important role in the immune responses to tuberculous mycobacteria as the major macrophage activation factor. The γ -IFN assay is an *in vitro* blood test developed in Australia in the late 1980s for the diagnosis of bTB in cattle in combination with the caudal fold SIT (Wood *et al.*, 1991; Wood and Rothel, 1994; Wood and Jones, 2001). As with the skin tests, the principle supporting the γ -IFN test is the detection of the host's CMI response against *M. bovis* infection (Pollock *et al.*, 2005). T-cells isolated from the peripheral blood of cattle release a measurable quantity of γ -IFN as early as 1 to 4 weeks post infection, when stimulated with bovine tuberculin or a similar *M. bovis* antigen. Briefly, the γ -IFN test is performed *in vitro* in two stages using heparinised whole blood samples collected on farm and sent to a testing laboratory for analysis which needs to be carried out within 28 hours of collection but ideally within 8 h (Ryan *et al.*, 2000; Buddle *et al.*, 2001). Samples are incubated with bovine PPD tuberculin and avian PPD tuberculin at 37°C and after 16-24 h of incubation, γ -IFN levels released into the plasma are quantified by a sandwich enzyme linked immunosorbent assay (ELISA), using a commercially available kit. The amount of γ -IFN produced by cattle T-cells is measured as colour development in the ELISA stage of the test and is expressed in optical density (OD) units (Wood and Rothel, 1994; Wood and Jones, 2001).

As the γ -IFN test and the SICCT test both compare the different immune responses to stimulation with both *M. bovis* and *M. avium* antigens, it is also true for the γ -IFN test

that if an animal has been infected with *M. avium* or other environmental mycobacteria, γ -IFN production will usually be greatest when T-cells are incubated with avian tuberculin and this can mask a positive *M. bovis* test result which is strongly indicated when bovine tuberculin stimulates more γ -IFN production than either avian tuberculin or the nil-agent control. Another issue is that, like the skin tests, the γ -IFN responses can be influenced by the prior application of tuberculin skin tests. Rothel *et al.* (1992) observed gradual increases in the OD values for the γ -IFN test between 7 and 59 days after a caudal fold SIT. Since then, numerous studies have investigated the effect of recent skin testing on γ -IFN responses and reached conflicting conclusions such as the caudal fold SIT can boost γ -IFN responses in *M. bovis* infected cattle and the SICCT test does not significantly boost γ -IFN responses and blood for Bovigam® testing can be taken as early as 3 days after SIT test without affecting the result of the assay (Whipple *et al.*, 2001) whereas data from experimentally infected cattle suggests that it may be safest to avoid performing the Bovigam® test in the 7 days following the SICCT test (Whelan *et al.*, 2004; Thom *et al.*, 2005). These discrepancies are likely due to the variable conditions under which each study was conducted, including the type of skin test applied.

The γ -IFN test using mammalian and avian PPD as antigens is marketed as a patented commercial kit under the name Bovigam® (Prionics, Switzerland). The same kit has been used for ante mortem diagnosis of TB in goats, sheep (Malone *et al.*, 2003), African buffalo (Grobler *et al.*, 2002) and could, theoretically, be applied to other species of the *Bovidae* family. The Bovigam® assay is not directly applicable to other mammals but the principle of this test has been adapted for ante mortem diagnosis of TB in humans (QuantiFERON® - TB, Cellestis Ltd, Australia; Katial *et al.*, 2001; Britton *et al.*, 2005), non-human primates and cervids, following the development of species-specific monoclonal antibodies against human, primate and cervine γ -IFN, respectively. A similar assay has been developed for the detection of γ -IFN in lymphocytes and whole blood of tuberculous badgers, although it requires further validation and evaluation (Dalley *et al.*, 2004). Chambers *et al.* (2008) evaluated the sensitivity and specificity of a lateral-flow immunoassay (BrockTB Stat-Pak) for detecting tuberculosis in Eurasian badgers (*Meles meles*). It was found that the assay was 49% sensitive and 93% specific against culture

for *M. bovis* at necropsy. However, the sensitivity was significantly higher (66 to 78%) in animals with more severe tuberculosis, indicating that the BrockTB Stat-Pak could have been useful of detection of badgers with the greatest risk of transmitting the disease. A commercial limitation related to the development of kits for new species of animals is precisely the fact that in each case new reagents have to be developed to detect the species-specific γ -IFN response.

The γ -IFN test is widely considered to be at least as sensitive as the skin test. Most importantly, it will detect a substantial proportion of infected cattle that escape detection by the tuberculin test (Neill *et al.*, 1994a; Monaghan *et al.*, 1997; Wood and Jones, 2001; Vordermeier and Goodchild, 2003, unpublished data, cited in de la Rua-Domenec *et al.*, 2005; Pollock *et al.*, 2005). The most likely reason for this is that the γ -IFN test identifies animal at an early stage of infection than the skin test does (Pollock *et al.*, 2005). Therefore, skin and γ -IFN tests detect slightly different subpopulations of tuberculous cattle (Neill *et al.*, 1994a; Gonzalez Llamazares *et al.*, 1999; Pollock *et al.*, 2005). The diagnostic performance of the conventional γ -IFN (i.e. Bovigam®) test for cattle has been evaluated in several studies conducted in Australia, Brazil, Ethiopia, UK, Republic of Ireland, Italy, New Zealand, Spain and the USA (Wood and Jones, 2001). In these trials, Se varied between 73.0% and 100% (median = 87.6%) and the median Sp was 96.6%, with a range of 85.0%-99.6%. As discussed for the skin test, the results gained in each case were influenced by the characteristics of the cattle populations in which the test was evaluated but were also found to be affected by issues relating to the test itself. For instance, different cut-off values were adopted to classify animals as test positive which affected the interpretation of the results and the batch and source of PPD tuberculin used as test antigen also influenced the results. In addition, the “gold standard” chosen to determine the true *M. bovis* infection status of the animals in the study affected the rate of false-positive results recorded. Despite the limitations of the methods available to evaluate bTB diagnostics tests, direct evidence that this test does accurately detect bTB infection comes from the fact that γ -IFN test-positive, skin test-negative cattle that are not immediately slaughtered were found to be far more likely to become reactors at subsequent skin tests than γ -IFN test-negative, skin test-negative animals (Pollock *et al.*, 2005).

The advantages of the γ -IFN test over the SICCT is that it has a better Se than the SICCT test even though a small portion of cattle infected with *M. bovis* that are reactors to the skin tests are not detected by the γ -IFN test (Pollock *et al.*, 2005) and because the test is laboratory based, it enables more objective and standardised interpretation of results and there is no need for a second visit to the farm to read the test results (Pollock and Andersen, 1997; Vordermeier *et al.*, 1999; Pollock *et al.*, 2000; Buddle *et al.*, 2001; Waters *et al.*, 2004). In contrast, the disadvantages of the γ -IFN test over the SICCT test is a lower Sp because non-infected cattle may give a non-specific response to the test believed to be due to the innate γ -IFN production by natural killer (NK) cells in peripheral blood when stimulated *in vitro* with certain antigens of the MTB complex (Vordermeier *et al.*, 2001a; Olsen *et al.*, 2005). On the practical side, there are logistical difficulties associated with the need to keep the blood samples in a stable environment (approx. 10-26°C) and incubate them with antigen within a few hours of collection (Rothel *et al.*, 1992) since delays in processing of the blood samples can significantly reduce the test sensitivity (Gormley *et al.*, 2004) and the technical requirements of the test mean that there is a relatively high cost associated with the laboratory component of the test.

One of the major concerns about the γ -IFN relates to its specificity. As already said, the median specificity of the conventional γ -IFN test using PPD tuberculin is 96.6%. This may seem high and only marginally lower than that of the SICCT test (99.5%). However, in a test area where the prevalence of bTB at animal level is low (as is often the case in developed countries with long-established test and slaughter schemes), this would result in the slaughter of unacceptably high numbers of non-infected cattle (Monaghan *et al.*, 1997; Lauzi *et al.*, 2000; Pollock *et al.*, 2001). This is the main argument against using the Bovigam® test for primary bTB screening. Therefore, the most beneficial application of the γ -IFN test to date is as an ancillary test in bTB breakdowns or in persistently infected herds alongside the skin tests, where removal of a small number of false-positive is a secondary concern compared to effective eradication of the disease from the herd (Neill *et al.*, 1994a; Archetti *et al.*, 1996; Monaghan *et al.*, 1997; Vordermeier *et al.*, 2001a; 2004).

In countries where the SIT is the primary screening test (e.g., USA and New Zealand), the γ -IFN is the test of choice for re-testing of skin test reactors from TB-free cattle herds in areas of low TB prevalence (Buddle *et al.*, 2001; Wood and Jones, 2001; Anon, 2005a). In the USA, the γ -IFN test was an approved supplemental test for cattle from 2001 but problems with consistency of kit performance had recently led to this application of the test being temporarily suspended in May 2017. In New Zealand, the γ -IFN test has also been approved for use in cattle as an ancillary test to complement the caudal fold SIT, both as a serial test of skin test reactors and as a parallel test of skin test-negative animals in infected herds. Additionally, the γ -IFN test is used in parallel with the SIT for pre-movement testing of cattle from infected herds. In July 2002, the γ -IFN test was formally recognised in the European Union as a supplementary parallel test, i.e. to enable the detection of the maximum number of infected cattle in a herd (Anon, 2004b). The test is now a standard tool in the European countries with endemic bTB problems, like Great Britain, the Republic of Ireland, Italy and Spain.

1.2.3 Antibody-based diagnostics

The humoral immune response against *M. bovis* is very complex. The titre of antibodies (Ab) changes significantly during the infection and they are mainly produced in advanced stages of the infection (Pollock and Neill, 2002), although an early antibody response has also been reported in experimentally infected cattle (Water *et al.*, 2006c) which suggests that animal infection models may not follow the same pathway of disease development due to the high microbial challenge given. Despite these limitations, several antigens have been identified from experimental studies as potential diagnostic targets (e.g. ESAT-6, CFP-10 and MPB70), although in domestic livestock and wild animals an antibody response is mainly triggered against MPB83 (Amadori *et al.*, 2002; Lyashchenko *et al.*, 2008; McNair *et al.*, 2001; Wiker, 2009). It was also found that responses against MPB83 are detected earlier in the course of experimental infections, with an increase in the antibody response being detected at 3-4 weeks post infection (O'Loan *et al.*, 1994; Waters *et al.*, 2006a; 2010). In contrast, an antibody response against ESAT-6 and MPB70 were only detected at 12 weeks or 20 months after experimental infection, respectively (Lyashchenko *et al.*, 1998; Fifi *et al.*,

1992). Therefore, the highest and earlier response triggered against MPB83 protein suggested that this protein is a good candidate as diagnostic antigen for the development of serological diagnostic tests.

Enzyme-linked immunosorbent assay (ELISA) are most commonly used to detect antibody responses since these methods allow many samples to be tested in a short time and they are simple, rapid and inexpensive and allows the standardization of the technique in different laboratories (Cho *et al.*, 2007). The ELISA has been extensively used to evaluate the humoral response against *M. bovis* in a number of animal species. Initially, PPD were used as antigen; however, its Se was low (Amadori *et al.*, 1998; Lilenbaum *et al.*, 1999a) and so it was replaced by the use of native or recombinant specific antigens such as ESAT-6, MPB70 or MPB83, but these also resulted in relatively low Se and Sp values (Waters *et al.*, 2011). Thus, great efforts have been focused on finding new antigens that would help to improve these results and several studies have focussed on the use of chimeric proteins formed by the combination of multiple antigens, resulting in Se and Sp values of over 65% and 95%, respectively (Liu *et al.*, 2007; Souza *et al.*, 2012; Waters *et al.*, 2011).

In the case of wild animals, it is very important to use a test based on a single sampling as holding the animals for any length of time is not practical. Indirect ELISA tests based on bovine PPD have also been developed and validated for wild animals. Sera from European wild boars were absorbed with *Mycobacterium phlei* suspension to remove non-specific anti-*Mycobacterium spp.* antibodies, and produced a reagent that showed a Se of 72% and a Sp of 96% (Aurtenexe *et al.*, 2008; Fernandez *et al.*, 2009). Several studies have shown that the response of different species to *M. bovis* is not constant since the different species appear to respond to different antigens and therefore several proteins may be required to produce a test with higher Se. In this line, a multi antigen print immunoassay (MAPIA) has been developed to characterise the antibody response. This test consists of a cocktail of antigens applied to nitrocellulose membranes in narrow bands. The strips are incubated with serum samples and then is followed by immunodetection using standard chromogenic methods (Lyashchenko *et al.*, 2000; 2008; Veerasami *et al.*, 2012). Also, to address this problem, other researchers have

developed and tested a multiplex chemiluminescent immunoassay that can simultaneously detect antibody direct to more than 20 antigens in a single well in a 96-well plate format that has been commercialised as Enferplex TB (Whelan *et al.*, 2008; 2010b; 2011).

Lateral-flow (LF) rapid tests based on immunochromatographic techniques have also been developed and evaluated for different species, and are also commercially available. The LF devices offer many advantages since they are easy to perform, stable at room temperature, and do not require high logistical demands. However, in trials the Se of these tests were found to range from 45% to 100%, depending on the species being tested (Buddle *et al.*, 2010; Greenwald *et al.*, 2009, Lyashchenko *et al.*, 2008; Michel and Simoes, 2009). To avoid the complexity of the development of a multi antigen system and the possible loss of Sp, several systems based on a single antigen have been developed. The SeraLyte-*Mbv* system is based on advanced chemoluminescence chemistry and optics for the highly sensitive detection of antibodies (Green *et al.*, 2009), uses MPB83 as antigen and is reported to have high Se and Sp. In a similar way, a novel serologic technique based on MPB83 recombinant antigen has been reported and assessed in a *M. bovis* naturally infected alpaca herd in Spain. This new assay used the MPB83 protein as antigen on the plate and as enzyme-conjugated antigen for the antibodies detection, for this reason, the test has been called double recognition ELISA (DR-ELISA), because the antibodies present in the sample have to twice recognize the specific antigen (Bezoz *et al.*, 2013). This assay offers many advantages with regards to other ELISA tests because it is able to recognize not only IgGs but also other immunoglobulin classes, such as IgMs (Ventoe *et al.*, 2012) allowing for early detection of the infection. In addition, it is a multi-species assay since it uses enzyme conjugated antigen instead of enzyme-conjugated species-specific secondary antibody and therefore, it can be used with sera from any animal infected by *M. bovis*. Moreover, it is highly specific due to the double recognition of the MPB83 protein. These characteristics render DR-ELISA a potentially valuable tool for early detection of antibodies against *M. bovis* and subsequent diagnosis of the TB infection although it has not yet been evaluated in cattle. Other techniques, such as the latex bead agglutination assay based on the detection of antibodies against one antigen (ESAT-6) have been

tested to differentiate between bTB and *M. paratuberculosis*-infected animals and showed adequate Se and Sp values in trials, although the number of samples so far reported is low (Koo *et al.*, 2004).

An increase of the Se of the serological assays due to a recent intradermal tuberculin test (booster effect) has been reported when sera samples are taken in a short interval after PPD injection are used. The booster effect was firstly reported in goats naturally infected with *M. bovis* (Gutierrez *et al.*, 1998) and in studies using experimentally infected cattle (Hanna *et al.*, 1992; Harboe *et al.*, 1990; Koo *et al.*, 2005; Palmer *et al.*, 2006; Waters *et al.*, 2006b). Nevertheless, few studies have been performed to demonstrate the effect in naturally infected cattle (Waters *et al.*, 2011). Therefore, further studies testing different sampling times after PPD injection should be carried out in naturally infected cattle to confirm an increase of the Se of the serological assays based on this booster effect and to evaluate the best time after PPD injection to collect the sera samples. Hence assays based on the detection of specific antibody might show a high potential for the diagnosis of bTB although new tests with improved Se and Sp under field conditions have to be developed and validated. Despite this, a combination of methods based on the measurement of the delayed hypersensitivity reaction and γ -IFN production, together with serological tests could increase the level of detection of the pathogen and help to control bTB (Schiller *et al.*, 2010).

1.2.4 Bacterial isolation

Isolation of *M. bovis* is considered “gold standard” for bTB diagnosis and characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis*. However, the long period required for the isolation and biochemical identification is one of the critical limiting factors since it may require more than twelve weeks to complete the final diagnosis, and in addition this test has low sensitivity (Collins *et al.*, 1994). To achieve cultural isolation, the samples collected from the animals are decontaminated with substances such as NaOH, H₂SO₄, oxalic acid, or quaternary ammonium compounds, to eliminate competitive microorganisms. Unfortunately, the toxic effects may affect mycobacterial viability, interfering with success rate of culturing

the organism (Ambrosio *et al.*, 2008; Medeiros *et al.*, 2010; Young *et al.*, 2005). Another major limitation for systematic cultivation of *M. bovis* from animals is the difficulty of obtaining samples. These are usually only available following post mortem examinations or from slaughterhouses and therefore are not useful ante-mortem screening tests. To overcome this limitation, studies have suggested the use of nasal swabs as an alternative to reduce contamination of samples and increase the sensitivity of the method (Ambrosio *et al.*, 2008). However, due to the fact that non-pathogenic mycobacteria are widespread in the environment, the identity of every isolate needs to be confirmed. In particular it is necessary to distinguish *M. bovis* from the other members of the MTB complex (Richter *et al.*, 2004). This underscores the need for more sensitive, accurate and faster methods to assist in the control of this zoonosis.

1.2.5 Molecular diagnosis

Polymerase chain reaction (PCR) has been successfully applied to detect members of the *M. tuberculosis* complex and is especially useful for the direct detection of *M. bovis* in bovine tissue samples as an alternative to culture which is problematic for the reasons discussed in section 1.2.4 (Zumarraga *et al.*, 2005). However, validation studies have shown that PCR assays to detect bTB are currently less sensitive than culture techniques in terms of a higher microbial load is required to reproducibly give a positive result. This lack of sensitivity is due to the robust characteristic of the mycobacterial cell walls which are resistant to both chemical and mechanical methods of cell lysis, meaning that DNA is not easily released from cells for detection by PCR. Therefore, before PCR can be adopted as a primary diagnostic method further steps would be required to improve PCR sensitivity (Schiller *et al.*, 2010).

Another issue associated with PCR-based diagnostics is that variation in the methods used will affect both the Se and Sp of PCR-based diagnostic tests and therefore there is also a need to standardize PCR methods (Schiller *et al.*, 2010). Distinct in-house PCR methods have been proposed for the rapid detection of low levels of *M. bovis* DNA. These methods have mainly been used to confirm the aetiology of macroscopic lesions detected at slaughterhouse inspection and to detect *M. bovis* in milk specimens (de

Kantor and Ritacco, 2006). The use of PCR for diagnosis of bTB infection has been extensively evaluated in detecting mycobacteria in milk, fresh tissues, and tissues fixed in formalin and embedded in paraffin with variable degrees of success. The variation in the results regarding the Sp and Se of these tests are reported to be due to many factors such as variation in the type of sample being tested, the use of several methodologies for sample preparation, the use of both different DNA amplification systems and methods to detect the product amplified (OIE, 2009), and these problems will only be settled by reliable protocols and standardized by several laboratories.

Therefore, although PCR tests have been introduced as a standard method for diagnosis of many different bacterial pathogens, for mycobacteria it is still recommended to use culture in parallel to confirm a viable *M. bovis* are present in a sample (Warren *et al.*, 2006). This is especially true when an indication is required that the cells being detected are viable since PCR will amplify DNA extracted from both live and dead cells. Nevertheless, it has already been shown that PCR is more sensitive than culture for the detection of *M. bovis* in bovine milk (Zumarraga *et al.*, 2005), which may not be predicted given the problems achieving efficient cell lysis. However, this may be attributable to the requirement for chemical decontamination of the sample before culture, which, as described in section 1.2.4, may kill a high proportion of bacteria and reduces the sensitivity of the culture-based detection (Meikle *et al.*, 2007).

1.2.6 Detection of volatile signature molecules using an e-nose

Fend *et al.* (2005) have investigated the potential of chemical sensors to diagnose *M. bovis* infections based on the detection and analysis of volatile organic compounds (VOCs) in serum samples from infected calves, an approach colloquially known as “electronic nose” (“EN” or “e-nose”). The method has the advantage of being rapid and cost-effective and therefore provides an attractive means of detecting cattle naturally infected with *M. bovis*. Although the initial results were promising, further and larger studies are needed to validate this technology as a robust, sensitive, specific point of care test. Electronic nose is the colloquial name for an instrument made up of chemical sensors combined with a pattern recognition system (Gardner and Bartlett, 1994). The

key function of an EN is to mimic the human olfactory system. In the EN, the human olfactory receptors have their analogue in chemical sensors that produce an electric signal (similar to nerve cells). These signals are subsequently analysed by pattern recognition software, that corresponds to the cerebral cortex of the brain and is able to classify and memorize odours (Bartlett *et al.*, 1997; Pearce, 1997). The sensors are characterized by a partial specificity, i.e., they respond to certain groups of chemicals, such as alcohols and aldehydes, etc., rather than a single compound (Gardner and Bartlett, 1994). The partial specificity generates a unique signature (pattern) of the sample. The successful analysis of infectious disease-related VOCs is based on the following principles of cell biology. The bacterial cell membrane consists primarily of amphipathic phospholipids, carbohydrates and many integral membrane proteins that are distinct for different cell types. In disease formation, both host and invading cells can undergo structural changes, one example of which would be oxidative stress, i.e., a peroxidation of the cell membrane that causes VOCs to be emitted (Kneepkens *et al.*, 1994). Some of the VOCs appear in distinctively different mixture compositions (Baumbach *et al.*, 2010; Barbieri *et al.*, 2006). What is particularly significant about this approach is that each type of disease has its own unique pattern of VOCs; therefore, the presence of one disease would not mask other disease types (Dummer *et al.*, 2011). These VOCs can be detected directly from: (i) cultured cells (i.e., the mixture of VOCs trapped above the cells in a sealed vessel) (Zhu *et al.*, 2010; Barbieri *et al.*, 2006); (ii) urine (Banday *et al.*, 2011) or (iii) exhaled breath (Baumbach *et al.*, 2010; Lechner *et al.*, 2007). In regard to exhaled breath, the principle is that the disease-related changes in blood chemistry are reflected in measurable changes to the breath through exchange via the lungs. In certain instances, breathing testing offers several potential advantages, such as (a) breath samples are non-invasive and relatively easy to obtain, and (b) breath testing has the potential for direct, inexpensive and eventually real-time monitoring. Peled *et al.* (2012), explored the utility of breath testing for the detection of *M. bovis* infections in cattle. They analysed breath samples collected from cattle using gas-chromatograph/mass-spectrometry (GC-MS) to identify the VOC patterns linked with the disease condition. Based on the detected VOC patterns, a nanotechnology-based array of sensors, termed Nano Artificial NOSE (NA-NOSE) (Hakim *et al.*, 2011; Ionescu *et al.*, 2011), was tailored for the detection of bTB disease from exhaled breath. NA-NOSE

is an artificial olfactory system based on an array of cross-reactive, nanomaterials-based, chemical gas sensors, which can identify and separate different gaseous mixtures, even if their constituent analytes are present at very low concentrations and their differences are very subtle. GC-MS analysis revealed the presence of two VOCs associated with *M. bovis* infection and two other VOCs associated with the healthy state in the exhaled breath of *M. bovis* infected and not infected animals, yielding distinctly different VOC patterns for the two study groups. Based on these results, a custom-made nanotechnology-based array of sensors (NA-NOSE) was then tailored for detection of *M. bovis* – infected cattle via breath. The system successfully identified all *M. bovis* – infected animals, while 21% of the not infected animals were classified as *M. bovis*-infected. The NA-NOSE has advantages over the GC-MS technique because it is faster, cheaper and portable. This method might also hold potential as a screening test for other diseases, such as bovine brucellosis and paratuberculosis.

1.3 Bacteriophage: a new tool for the investigation of bovine TB.

1.3.1 Bacteriophage characteristics

Bacteriophage are viral parasites that infect bacteria. Like all viruses, when outside the host they are metabolically inactive and therefore they are described as obligate parasites. The virus structure consists of the nucleic acid surrounded by a protein coat (capsid), and in some instances the capsid may contain lipid layers or even be surrounded by a lipid envelope. The nucleic acid most commonly consists of double stranded DNA, but some phage have single stranded DNA genomes. Others have RNA genomes, and phage with both ssRNA and dsRNA have been identified (reviewed in Rees *et al.*, 2014). Unique among viruses is the presence of a complex tail structure on some bacteriophage that is involved in the recognition of the host cell surface and delivery of the nucleic acid into the host cell during the first stage of infection. The length and complexity of the tail structure is variable, however, and some phage belonging to the Tectiviridae group do not have tail structures at all. These three characteristics (capsid structure, tail structure/presence, and nucleic acid type) are used as a basis for morphological characterisation of phage. The majority of phage described

to date and used to develop diagnostic methods fall into two morphological groups – the Myoviridae and the Siphoviridae – both of which have ds DNA genomes packaged into icosahedral capsid. The Siphoviridae have simple, long, noncontractile tails; the Myoviridae have rigid, contractile tails and additional tail fibers, and complex base plate structures (Fig. 1.2).

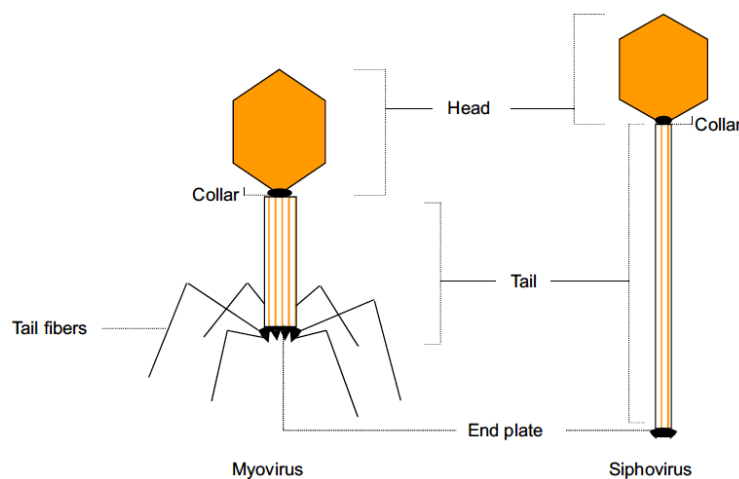


Fig. 1.2 Bacteriophage structure (based on Rees, Swift and Botsaris Book Chapter. Encyclopaedia of Food Microbiology, 2014)

Bacteriophage particles have evolved to be relatively robust and are capable of existing for long periods of time between release from the parent cell and contact with new host cells. In the presence of an appropriate host cell, the phage can attach to the bacterial cell surface via interaction with a specific receptor molecule and the viral nucleic acid enters the cell (reviewed in Rees *et al.*, 2014). When this happens, the phage enters what is known as the eclipse phase, i.e. the time during which the phage DNA is not packaged into a capsid but rather is replicating inside the host cell and new phage particles have not yet been formed. During this period, the bacteriophage takes over the host's metabolic machinery to replicate the viral genome and synthesize new capsids and tails. The phage also encodes a number of proteins required for the assembly of these components into new mature phage particles, which then need to be released from the infected host cell. This is normally achieved by phage-induced breakdown of the bacterial peptidoglycan layer, resulting in the loss of the structural

integrity of the cell wall, causing the cell to rupture and release progeny bacteriophage. This process is often mediated by a two-component system, composed of a peptidoglycan digesting enzyme (lysin) and a transport protein (holin) (reviewed in Fischetti, 2008). This system is required to allow the lysin protein to cross the inner membrane of the cell and access the peptidoglycan present in the periplasm of Gram-negative bacteria or in the outer layer of the cell wall of Gram-positive bacteria. Not all phage require specific genes to achieve phage lysis, however. Some, such as ØX174, weaken the cell wall and induce lysis by interfering with host cell wall synthesis pathways. Others, such as the filamentous phage M13 or Fd, create a persistent infection and extrude phage particles from the host cell in an ATP-dependent manner, and in this case, the host cells do not lyse. These examples are exceptions, however, and the majority of phage encode lytic enzymes that can be exploited as biocontrol agents or to develop detection methods (Fischetti, 2008).

1.3.2 Detection by Phage amplification

The phage amplification technique for detecting mycobacteria relies on two key characteristics: the specificity of bacteriophage to target cells and the ability of a potential virucidal agent to rapidly inactivate free extracellular phage, while remaining non-destructive to bacterial cells. A variety of compounds can be used as the virucide, including chemicals, such as ferrous ammonium sulphate, and plant extracts, such as tea and pomegranate rind. These attributes allow the detection of specific groups of bacteria on the basis of the ability of the host cell to protect a phage that has infected it from destruction by the virucide which will allow the production of new progeny phage particles (see Fig. 1.3) so that a positive indication of the presence of bacteria is the formation of plaques at the end of the assay (Swift and Rees, 2013).

To carry out the phage amplification assay, the sample containing the target cell is first infected with the bacteriophage. An incubation period then follows to allow time for cell infection and for the phage to enter the eclipse phase. At this point, any exogenous phage are destroyed by the addition of a virucide. Only the phage in the eclipse phase will avoid inactivation and can replicate inside the host cell. To detect the phage

released from this primary infection, a phage-susceptible, non-pathogenic variant is used as a host strain (termed “sensor strain”). The sensor strain is added to the sample after the virucide treatment, and the whole sample plated in soft agar and poured into a Petri dish. The sensor cells provide a lawn of phage-sensitive cells that will support phage replication and the formation of plaques (areas of cell lysis) in the bacterial lawn (Rees *et al.*, 2014).

1.3.3 Detection of *Mycobacteria* by phage amplification

*FastPlaqueTB*TM (FPTB) was developed as a low cost commercial test produced for the detection of *M. tuberculosis* in human sputum samples. The bacteriophage employed is the broad host range lytic mycobacteriophage D29 (Rees *et al.*, 2014). Mycobacteriophage D29 has a head of uniform size (average diameter 65 nm) and regular shape and a tail of variable length. The stability of the phage is optimal between pH 9 and 10. The virus contains double stranded DNA and six structural polypeptides, three major and three minor. The molecular weights of these six polypeptides, as determined by SDS-PAGE, are 150 KDa, 138 kDa, 130 KDa, 66 KDa and 240 KDa. The virus contains no lipid as shown by (a) the lack of structural changes after inactivation of the bacteriophage with chloroform, (b) the absence of lipids containing [³²P] phosphate or [³⁵S] sulphate in labelled viruses and (c) the absence of an electron paramagnetic resonance spectrum in bacteriophage which had been incubated with a nitroxide-containing fatty acid (Schäfer *et al.*, 1977).

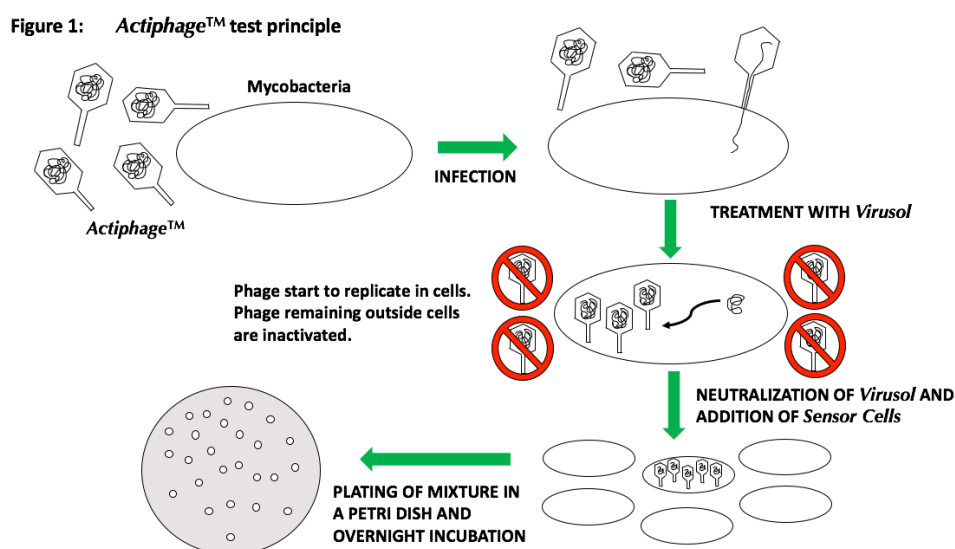
The *FASTPlaqueTB*TM assay used processes similar to those used for culture of mycobacteria from sputum. Thus, a pre-treatment of sputum with *n*-acetyl-L-cysteine-sodium hydroxide was carried out before the sample was washed, and then incubated overnight in a recovery medium to resuscitate the *M. tuberculosis* bacilli, before the *FASTPlaqueTB*TM assay was carried out. A trial was conducted in collaboration with the South Peninsula Municipality Health Services (Cape Town, South Africa; Albert *et al.*, 2001). They evaluated the performance of the *FASTPlaqueTB*TM assay compared to testing samples using Acid Fast Bacterial (AFB) smear microscopy (i.e. direct visualisation of mycobacteria in sputum) or culture on Löwenstein-Jensen slopes by

testing 1,962 decontaminated sputum specimens that were obtained from 853 new tuberculosis suspects. The FASTPlaqueTB™ assay detected *M. tuberculosis* in 73.1% of all cases that were given a clinical diagnosis of tuberculosis and the Sp of the assay was determined to be 99.0%. Performance of the FASTPlaqueTB™ test was significantly better than the concentrated AFB smear microscopy (64.2% sensitivity and 97.3% specificity). In specimens with very low numbers of AFB, false-positive smear results can be given making the diagnosis of tuberculosis difficult to confirm. In the study, 35% of the tuberculosis cases had two negative sputum smears but the FASTPlaqueTB™ assay gave a positive result in either one or both of the specimens tested in 55.3% of these cases, suggesting that the FASTPlaqueTB™ can successfully detect low numbers of cells (Trollip *et al.*, 2001). However, the fact that a positive detection event depended on the number of plaques detected alone, with no other direct confirmation of the presence of *M. tuberculosis*, then this assay fell out of popularity with the advent of molecular diagnostic techniques which replaced both AFB smears and culture as the standard diagnostic techniques for human TB infections.

An advantage of phage amplification technology is its adaptability. It can be adapted for use in detection of many different types of mycobacteria, taking advantage of the broad host range of the bacteriophage D29. Foddai *et al.* (2009) optimized the phage amplification assay conditions to permit accurate quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle and, is associated with the development of Crohn's disease in humans. It also has been shown that phage amplification assay can be used for the detection of MAP in raw milk and cheese samples (Botsaris *et al.*, 2010; Botsaris *et al.*, 2013). Unlike human sputum samples, raw milk is likely to contain other environmental mycobacteria in addition to the pathogens that are being targeted. Hence, infection by a broad host range phage alone is not sufficiently discriminating to allow for identification of the bacterium being detected. This led to the development of the phage-PCR method where PCR is used to identify the cell being detected following the phage assay. The PCR assay can be species specific, or it can have a multiplex PCR format for the simultaneous identification of different species (Stanley *et al.*, 2007). The combination of phage amplification with PCR

has been shown to deliver a high specificity and sensitivity, with less than 10 cells per sample being routinely detected (Stanley *et al.*, 2007, Swift *et al.*, 2013).

Figure 1.3: Principle of the phage amplification assay



The phage are added to the sample. If any viable mycobacteria are present the phage will inject their nucleic acid inside the cell and infection occurs. The virucide is then added to inactivate any phage that have not infected any mycobacteria. Non-pathogenic mycobacteria (Sensor cells) are added and the mixture is plated with soft agar. The Sensor cells will provide a lawn of phage-sensitive cells that will support the phage replication and the formation of plaques.

(From Actiphage™ Instructions for use; PBD Biotech Ltd).

1.3.4 Detecting bovine TB in blood using phage

Swift *et al.*, (2016) have developed a sensitive and rapid phage amplification method for detection of MTC in bovine peripheral blood mononuclear cells (PMBC). In this case an isothermal DNA amplification protocol using recombinase polymerase amplification (phage-RPA) was used to identify the mycobacteria detected in the blood samples and the authors reported the detection of viable *M. bovis* cells within 48 hours, with a limit of detection of approximately 10 *M. bovis* bacilli per ml for blood. When blood samples from SICCT–positive animals were tested, viable MTC bacteria were detected in 66%

(27/41) of the samples. Of the 41 animals sampled, only 13 had visible lesions. Over 50% of the animals with no visible lesions (NVL) had detectable mycobacteremia indicating that this is established before the disease has developed to the point where lesions are visible. Importantly, no MTC DNA was detected in the blood samples from TB negative herds. The high frequency of viable MTC detection in the peripheral blood of SICCT-positive NVL animals has changed the paradigm of the disease. Mycobacteremia has only rarely been reported in *M. bovis*-infected cattle, and its occurrence was expected to be associated with disseminated and advanced clinical disease, as it appears to be the case in humans. In addition to its diagnostic applications, the high frequency of *M. bovis* detection in bTB infected animals revealed by this new method provides useful insights into bTB pathogenicity.

1.3.5 Commercial developments and aims of this project

*FastPlaqueTB*TM was discontinued as a human product by Lab 21 in 2012. However, before this the company had worked with researchers at the University of Nottingham to establish new applications of the technology by combining phage with PCR and, as discussed in the literature review, allow the detection and identification of different types of mycobacteria in a range of samples types. Since there is an urgent need for new tests that allow to definitively diagnose infected animals this work has led to the development of a new version of the method, named *Actiphage*TM Rapid, which was patented by the University of Nottingham. This patent led to the establishment of PBD Biotech Ltd. which is commercialising both the old form of the phage amplification assay (now named the *Actiphage*TM test) for detection of mycobacteria in milk and blood and also the new form of the assay – named *Actiphage*TM Rapid - as an assay for the rapid detection (6 h) of mycobacteria in blood, milk and other sample types.

To carry out the *Actiphage*TM Rapid assay, the sample is prepared, loaded in a specialised sample tube and is then mixed with a bacteriophage (*Actiphage*TM) and incubated. If any target mycobacterial cells are present, they will be rapidly infected by the target specific bacteriophage added to the specimen. Inside the mycobacterial cell

the phage continues to replicate until new progeny phage are released as the cells break open. Following filtration, the genetic material released from the viable mycobacterial cells is collected and the identity of the mycobacteria is determined by conventional nucleic analysis such as PCR.

PBD Biotech Ltd. sponsored this studentship to carry out the research required to start producing both the original kit format (renamed Actiphage™) and the new Actiphage™ Rapid kits. Hence the aim of this project was to investigate the practical issues surrounding the production of bacteriophage for diagnostics tests, including the best method for freeze-drying the phage and also investigating methods to reduce cost and allow scale up of production. The best methods for the production and delivery of each component of the kits were also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 General media and reagents

2.1.1 Middlebrook 7H9 Broth Base

Middlebrook 7H9 Broth base was prepared by adding 180 ml of RO or distilled water to 0.93 g of Middlebrook 7H9 Broth Base powder in a Duran bottle and mixing until the powder was completely dissolved. The resulting solution was autoclaved at 121°C, under a pressure of 15 psi, for 15 min. Once cooled to room temperature, the medium is ready for use.

2.1.2 Preparation of Media Plus

To prepare Media Plus, 20 ml of Middlebrook OADC Supplement was added to 180 ml of sterilized and cooled to room temperature Middlebrook 7H9 Broth Base (1:10 dilution rate).

2.1.3 Nutrient Agar No. 2

Nutrient Agar No.2 was prepared by mixing 2.6 g of powder with 120 ml RO or distilled water and autoclaving at 121°C, under a pressure of 15 psi, for 15 min and then held at 50 - 55°C for no more than 48 h.

2.1.4 *M. smegmatis* culture

One colony of *M. smegmatis* was inoculated with the required volume of Medium Plus and incubated with shaking (150 – 200 rpm) at 37°C for 1 – 2 days until the culture reached an optical density at 600 nm of no more than 1.

2.1.5 *M. smegmatis* – Agar plates

To prepare *M. smegmatis* – Agar plates, 6 ml of Media Plus and 750 µl of *M. smegmatis* culture were mixed in a Petri dish. To this was added 6 ml molten agar and the plates

swirled to mix thoroughly and to distribute the soft agar over the entire surface before allowing to set at room temperature. Plates were incubated base-side down to prevent the soft agar falling out of the Petri dishes.

2.1.6 Virusol

Add 5 ml of purified water to the Virusol vial and dissolve. Tablets that are broken can still be used. Once reconstituted Virusol can be stored for up to 7 days at 2-8 °C. Virusol solution must be mixed well prior to use to ensure all material is dissolved. Discoloration and precipitation may occur on storage. This does not affect the performance of reagent. Shake gently to re-dissolve before use.

2.1.7 PBS

PBS tablets (Oxoid) were dissolved in 100 ml of purified water and autoclaved for 15 min at 121°C, under a pressure of 15 psi. This solution was prepared in advance and stored at room temperature until required.

2.2 Protocols

2.2.1 Titration of phage

M. smegmatis – Agar plates were prepared as described in section 2.1.5.

The phage suspension was serial diluted in Media Plus to a 10^{-7} dilution. The base of the Petri dishes used to prepare the *M. smegmatis* plates was divided into four sections and each section was labelled respectively -2, -5, -6, -7. Five 10 µl drops of each 10^{-2} , 10^{-5} , 10^{-6} , 10^{-7} dilution were placed on the respective area of the *M. smegmatis* – Agar plate. The plates were left near a Bunsen burner until the spots were dry and then they were incubated overnight at 37 °C. The next day the plates were checked for the presence of plaques and the plaques were counted on the dilution that gave an average of 10 plaques per spot. The titre was determined using the equation

Titre (pfu ml⁻¹) = (mean counted plaques / dilution factor) x 100

If the titre was at least 10¹⁰ pfu ml⁻¹, the phage suspension was diluted in Media Plus to a 10⁻⁹ dilution and 10⁻⁸ and 10⁻⁹ dilution were plated to check the exact titre of the suspension.

2.2.2 Preparation of D29 for Freeze drying.

Method 1: Aliquots (10 µl) of phage stock diluted to approximately 1 x 10¹ pfu ml⁻¹ in Media Plus were spotted on the surface of an *M. smegmatis*– Agar plate (section 2.1.5). The plates were incubated at 37 °C overnight. A 2-mm biopsy punch was used to excise 100 plaques from the agar and these were added to 5 ml of Media Plus for 10 min to allow the phage to elute from the small quantities of agar. The resultant phage suspension was transferred into a 15 ml sterile centrifuge tube and centrifuged at 4,400 x g for 3 min to remove whole cells and agar debris. The supernatant was poured into a sterile centrifuge tube and the pellet was discarded.

Method 2: The phage stock was titred (section 2.2.1) and used to calculate the volume and dilution of phage required to obtain 500 plaques plate when 0.1 ml of phage suspension was added to a 90 mm Petri dish containing 6 ml of Media Plus and 750 µl of a culture of *M. smegmatis* containing approximately 1 x 10⁸ cfu ml⁻¹ (section 2.1.4). To this were added 6 ml of Nutrient Agar No. 2 and the content of the plates was mixed by swirling. The agar was allowed to set and the plates incubated at 37 °C overnight. The next day the presence of plaques was confirmed to be approx. 500 plaques per plate, as predicted. The agar was then cut into cubes of less than 3 mm using a sterile scalpel and the content of one plate was transferred into a sterile beaker and eluted in 7 ml of Media Plus for 10 min. The eluate was transferred to a 15 ml centrifuge tube and centrifuged at 4,400 x g for 3 min. The supernatant was transferred into a clean centrifuge tube and the pellet was discarded. The titre was checked before freeze drying.

2.2.3 Blood sample preparation: purification of PBMCs

Blood samples were provided in heparin vacutainers (minimum volume required was 10 ml) and stored at room temperature (+18-25 °C) for no longer than 2 days before testing. Leucosep tubes were prepared by filling each one with 3 ml of Ficoll and centrifuging at 1000 x g for 3 min at room temperature (approx. +18-25 °C) and stored in the dark until required. To prepare the PBMCs, 2 ml of blood was removed from the vacutainer using a pipette and gently mixed with 2 ml of PBS in a 15 ml Falcon tube. This was then layered onto 3 ml of Ficoll in the Leucosep tube and centrifuged at 800 x g for 15 min at room temperature (approx. +18-25 °C) with no brake in deceleration phase in a centrifuge rotor with biocontainment lids. After centrifugation, the plasma layer was carefully removed using 10 ml pipette and the PBMC layer was then harvested using a Gilson pipette and filter tips. This was transferred to a clean 15 ml Falcon tube containing 6 ml of PBS. Finally, the washed PBMCs were recovered by centrifuging at 100 x g for 10 min. The supernatant was removed using a pipette and the pellet resuspended in 1 ml of Actiphage™ Media Plus to lyse the PBMCs and release intracellular bacteria. This was then transferred into a sterile Eppendorf tube and centrifuged at 13,000 x g for 3 min to recover the mycobacteria. Finally, the pellet was resuspended in 0.1 ml of Actiphage™ Media Plus and transferred to the Rapid Tube prior to testing using the Actiphage™ Rapid assay.

2.2.4 Milk sample preparation

Milk samples were provided by customers and were sent to the UoN laboratories for testing in chill boxes. Samples (50 ml) were centrifuged in a conical centrifuge tube for 15 min at 2,500 x g at room temperature (approx. +18-25 °C). The cream layer was removed (peeled off) using a sterile spatula and the milk layer aspirated, leaving the pellet undisturbed in the bottom of the tube. The somatic cell pellet was resuspended in 3 ml Actiphage™ Medium Plus and then recentrifuged for 10 min at 2,500 x g at 5°C to collect the mycobacterial cells. The pellet was then resuspended in 1 ml Actiphage™ Medium Plus ready to perform the Actiphage™ assay.

2.2.5 Actiphage™ Rapid Blood procedure

A Negative control was prepared by dispensing 100 µl of Actiphage™ Medium Plus into a Rapid Tube. A positive control was prepared by dispensing 100 µl of an active growing liquid culture of target mycobacteria (approx. 10^3 cfu ml⁻¹ grown aerobically at 37 °C in Actiphage™ Medium Plus) into a Rapid Tube. To carry out the assay the samples prepared as described in section 2.2.1 (100 µl) were added to the top section of the Rapid Tubes. The Actiphage™ reagent (10 µl) was added directly into the sample using a pipette with filter tips and mixed carefully by pipetting. This was incubated for 3.5 h at 37 °C and then the sample was centrifuged at 13,000 x g for 3 min. The flow through which contains the released mycobacterial genomic DNA was collected in the bottom sample tube. This DNA was either stored frozen at -20 °C until required or immediately further purified using a Zymo DNA Clean and Concentrator kit, eluting into a final volume of approx. 10 µl water.

2.2.6 Actiphage™ procedure

A Negative control was prepared by dispensing 1 ml of Actiphage™ Medium Plus into a sterile reaction vessel provided in the kit. A positive control was prepared by diluting an *M. smegmatis* culture to 10^2 cfu ml⁻¹ in Actiphage™ Medium Plus and dispensing 1 ml into a sterile reaction vessel provided in the kit. To each sample the prepared as described in section 2.2.2, 0.1ml of Actiphage™ reagent was added and the sample gently inverted to ensure good mixing. These were incubated at 37 °C for 60 min before addition of 0.1ml of the Virusol solution to each reaction vessel. The samples were mixed well by inverting and rolling the reaction vessel for 5 min at room temperature (20-25°C). Then 5 ml of Actiphage™ Medium Plus was added to vessel and mixed by inverting the reaction vessel once. A sample of *M. smegmatis* culture (1 ml of 10^8 cfu ml⁻¹) was added to each vessel and the mixture transferred into an empty pre-labelled sterile Petri dish. This was then mixed with 6 ml of molten Actiphage™ Agar (55°C) and mixed well by swirling in both directions, ensuring that the entire bottom surface of the plate is covered before allowing to set at room temperature. Petri dishes were incubated at 37°C overnight (18-24 h). The results were recorded by counting the

number of plaques formed. For further molecular analysis agar was extracted from five plaques and transferred into an Eppendorf tube. DNA extraction was performed using a Zymo Gel DNA Recovery Kit eluting in approx. 10 µl water. Molecular analysis.

2.2.7 Freeze drying phage

The freeze dryer currently used is Christ Alpha 1-2 LD-plus Freeze dryer with sealing device. The product is placed in the vacuum drying chamber for drying. The ice condenser can also be described as a vapour pump as the moisture which evaporates under vacuum during drying freezes onto the ice condenser. Consequently, the vacuum pump is only intended to remove the air from the drying chamber (= gas pump) but not the vapour. In order to start the sublimation process, energy must be supplied to the product. This takes place during drying in round bottle flasks, wide neck filter bottles or, as in this case, 3 ml glass vials, due to the much warmer environment (direct heat contact), on unheated shelves by means of heat radiation from the environment and directly by means of the shelves when heatable. With increasing pressure the rate of sublimation rises and the drying period is shortened.

The water vapour generated during the main phase is not pumped off by the vacuum pump but collected by the ice condenser. The purpose of the vacuum pump is to lower the partial pressure of the non-condensable gases so that the water vapour can be transported from the product to the ice condenser. However, small quantities of water vapour are also pumped off by the vacuum pump. Therefore, the vacuum pump is equipped with a gas ballast device. If the gas ballast valve is open the extracted condensable vapours will be emitted via the exhaust pipe together with air. For this reason, the gas ballast valve must be open during the main drying phase. During the main drying phase the moisture is removed by sublimation. The bound moisture is removed by desorption during final drying. However, the freeze drier currently used does not include the “final drying” option.

The following diagram (Fig. 2.1) shows the predicted drying process for a product containing approx. 10% solid matter. During the first quarter of the main drying phase

50 % of water content is condensed. During the next quarter of the main drying phase 50% of the remaining water is condensed. This continues until the drying curve approaches the axis asymptotically.

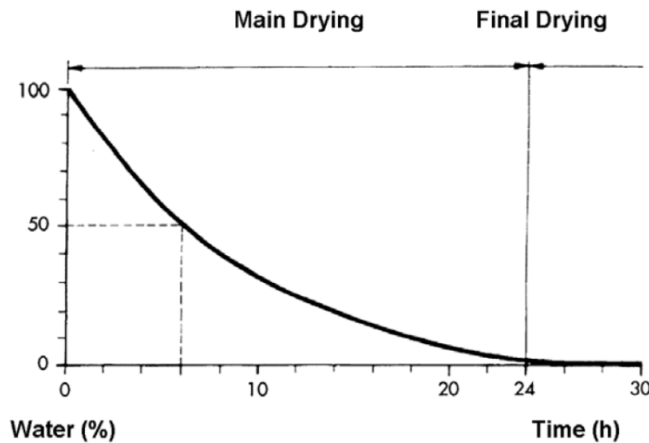


Fig. 2.1 Drying process of a product containing approx. 10% solid matter (From "Operating Manual Freeze Dryer Alpha 1-4 LSC / ALPHA 2-4 LSC". Christ Freeze Dryers Rotational-Vacuum-Concentrators. Version 04.2003 – English.)

This typical drying curve is due to the fact that the area of sublimation recedes into the product and the water vapour still to be extracted has to pass through the already dried layers. During the drying process the inner resistance increases. Thus, the drying curve is primarily determined by the latent heat of sublimation and the water vapour transport speed. In order to increase the specific heat conduction properties of the product to be dried and to keep water vapour volume as low as possible it is necessary that drying takes place as close as possible to the eutectic point. The drying time depends heavily on the drying vacuum. The nearer the vacuum is to the solidification point in accordance with the vapour pressure curve above ice, the shorter the drying time is.

CHAPTER 3

RESULTS

The work in this chapter describes the investigations that were carried out to allow a new kit to be produced containing the Actiphage™ components. This required both investigations of how to prepare key reagents in a stable form and also the design work to produce the packaging. Section 1 deals with experiments carried out to produce reagents, Section 2 describes the work carried out to source the other kit components and finally Section 3 describes the design process for the packaging.

3.1 Production of the Actiphage™ reagent

3.1.1. Introduction

For a phage-based diagnostic test a key component is the phage reagent itself. For both the Actiphage™ and Actiphage™ Rapid kits this constitutes freeze dried phage suspension. The drying process takes place by avoiding the liquid state through sublimation, i.e. direct conversion from solid (ice) to gaseous (vapour) state. This happens under vacuum, when the temperature in the product is less than -10°C. The aim of freeze drying is to obtain an easily water-soluble product which will have the same characteristics as the original product after addition of water. As the drying process takes place in frozen state at very low temperatures it is possible to dry e. g. albumen which will remain water soluble. Also, most of the other chemical compounds will be quantitatively and qualitatively unchanged. Through freeze-drying the product, mainly of biological origin – such as tissues, tissues extracts, bacteria, vaccine, sera and, of course, bacteriophage – is transformed into a dry product. During this process enzymatic, bacterial and chemical changes are largely avoided.

The principle of sublimation can be explained using the phase diagram of water (Fig. 3.1).

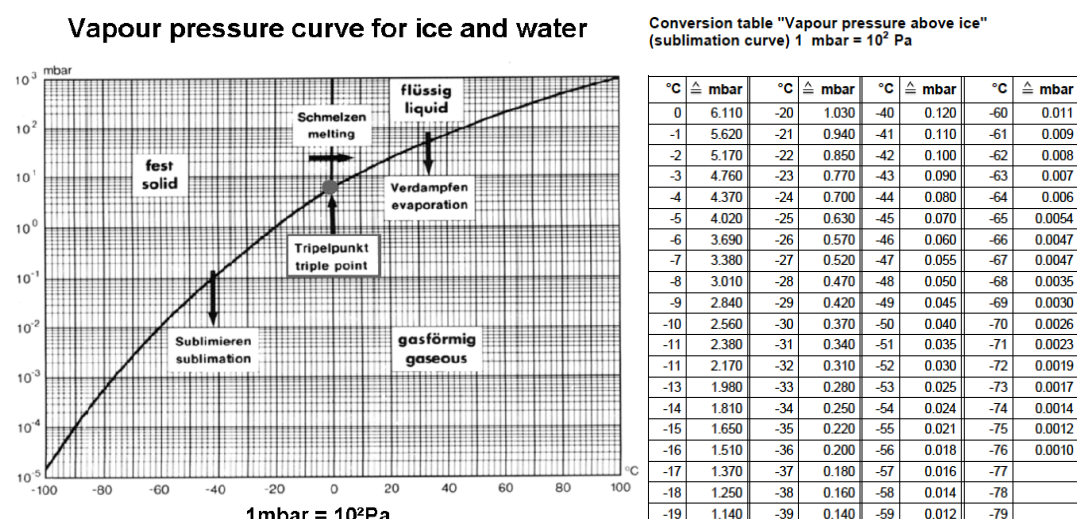


Fig. 3.1 Phase diagram of water and Conversion table (From "Operating Manual Freeze Dryer Alpha 1-4 LSC / ALPHA 2-4 LSC". Christ Freeze Dryers Rotational-Vacuum-Concentrators. Version 04.2003 – English.)

As can be seen in figure 3.1, if the atmospheric pressure is higher than 6.11 mbar, water passes through all three phases (solid, liquid, gas) when the temperature is lowered or raised. At 6.11 mbar the melting pressure curve, vapour pressure curve and sublimation pressure curve meet in one point called triple point. At this point all three phases occur simultaneously. Below this point, i.e. the pressure is lower than 6.11 mbar (see table in Fig 3.1), the ice is converted directly from a solid to a gaseous phase on reaching the sublimation pressure curve (vapour pressure curve above ice).

As soon as sublimation of water vapour from the frozen product begins, heat is extracted and consequently the product continues to cool down. As the plan was to freeze dry small aliquots of phage suspension, this cooling effect meant that they could be frozen directly inside the ice condenser chamber of the freeze-dryer whereas larger quantities would have needed to be pre-frozen to assist the freeze-drying process. The maximum rate of sublimation is reached at the start of drying process and the duration of the main drying phase depends mainly on the layer thickness of the product, the solid content of the product, the heat supplied to the product during the drying process and the pressure inside the drying chamber during the drying process.

The end of the main drying phase is reached when the product temperature is nearly the same as the shelf temperature and the vacuum and the ice condenser temperature can be used as a rough indication when the end of drying is reached. The ice condenser reaches the temperature of approximately -55°C to -85°C and the pressure in the drying chamber decreases according to the ice condenser temperature, however the actual drying time required for each product has to be determined empirically.

To maintain the product in a dry state it can be sealed with rubber stoppers inside the ice condenser chamber while still under vacuum. This is facilitated by moveable shelves which are used to press down on the rubber stoppers at the end of the drying process. The vacuum can then be released to allow the product to be removed from the chamber.

For the Actiphage™ Rapid Assay, 10 µl of Actiphage™ reagent are required per test since it is known from the original research carried out by the company that this concentration of phage is required to ensure that all cells in the sample are infected within the incubation period. Therefore, five vials of freeze dried phage of a titre of 10^9 pfu ml⁻¹ that are reconstituted in 100 µl of Media Plus need to be provided in each kit to allow 50 tests to be performed. For the Actiphage™ assay larger volumes of phage are required per test (0.1 ml of a phage reagent of 10^9 pfu ml⁻¹) therefore for these kits sufficient phage to allow reconstitution in 1 ml of Media Plus is needed. To allow the freeze drying of small aliquots, a phage stock of 10^{10} pfu ml⁻¹ is required which can then be reconstituted in a larger volume to generate the Actiphage™ reagent with a titre 10^9 pfu ml⁻¹.

3.1.2 Results

First, to establish a simple and reproducible method to produce the phage for freeze drying, two different phage preparation methods were compared. For method 1 (section 2.2.2) small aliquots of phage stock were spotted on the surface of an *M. smegmatis* – Agar plate (section 2.1.5) to produce discrete plaques. These were extracted using a 2-mm biopsy punch and 100 plaques were transferred into a tube

containing 5 ml of Media Plus and the phage allowed to elute for 10 min. Agar debris and any remaining cells were removed by centrifugation and the number of phage recovered determined by phage titration (section 2.2.1). The idea behind this method is that a reproducible phage stock could be produced for freeze drying as a precise number of plaques were used to prepare the phage. When this method was used the titre of the phage stock obtained was 10^9 pfu ml⁻¹, which was sufficient if there was no loss of phage during the freeze drying process but did not give any margin for error since the titre of the reconstituted freeze dried ActiphageTM product needed to be at least 10^9 pfu ml⁻¹. Therefore, it was decided to freeze dry these stocks using 110 µl aliquots which were reconstituted in 100 µl to allow for any reduction in titre during the freeze drying process.

To try and increase the titre of the phage stocks method 2 was developed based on observations made by other researchers in the laboratory (A. Kubala, UoN, personal communication). For this method, the phage stocks were diluted to a titre of 5×10^3 pfu ml⁻¹ and 0.1 ml of this was added to a 90 mm Petri dish containing 6 ml of Media Plus and 750 µl of *M. smegmatis* culture (section 2.1.4) and the contents set by addition of 6 ml of Nutrient Agar No. 2. After overnight incubation at 37 °C the density of the plaques per plate was confirmed to be approximately 500 plaques per plate on the basis of visual observation that semi confluent lysis had occurred. The agar was then cut into small cubes and transferred into a sterile beaker to which 7 ml of Media Plus was added to elute the phage. After 10 min, the eluate was centrifuged at 4,400 x g for 3 min to remove any agar debris and any remaining intact cells and the supernatant titred (section 2.2.1) to determine the number of phage produced. Although this was a less precise method, which might be an issue for reproducibility, the titre of the phage stock obtained using this method was always at least 10^9 pfu ml⁻¹, and occasionally produced a titre of 10^{10} pfu ml⁻¹, which was clearly more favourable for producing the ActiphageTM reagent. It was also decided to freeze dry this product in aliquots of 110 µl of this were freeze dried and reconstitute in 100 µl to allow for any drop in titre. In addition to producing phage stocks of a higher titre, this method was also simpler than method 1 and therefore is more practical when large amounts of product need to be produced.

Next experiments were carried out to determine if the phage stocks using either of these methods could be successfully freeze dried. It was decided to use small aliquots as this overcomes the need to pre-freeze the samples that might have resulted in loss of phage titre. To do this 110 μl aliquots of phage were placed in the freeze drier overnight and the drying process allowed to continue until a final reading of 0.014 atm was reached (this value was the reading achieved after 18 h of freeze drying of the samples rather than an empirically determined reading). The freeze-dried phage samples were then resuspended in 90% of the initial volume in Media Plus and the titre of the reconstituted ActiphageTM reagent determined as described in section 2.2.1. In both cases no drop in phage titre was detected after freeze drying (Table 3.1) and therefore it was concluded that the approach of drying small aliquots of phage was a simple and effective method to create the ActiphageTM reagent to be included in the kits (see Fig 3.2).

Table 3.1 ActiphageTM titre before and after freeze drying

Batch Number	Freeze dried volume (μl)	Method employed	Titre before freeze-drying (pfu ml^{-1})	Titre after freeze-drying (pfu ml^{-1})
PH001-2018	110	1	1.0×10^9	1.0×10^9
	110	2	1.0×10^{10}	1.0×10^{10}
PH002-2018	110	2	4.0×10^9	3.7×10^9
				3.3×10^9
				3.3×10^9
				5.0×10^9
				3.3×10^9
01061801	110	2	2.06×10^9	1.8×10^9
				1.9×10^9
				1.84×10^9
				1.56×10^9
				1.7×10^9
01061802	1100	2	2.50×10^9	2.5×10^9
				2.5×10^9
				2.5×10^9
				2.5×10^9
				2.5×10^9

Figure 3.2: Finished Actiphage™ reagent



Image of the Actiphage™ (freeze-dried phage) vial showing finished labelled product (see section 3.x) containing freeze-dried mycobacteriophage D29

However, as titres of 10^9 pfu ml⁻¹ were routinely being produced by both of the preparation methods, and this was not sufficiently high enough titre to produce the larger volumes of Actiphage™ reagent required for the Actiphage™ kits, one way to overcome this was attempting to directly freeze dry 1 ml aliquots of phage. This approach was tried and it was noted that the release of dissolved gases in the early stage of the freeze drying process led to the phage suspension in the bottles boiling. However, the same end point reading was produced after 18 h of drying and the reagent was again resuspended in 90% of the initial volume of Media Plus to allow for any loss in phage titre.

At the end of the drying process, again no loss in titre was recorded (Table 3.1) but when the phage were tested by carrying out the Actiphage™ assay (section 2.2.6), the breakthrough occurred in the negative control indicative that viable *M. smegmatis* cells were present in the sample. One reason why cells were able to survive in this sample compared to the smaller freeze dried samples could be that the product formed a thicker cake in the bottom of the vial, whereas when the freeze-dried volume was 110 µl the pellet is very thin and there does not provide a matrix that might protect the bacterial cells. Therefore, this approach was not seen as an appropriate method to overcome the fact that the phage titre was lower than that required.

Therefore, to try and improve the phage production method to ensure that a stock with a titre of 10^{10} pfu ml⁻¹ was routinely obtained a third method of phage production was

developed. This was a modification of method 2, but focussing on ensuring that (a) all of the phage were eluted from the agar and (b) all of the contaminating *M. smegmatis* cells were removed from the sample. To do this 10 agar plates were prepared as described for method 2 with 500 plaques per plate and another set of 10 plates were prepared containing a predicted number of 1000 plaques per plate. After incubation overnight at 37 °C, the plates were examined to confirm that the predicted number of plaques had been produced. This time the agar was cut with a sterile scalpel into cubes of approx. 1 mm and the content of each plate was then transferred to a sterile beaker with 8 ml of Media Plus. The content of each beaker was then stirred for 30 min using a magnetic stirring bar to help the phage elute from the agar matrix. The supernatant (Media Plus containing the phage) was then centrifuged at 4,400 x g for 10 min as before, but then was filter sterilized using a 0.22 µm filter to eliminate any cells remaining. The smaller size of the cubes, a bigger volume of Media Plus used for elution, longer time of elution, mixing during elution and longer time of centrifugation were all measures predicted to help the phage to elute from the agar into the Media Plus and as expected the titre of the phage stocks obtained from both sets of plates was 10¹⁰ pfu ml⁻¹ indicating that even when the number of plaques was slightly lower than optimal this method still allowed efficient phage elution.

Once the reagent had been prepared it was important to determine the shelf life of the freeze dried ActiphageTM reagent. It was known that the original FASTplaqueTB freeze dried reagent was stable for many years when stored at + 5 °C (Dr C Rees, UoN, personal communication), so it was hoped that the new reagent would also be stable for this amount of time. First a test was carried out to see if the ActiphageTM reagent was stable at room temperature. To do this, a sample of a batch of reagent with an initial phage titre of approximately 1 x 10⁹ pfu ml⁻¹ after reconstitution was left at room temperature for a period of one month. After this time interval, the vials left at room temperature were reconstituted in 90% of the initial sample volume used for freeze drying (100 µl Media Plus) and the phage titre again determined using the method described in section 2.2.1. In this case no viable phage particles were detected meaning that that no phage survived.

To determine the shelf life of the ActiphageTM reagent stored at + 5 °C, the titre of 3 vials from each production batch was checked monthly to determine if the phage titre was lower than originally recorded. Regarding a batch that was manufactured in January 2018, it was found that 6 months later (June 2018), the titre determined for all the replicate samples was 10-fold lower (10^8 pfu ml⁻¹ instead of 10^9 pfu ml⁻¹). However, in contrast, no drop of titre was detected when the titre of 5 vials from a batch of phage made in March 2018 was tested three months later (June 2018); for this batch, the titre was 10^9 pfu ml⁻¹ both times. This suggests that there is some variability in the properties of the different batches of phage, or that the ActiphageTM reagent stored at + 5 °C is only stable for a period of 3 months.

3.2 Preparation of the Virusol reagent for the ActiphageTM assay

3.2.1 Introduction

Virusol is used in the ActiphageTM test to inactivate all the bacteriophage that have not infected any target mycobacteria present within the sample. McNerney *et al.* (1998) and Stewart *et al.* (1998) both showed that the treatment of mycobacteriophage D29 with Ferrous ammonium sulphate solution (FAS) renders the phage unable to infect its host bacterium, and they have demonstrated the use of FAS as a simple method for inactivation of exogenous phage without adversely affecting the replication of phage within a host bacteria. While the uptake and metabolism of iron by mycobacteria has been well documented (Wheeler and Ratledge, 1994), the mode of inactivation of the phage by FAS is not known. Inactivation of coliphage Q β by ferrate ions has previously been investigated and it has been suggested that an oxidative intermediate generated by decomposition of the ferrate ion is responsible (Kazama, 1995). Since the inactivation is enhanced by the presence of polyphenols extracted from plants (Siqueria *et al.*, 2003; Stewart *et al.*, 1992), this suggests a role for the generation of reactive oxygen species as the basis for D29 and other phage inactivation by FAS.

At the beginning of this work information was supplied about the composition of the original FAS tablets provided in the *FAST*plaqueTB kits (Dr C Rees, UoN, personal communication) and also the specification of these tablets (Table 3.2).

Table 3.2: Virusol tablet specification from *FAST*plaqueTB kits

Virusol tablet specification	
Colour	Pale green
Shape	Circular
Diameter	8 mm ($\pm 5\%$)
Thickness	5 mm ($\pm 10\%$)
Compression weight	424 mg
Average weight	Not more than $\pm 10\%$ from target compression weight
Uniformity of weight	Not more than $\pm 10\%$ of mean (based on average weight of 20 single tablets)
Disintegration time	≤ 5 min. (in 5 ml distilled water)

Ferrous ammonium sulphate hexahydrate (FAS) is the main component of the tablet. In addition, it was known that the tablets contained Sodium starch glycolate, Citric acid (anhydrous) and Polyethylene glycol (PEG), molecular weight 6000 (NB: exact formulation cannot be reported in this thesis due to confidential nature of the information). Citric acid anhydrous (Table 3.3) is a weak organic acid and is used as an excipient to adjust pH of formulations and, because of its antioxidant properties, it helps to maintain the stability of active ingredients.

Table 3.3 Chemical and physical properties of citric acid anhydrous

Citric Acid Anhydrous	
Physical form	Crystalline
Molecular formula	$\text{HOCOCH}_2\text{C}(\text{OH})(\text{COOH})\text{CH}_2\text{COOH}$
Formula weight	192.13 g/mol

A disintegrant is an excipient that is incorporated into the formulation of tablets to promote their disintegration when they come into contact with liquid or fluid matter. Several types of disintegrants have been used for many years and may be distinguished according to their mode of action: (a) those that enhance the action of capillary forces that promote the absorption of water (b) those that swell on contact with water and (c) those that release gases leading directly to the disintegration of the tablet (LFA Tablet Presses Articles, An Overview of Disintegrants). The general purpose of incorporating one or more disintegrants in the product formulation is to increase the surface area of the product and soften the binding matter that holds together the solid particles that make up the product. The net effect is that a tablet when exposed to aqueous media disintegrates first into granules, and then into fine particles. The rate of dissolution in the media increases as the particle size reduces and is greatest when the tablets reduced to fine particles.

Starch, in the form of potato and corn starch, has been a common and widely used disintegrating agent, since the beginning of the 20th Century. Natural starches have some limitations as tablet disintegrants, however, and these have stimulated the discovery of modified starches that have more appropriate qualities. Starches function by promoting wicking of water and, to a lesser extent, by their particles becoming deformed during compression and restored on absorption of water. Starch is a polymer (polysaccharide) of high molecular weight. The starch molecules naturally arrange themselves into crystalline agglomerates or granules of different sizes that are visible under an optical microscope (LFA Tablet Presses Articles, An Overview of Disintegrants).

According to Lowenthal & Wood (1972), large agglomerates are required for starch to be an effective disintegrant. Starches also perform best if a low compression pressure is employed for the tablet making. The concentration of starch in the formulation is also important. If the concentration is low the starch will create an insufficient number of channels for wicking of water or body fluid. On the other hand, if the concentration is too high, the material will be too difficult to compress into a tablet.

Starch can be chemically modified by carboxymethylation to increase cross-linking between molecules. Such modified starch yields a higher degree of swelling when it absorbs water, leading to faster disintegration of the tablet. Sodium starch glycolate is an example of a such a starch derivative that can absorb 20 times its weight in water. Modified starches and starch derivatives swell extensively with minimal gelling, and optimum concentration levels of 4-6% by weight. When fully hydrated the starch forms a sticky and gelatinous matter that continues to aid the disintegration process as it helps to hold keep the tablet particles together. Due to their high swelling capacity, modified starches are highly efficient even in low concentrations (LFA Tablet Presses Articles, An Overview of Disintegrants).

The growing demand for faster and more rapid disintegrating formulations has stimulated the development of what the industry calls “superdisintegrants”. These derivatives are developed to have greater effectiveness even at low concentrations. They are also effective intragranularly. Superdisintegrants function principally by swelling on absorbing water. Sodium starch glycolate (Table 3.4) is an example of a superdisintegrant.

Table 3.4 Chemical and physical characteristics of sodium starch glycolate

Sodium starch glycolate	
Appearance	White free flowing powder
Solubility	Readily soluble in water to form colloidal solution
pH (in water at 25°C)	5.50 – 7.50
Linear formula	$(C_2H_4O_3)_x \cdot (Na)_x$

(HiMedia Laboratories, Sodium Starch Glycolate Certificate of analysis, Catalogue number GRM7519).

A binder is a chemical compound that has an adhesive property, and is one of the more important excipients that are included in tablet formulations. In simple terms, binders or adhesives are the substances that promotes cohesiveness. A binder is therefore used for transforming powder into granules through a process known as granulation. In these tablets, the role of Polyethylene glycol 6000 (PEG) is as a binder.

Table 3.5 Characteristics of PEG6000

PEG 6000	
Physical form	Powder
Molecular formula	$H(OCH_2CH_2)_nOH$

(Product label, Alfa Aesar, Catalogue number A17541)

In tablet making, granulation is the process by which small powdery particles are agglomerated into larger entities called granules. In the agglomeration, the active ingredient and excipients are bound together by the binder which gives the granule strength. Binders can also form intergranular bonds that fuse the granules together, forming bridges between adjacent granules. The bridges may result from the melting or softening of the binder during the compression process. Rather than add the binder to the powder before the granulation process, a relatively recent innovation is to mix a dry binder directly with the powder before the tablet compression step. This approach is

known as direct compression (LFA Tablet Presses Articles, Binder Types and Properties Used in Tablet Manufacturing).

Flow or fluidity and compressibility are the essential characteristics required for powders/granules that are intended for tablet compression. Fluidity is necessary for the production of tablets of consistent weight and uniform strength. Compressibility is required to form a stable, intact and compact mass when pressure is applied. Traditionally granulation has been the means to achieve both fluidity and compressibility of the tablet material. Granulation has also been used to improve the appearance, mixing properties, to avoid dustiness, to densify material, to reduce segregation, and in general to either eliminate undesirable properties or to improve the physical characteristics of fine powders and the compounds are added in low concentrations - typically about 2-10% by weight.

Direct compression offers a simpler route to tablet manufacture, product stability and high efficiency, and the method has therefore increased in popularity in recent years. Direct compression is suited to heat or moisture sensitive ingredients (such as FAS). It is also well suited to powders which can be mixed well and do not require further granulation steps prior to introduction to the tablet press. For direct compression, directly compressible binders are required which should exhibit adequate powder compressibility and flowability (LFA Tablet Presses Articles, Binder Types and Properties Used in Tablet Manufacturing). Shah *et al.*, (1977) evaluated PEG 6000 as a binder for the direct compression of 11 tablet formulations. Six formulations compressed quite well and the resulting tablets were satisfactory in all respects. Five of the six formulations were stable after a shelf life of 3 years.

3.2.2 Preparations carried out to produce FAS tablets

Having established the key factors in tablet making from the literature review, and having been provided with the original formulation of the FAS tablets, this information was used to design a production process. First the nature of the FAS itself needed to be considered. In addition to being sensitive to oxidation, FAS is highly hygroscopic

therefore there was a concern about accurately weighing the amount of dry FAS ingredient that may have taken up different amounts of water when making the tablet mix. To solve this problem the FAS powder was dried at 30°C for approximately 30 min before the required amount was weighed out.

Table 3.6 Chemical and physical characteristics of FAS

Ferrous ammonium sulphate hexahydrate	
Appearance	Light green crystalline powder
Molecular weight	392.14 g/mol
Molecular formula	H ₈ Fe N ₂ O ₈ S ₂ .6H ₂ O
Linear formula	(NH ₄) ₂ Fe(SO ₄) ₂ .6H ₂ O

(Across Organics, Certificate of Analysis, Ferrous Ammonium Sulphate, Catalogue number 20137)

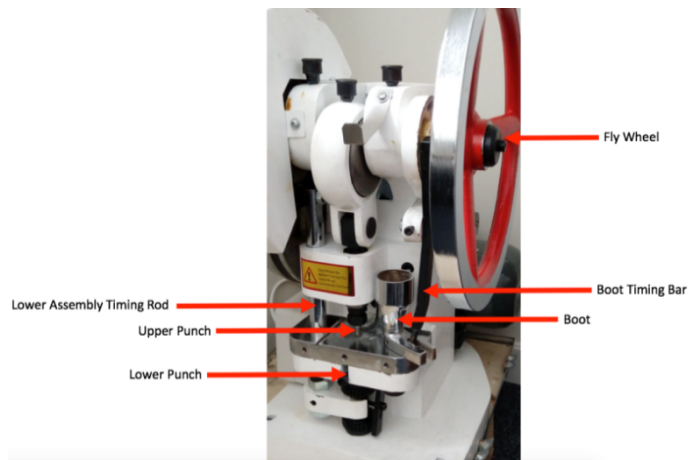
After the raw materials had been weighed, they were passed through a sieve (mesh size approx. 1 mm), to eliminate any lumps that can cause problems during the compression stage, and were then placed in a zip-lock bag suitable for 1 kg of material and then this was shaken until the raw materials were uniformly mixed. The compression of the mix was then carried out using a TDP 5 Desktop Tablet Press (Fig. 3.3 a,b,c).

Figure 3.3 TDP 5 Desktop Tablet Press and main components

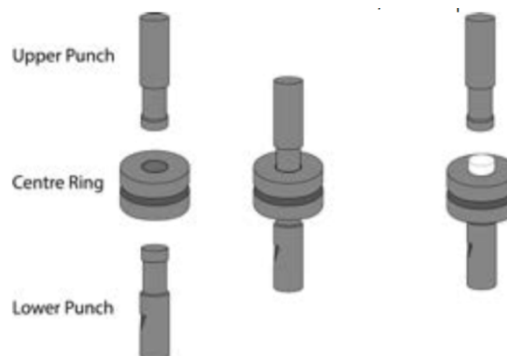
Panel a



Panel b



Panel c



The tablet press operates by pouring the mix into the boot using the funnel (panel a) and, as the Flywheel is manually or automatically operated, the Boot Timing Bar (panel b) moves the Boot back and forth over the machine's centre ring. As the Boot passes over the centre ring, the mix flows down to the hole located in the middle area of the centre ring. The Boot Timing Bar then moves the Boot and pulls it back out of the way. The top and bottom die punches meet in the middle to press the material under high pressure (Panel c). The Lower Assembly Timing Rod and the Lower Drift Pin Assembly Lifting Bar then raise the finished tablet and push it out of the die by lifting the Lower Drift Pin Assembly and the bottom die punch. The Boot Timing Bar then moves the Boot back so that the next granular matter is placed into the feeder for the next tablet. The

new tablet would then be pushed out of the way. The process repeats itself at full speed and it can punch out as many as 5000 tablets per hour.

3.2.3 Results

During the first production tests, jamming of the press and broken tablets were the main issues encountered. The problem of obtaining broken tablets was overcome by properly adjusting the ejection height. It was discovered that if this was slightly low, it caused the newly formed tablet to crash against the centre ring as the lower punch was slightly lower than the optimal height. After adjusting the ejection height, the number of broken tablets significantly decreased. However, a small number of broken tablets were found to be produced towards the end of the process which appeared to be mainly due to the fact that at this stage the amount of mix available for compression is small. It was noticed that the number of broken tablets can well be kept under control by cleaning the punches regularly after a production run. This prevents small quantities of the mix from accumulating and causing encrustation on the punches, that can lead to broken tablets.

To address the jamming issue, adjustments were made to the punch pressure as it was found that this was initially too high and also reducing the amount of mix being used. In addition, it was found that operating the machine manually was a better way to achieve the right pressure, which was achieved when a slight resistance was felt while operating the machine manually allowing the tablet to be ejected smoothly.

Due to the hygroscopic nature of the FAS, the tablets obtained from the production tests were kept in a sealed zip-lock bag. However, it was noted that this was not a good storage method, as a certain amount of mix was found to be accumulating at the bottom of the bag, suggesting material was being lost from the tablets. Since the purpose of the tablets is to allow a FAS solution of a given strength to be generated by dissolving a set amount of solid in given amount of water (1 tablet in 5 ml sterile water), then this was not ideal. To avoid this problem, after production, Virusol were immediately dispensed into glass vials (one tablet per vial) that would be used to prepare the FAS

solution. Glass vials were preferred because the required volume of water that is necessary to dissolve the tablet can be added directly into the vial and then solution is then ready to use (Fig. 3.4). Therefore, if any material was lost it would be retained within the vial and would still be present when the solution was prepared.

Figure 3.4 Final Virusol product

Panel a



Panel b



Panel c



Images showing (panel a) batch of tablets (panel b) labelled product in vial and (panel c) product after addition of 5 ml of distilled water to make Virusol solution. As can be seen from panel a, the colour and shape meet the specification and the tablets were quickly soluble after addition of water.

However, since it was important to weigh tablets after production to ensure that the weight of material being incorporated into each tablet was reproducibly the same, the question arose whether it is better to weigh the tablet in the vial or the tablet alone for quality assurance purposes. To answer this question the variation in the vials and the tablets was determined (Table 3.7). As can be seen from Table 3.7, the standard deviation of the weights of the tablets alone was 10-fold lower than the standard deviation of the weights of the tablet plus vials. Therefore, weighing tablets in the vials was not an appropriate method of analysis. To solve this problem, tablets were carefully removed from the vial before weighing and the change in weight of the vial ($D - A$) was used to check that there were no losses during the process of transferring the tablet from the vial to scales. As can be seen there are no significant losses so this method,

although a bit laborious, worked well to ensure that the quality assurance checks could be performed when the tablets were stored in separate vials to avoid losses.

Table 3.7: Analysis of tablet weights in vials

A	B	C	D	E
WEIGHT VIAL + CAP (g)	WEIGHT TABLET (g)	WEIGHT TABLET + VIAL + CAP (g)	C - B (g)	D - A (g)
8.553	0.419	8.972	8.553	0.000
8.294	0.432	8.728	8.296	0.002
8.523	0.433	8.959	8.526	0.003
8.535	0.447	8.982	8.535	0.000
8.427	0.444	8.873	8.429	0.002
8.303	0.414	8.717	8.303	0.000
8.300	0.432	8.736	8.304	0.004
8.479	0.408	8.893	8.485	0.006
8.524	0.423	8.957	8.534	0.010
8.273	0.416	8.689	8.273	0.000
8.227	0.436	8.663	8.227	0.000
8.421	0.427	8.853	8.426	0.005
8.349	0.437	8.786	8.349	0.000
8.312	0.412	8.724	8.312	0.000
8.304	0.402	8.706	8.304	0.000
8.305	0.401	8.707	8.306	0.001
8.407	0.437	8.845	8.408	0.001
8.157	0.405	8.562	8.157	0.000
8.502	0.423	8.926	8.503	0.001
8.368	0.429	8.8	8.371	0.003
AVERAGE (g)	AVERAGE (g)	AVERAGE (g)	AVERAGE (g)	AVERAGE (g)
8.378	0.424	8.804	8.380	0.002
ST. DEV. (g)	ST. DEV. (g)	ST. DEV. (g)	ST. DEV. (g)	ST. DEV. (g)
0.114	0.014	0.120	0.115	0.003

3.3 Sourcing other kit media components

The two Actiphage™ kits required slightly different components, Actiphage™ Medium and Actiphage™ Growth Supplement are provided with both Actiphage™ and Actiphage™ Rapid, whereas Actiphage™ Agar is provided only with the Actiphage™ kit.

3.3.1 Actiphage™ Growth Supplement

Actiphage™ Growth Supplement is based on Middlebrook OADC Enrichment (Table 3.8) with additional supplementation including calcium chloride to promote phage binding to host cells, however the precise formula of this is confidential. The largest commercial supplier of OADC is Becton Dickinson (BD), but as shown in Table 3.9 the cost of this

reagent is very high. In addition, if purchased from this company the supplements would have to be added as sterile solutions after manufacture which would increase the risk of contamination. Therefore, it was decided to identify a new source of Actiphage™ Growth Supplement and then to confirm that it performed in the same way in the assays as the OADC produced by BD.

Table 3.8 Formulation of BD OADC

Ingredient	g l⁻¹
Oleic acid	0.6
BSA	50
Dextrose	20
Catalase	0.03
Sodium chloride	8.5

First quotes were obtained from different companies for production of the Actiphage™ Growth Supplement and the range of prices quoted is shown in Table 3.9. As can be seen from Table 3.9 the price offered by Boval is very competitive. However, when a batch was ordered from this US-based company there were custom clearance issues when entering the UK as the product contains bovine albumin and there are restrictions on movement of these products. The product, which needs to be stored refrigerated at 2 - 8°C, was kept at room temperature for nearly two weeks. As the OADC supplement contains the enzyme catalase and also bovine albumin, keeping the product not refrigerated could potentially lead to diminished product performances. However, when the product was finally delivered and tested, no negative effects on product performance, or contamination, were detected (data not shown). However, to avoid this potential problem in the future, despite a higher cost and a relatively long delivery time (4 - 6 weeks) Abtek Biologicals was chosen as the primary supplier of Middlebrook OADC Enrichment. This company also offered the possibility of discounts on large orders and of purchasing custom made vials to suit the kit format.

Table 3.9 Middlebrook OADC price comparison

Middlebrook OADC Enrichment		
Supplier	Price	Amount
Abtek Biologicals (with supplements)	80.00 GBP	1-25 packs of 10 x 25 ml vials
	72.00 GBP	Over 25 packs of 10 x 25 ml vials
Boval (with supplements)	22 USD	100 ml
Sigma Aldrich (no supplements)	134.00 GBP	50 ml
BD (no supplements)	240.03 GBP	500 ml
	257.73 GBP	6 X 100 ml

3.3.2 ActiphageTM Media base

A similar approach was taken to identify a source of Middlebrook 7H9 Broth Base that is a growth medium specifically used for culture of *Mycobacterium* species and the standard formula is shown in Table 3.10. Again, quotes were obtained from different suppliers (Table 3.11). Interestingly BD was not the most expensive supplier, but Trafalgar Scientific offered a price approximately four times cheaper than the average price from other suppliers. The fact that a large quantity was purchased, and only a small amount of product is used in each kit, means that the long delivery time quoted (7-8 weeks) was not a problem. Middlebrook 7H9 Broth Base was supplied by Trafalgar Scientific (Leicester).

Table 3.10 Middlebrook 7H9 Broth Base standard formula

Ingredients	g l⁻¹
Ammonium sulphate	0.50
Disodium hydrogen phosphate	2.50
Potassium dihydrogen phosphate	1.00
Sodium citrate	0.10
Magnesium sulphate	0.05
Calcium chloride anhydrous	0.0005
Zinc sulphate	0.001
Copper Sulphate	0.001
Ferric ammonium citrate	0.04
L-Glutamic acid	0.50
Pyridoxine hydrochloride	0.001
Biotin	0.0005

Final pH (at 25°C) 6.6 ± 0.2

Table 3.11 Middlebrook 7H9 Broth Base price comparison

Middlebrook 7H9 Broth Base		
Supplier	Price (GBP)	Amount (g)
Trafalgar Scientific	36.00	500
Sigma Aldrich	179.00	500
Fisher Scientific	105.85	500
BD	150.85	500

3.3.3 Performance of ActiphageTM Media Plus

Once suppliers of the reagents were identified it was important to confirm that they performed in the same way as the BD reagents that were standardly used in the laboratory. First a quality check was performed on 10 vials from the first batch of Abtek's ActiphageTM Growth Supplement by placing 10 bottles in a static incubator at

37 °C for 24 h. There was no evidence of growth confirming that there was no evidence of contamination of the product. Then a series of five flasks containing (a) 45 ml 7H9 broth base, 5 ml Actiphage™ Growth Supplement, 10 µl *M. smegmatis* culture (section 2.1.4) (b) 45 ml 7H9 broth base, 5 ml BD OADC, 10 µl *M. smegmatis* culture (section 2.1.4) and (c) 45 ml 7H9 broth base and 5 ml Actiphage™ Growth Supplement (sterility check). These flasks were then incubated at 37°C with shaking for 18h.

After incubation, there was no visible difference in the level of growth of *M. smegmatis* between the Abtek and BD OADC samples. However, some clumping of the *M. smegmatis* was noticed in the flasks containing Abtek OADC. Due to the hydrophobic nature of the mycolic acid in the mycobacterial cell wall, cultures of these bacteria are known to clump together, however as the same inoculum was used for both sets of flasks then it was possible that this was being induced by the difference in the two media since unlike BD OADC, Actiphage™ Growth Supplement contains additional supplements and it was hypothesized that this could be mostly responsible for the clumping. To investigate this further, flasks were prepared containing (a) 45 ml 7H9 broth base, 5 ml Actiphage™ Growth Supplement, 10 µl *M. smegmatis* culture (section 2.1.4) (b) 45 ml 7H9 broth base, 5 ml BD OADC, 10 µl *M. smegmatis* culture (section 2.1.4) and (c) 45 ml 7H9 broth base, 5 ml BD OADC supplemented with the same substances added to the Actiphage™ Growth Supplement, 10 µl *M. smegmatis* culture (section 2.1.4). These were again incubated for 18 h at 37°C with shaking and after incubation, it was noticed that in the flask containing BD OADC original formula, no clumps were visible but there was clumping of the mycobacteria in the flasks containing Actiphage™ Growth Supplement and in the one containing BD OADC with the additional supplements. This led to the conclusion that the supplement was most likely to be responsible for the clumping.

It must be noted that Actiphage™ Growth Supplement was produced to the same formula that had been used in the original FPTB kits, and that this formula had been shown to perform well in the phage amplification assays. Therefore, to confirm that the clumping of the mycobacteria in the liquid cultures was a new problem, tests were carried out by other members of the research group to confirm that the same number

of mycobacterial cells could be detected using either the new formulation or the BD formulation with supplements that was routinely used in the UoN research group. In this case the two different media performed in exactly the same was (Dr C Rees, UoN, personal communication) and therefore it was decided that these reagents were suitable for inclusion in the Actiphage™ kits.

3.3.4 Packaging of Actiphage™ Media base and Agar

The next problem to be addressed was identifying a practical way of delivering the correct amount of powder required for each Actiphage™ kit. As the smallest fill weight that can be provided by the current sachets manufacturer is 2 g, and the content of one Actiphage™ Growth Supplement vial is 25 ml, the weight of the 7H9 Broth Base powder required to make 225 ml of Actiphage™ Medium base in the sachet is 2.35 g. In addition to the Actiphage™ Medium base powder (2.35g Middlebrook 7H9 Broth Base) the Actiphage™ kit also needs to contain 2.6 g Actiphage™ Agar. Again, the exact composition of the Actiphage™ Agar is confidential but it is a commercially sourced, standard microbiological media that has been shown to be compatible with the Actiphage™ assay, and in fact in comparative tests had performed better than the original FASTPlaqueTB agar (Dr C Rees, UoN, personal communication).

It was decided to package the required amount of Middlebrook 7H9 Broth Base into sachets. Two fill systems were suggested by the sachet manufacturer identified: the minimum weight fill system and the average weight fill system. The minimum weight system uses up more powder, as every Actiphage™ Medium and Actiphage™ Agar sachet would not have less than 2.35 or 2.6 g, respectively. By definition the average weight system has a percentage of sachets that are below the target fill weight and therefore uses less powder, leading to a better yield but runs the risk of some sachets having a less than optimal amount of powder. Based on the parameters provided by the manufacturer, a calculation was carried out to determine which of these two processes would be preferable. The results of the calculation of the weights that would be delivered into the sachets are shown in Table 3.12.

Table 3.12 Minimum Weight System and Average Weight System comparison

Minimum Weight System		Average Weight System	
<u>Medium</u>	<u>Agar</u>	<u>Medium</u>	<u>Agar</u>
Min: 2.35 g	Min: 2.6 g	Min: 2.15 g	Min: 2.4 g
Target: 2.55 g	Target: 2.8 g	Target: 2.35 g	Target: 2.6 g
Max: 2.75 g	Max: 3.0 g	Max: 2.55 g	Max: 2.8 g

As can be seen from Table 3.12, using the minimum weight fill system the desired weight of the powder in the sachet is the minimum weight, the target weight is 0.2 g greater and the maximum weight is 0.4 g greater. In contrast using the average weight fill system, the desired weight of the powder in the sachet is the target weight, and the minimum and maximum weight are both 0.2 g smaller and greater than the target weight which is within the tolerances normally used when preparing bacteriological media. This means that of the two fill methods, the one that gives less variation of the desired weight of the powder in the sachet is the average weight fill method and therefore the choice was made for the average fill system as the target weight using this system is the weight of the amount of powder that is needed to be in the sachet.

Moreover, this method leads to a better yield and therefore to a greater number of sachets. An image of the final product, plus the templates designed for the packaging that included all of the legally required safety information is shown in Figure 3.5.

Figure 3.5 Details of sachets containing Actiphage™ Medium and Actiphage™ Agar



Actiphage™ Medium and Actiphage™ Agar contain, respectively, dehydrated Middlebrook 7H9 Broth Base and Actiphage™ Agar, packed into 80 mm x 55 mm, 50 GK / 12 PE / 7 Alu / 23 Surlyn laminate sachets. The fact that the average weight fill method was used is stated on the sachets by the “e” mark next to the fill weight.

3.4 Kit design

The final task of this project was to determine the best way to design the packaging for the kits. In addition to the media and phage reagents described in section 3.2 and 3.3, the two kits also required some specialised plasticware.

3.4.1 Plastic disposables

For the Actiphage™ kit, Reaction Vessels are required, which are 10 ml polypropylene flip-top vials (Fig. 3.6A) in which the mixture composed of processed sample, reconstituted phage (Actiphage™ reagent), Virusol and *M. smegmatis* cells are placed at different stages of the assay. For the Actiphage™ Rapid kit, Rapid Tubes (Fig. 3.6B) are provided which are polypropylene microcentrifuge filter tubes containing a 0.22 µm cellulose acetate membrane with a 500 µl capacity. These Rapid Tubes are used to hold the sample and Actiphage™ reagent and to allow incubation of the sample until cell lysis has occurred. Centrifugation is used then to separate the genetic material released from the viable mycobacterial cells from any remaining intact cells (potentially non-viable cells). This specialist plasticware is the most expensive component of Actiphage™ Rapid kit but is an essential component to ensure that the DNA detected at the end of the assay does come from viable mycobacteria.

Figure 3.6 Images of specialist plasticware required for Actiphage™ kits



A) Reaction Vessel



B) Rapid Tubes

3.4.2 Kit components

The number of Actiphage™ Medium sachets provided for both the Actiphage™ Rapid and Actiphage™ kits, is two. Only one is necessary to make sufficient Actiphage™ Medium Plus to carry out 50 blood tests (standard kit size) but the volume of Actiphage™ Medium Plus that is necessary to perform 50 milk tests with the Actiphage™ kit is approximately 500 ml and two sachets are required. Rather than having different numbers of sachets in different kits it was decided that a spare sachet would be provided in the blood test kits since each sachet was very low cost it was decided to include a spare sample to cover the eventuality that the media becomes contaminated after reconstitution. Two 25 ml Actiphage™ Growth Supplement vials are also provided, to give a total volume of Actiphage™ Medium Plus of 500 ml, when added to 450 ml reconstituted Actiphage™ Medium.

3.4.3 Design of Box and inner trays for Actiphage™ kits

Box and inner trays for the two kits were designed based on the number and on the size of the components required for the kit. Regarding the vials, the base diameter and the height are of particular importance (Fig 3.7).

Figure 3.7 Images and dimensions of Actiphage™ vials



Actiphage™ Growth Supplement vials: approx. 75 mm high x 42 mm base diameter

Actiphage™ Reagent vials: 36 mm high x 18 mm base diameter

Virusol vials: 42 mm high x 20 mm base diameter (Virusol)

The first sketches of box and inner trays were planned on paper first and then using MS Power Point, final scale design was produced for the manufacture (Cyberpac, Bury St. Edmunds, Suffolk). The material used to manufacture boxes and inserts is corrugate cardboard. Initially, the preferred material chosen for the inserts was foam, but calculation of the potential production cost was too high.

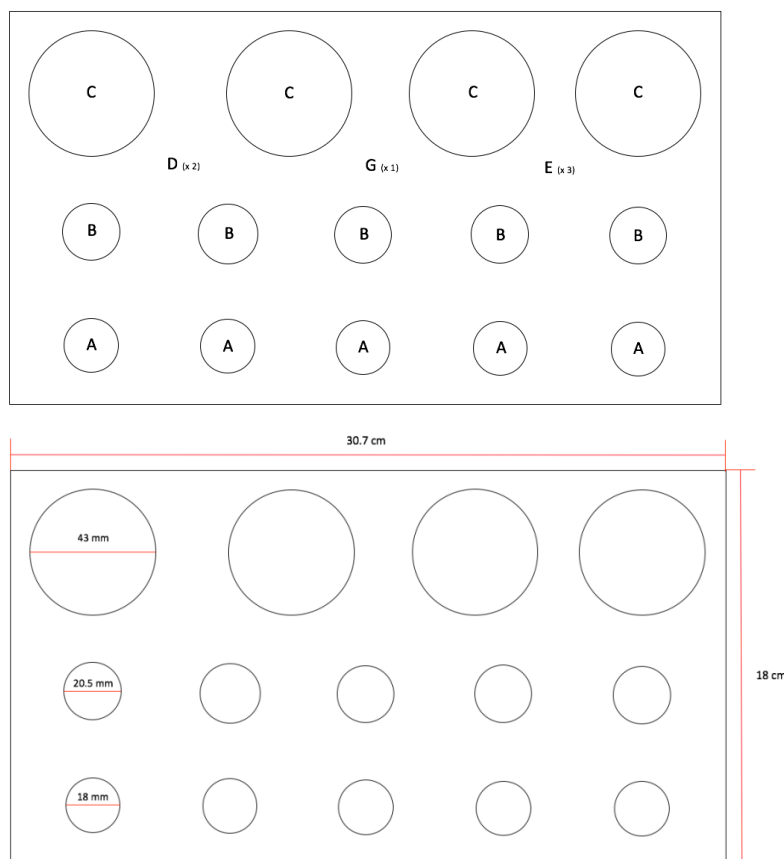
The box needed to contain 5 vials of Actiphage™ Reagent (A), 5 vials of Virusol (B) and 4 vials of Actiphage™ Growth Supplement (C). In addition, it needed Actiphage™ Medium (D) sachets (x 2), Actiphage™ Agar (E) sachets (x 3) and Instructions for use (G). In each case these letters correspond to the positions shown in the diagram of the insert design shown in Figure 3.8. On the sides of the inserts there are four flaps that, when the insert is placed in the box, are designed to be folded, to keep the insert in place.

As can be seen from the list of components size given in Figure 3.7, the height of the Actiphage™ Growth Supplement vials is much bigger than the height of the Actiphage™ or Virusol vials and therefore a design was needed to allow all these vials to be held at approximately the same height. To solve this problem, and therefore to have all the vials at approximately the same height, a foldable board and a flat approx. 1 mm thick board were designed (see Fig. 3.9D).

The inner tray was designed to be placed in a box of approximately 19 (W) x 32 (L) x 10 (H) cm with hinged lid (Fig. 3.10). The box was designed to accommodate the reagents but in addition the same box, without insert, was designed so it could be used to accommodate a zip-lock bag containing 50 Reaction Vessels. The box graphics (Fig. 3.10) were designed by Cyberpac using the PBD Biotech logo, Actiphage™ trademark name and images from the PBD Biotech website, with the company contact details placed on the sides of the box. The colours were chosen to respect as much as possible the colours of PBD Biotech.

Figure 3.8 Actiphage™ inner tray (sketch) and image of final product

A) Sketches of proposed Actiphage™ inner tray



B) Image of Actiphage™ inner tray

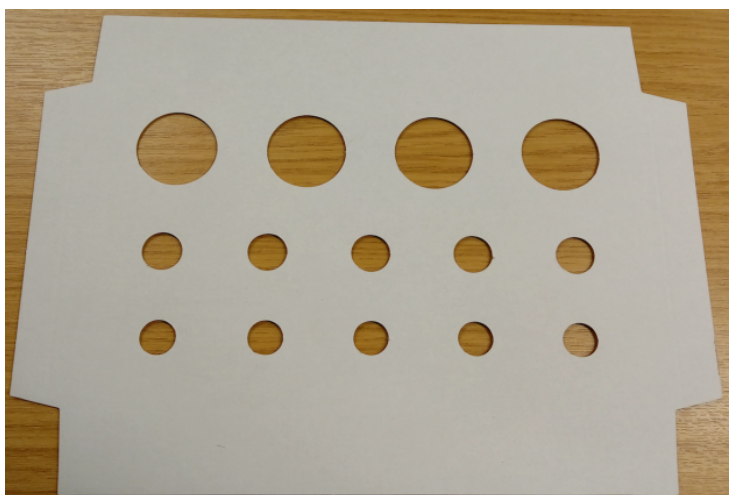
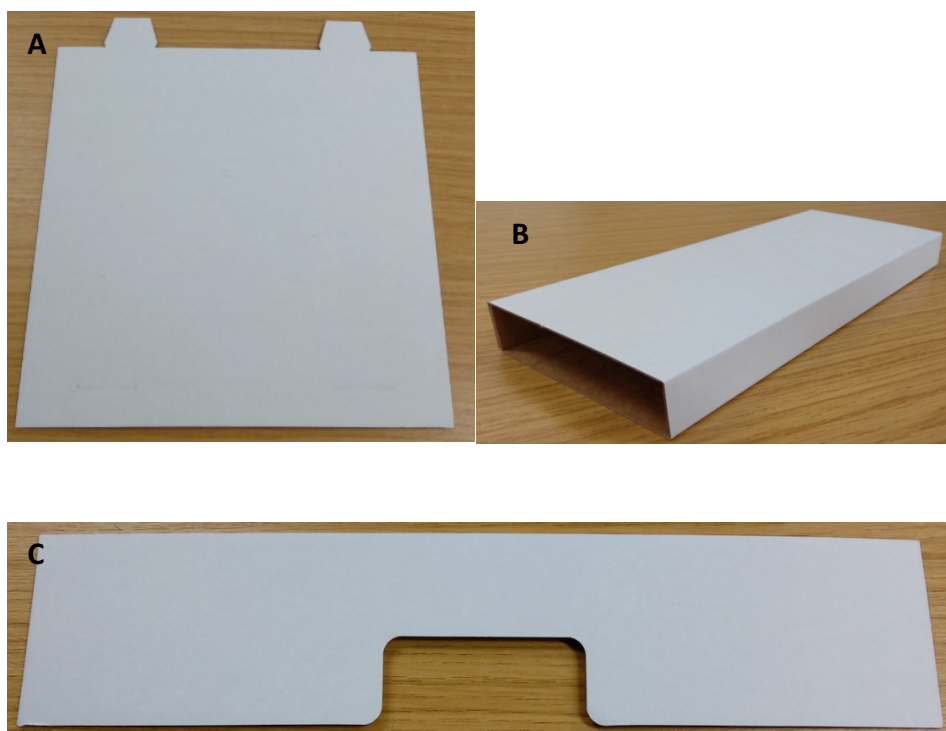
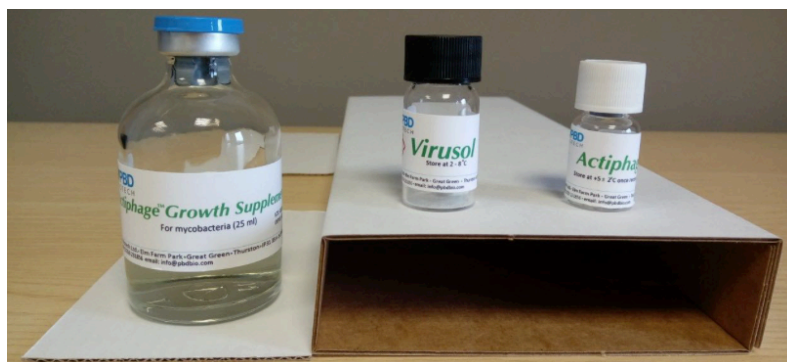


Figure 3.9 Details of Actiphage™ foldable board and flat board

Images showing the foldable board (A) unfolded and (B) folded and the flat approx. 1 mm thick board (C).



D Images showing the Actiphage™ vials placed on top of the card inserts.



The folded board when folded is approx. 3 cm high. This is placed inside the box, in the front and the flat approx. 1 mm thick board is placed immediately behind this and the vials are placed as shown.

Figure 3.10 Box design and final product

A) Actiphage™ box design

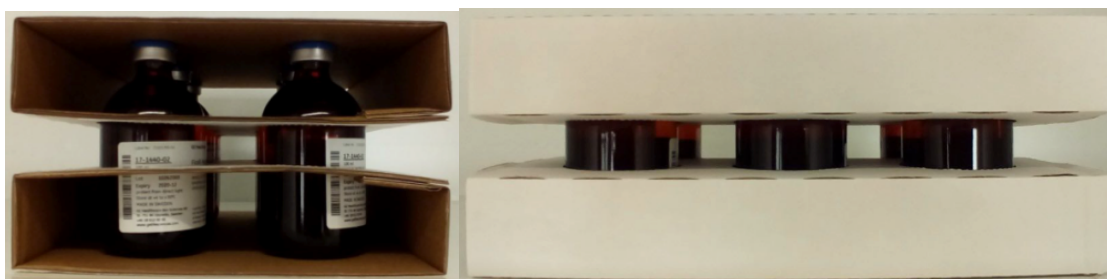


B) Actiphage™ inner tray and box C) Filled Actiphage™ kit

It was essential for the box graphic to be produced using vector images which, unlike bitmaps, are not based on pixel patterns, but they are realised using programmes that use mathematic equations and geometric primitives (points, lines and shapes) to create art that can be scaled infinitely, without any loss of quality or fidelity. Regarding the area for the product label, this was originally designed to be 102 (W) x 152 (L) mm (4" x 6") but was changed to be 152 (W) x 102 (L) mm (6" x 4"), (Fig. 3.13) because it was felt that it would be easier for the customer to read the product label if it is printed horizontally.

Once the box had been designed, it was filled with dummy components (the actual reagents were not available at this time), to allow it to be road tested by sending the boxes through the post. It was found that the sachets fell through the insert and ended up underneath the inner tray, so to solve this it was decided to change the design of the slot to be 55 mm (L) x 4 mm (W), to accommodate the sachets vertically rather than horizontally. Placing the sachets vertically, after transport, they remained in place. During the road test, it was also noticed that the 18 mm base diameter vials were loose, as on despatch, some were scattered all over the box. This problem was solved by slightly narrowing (in both the inner trays) the size of the slots in which these vials are placed. However, a more secure solution if this problem occurred again, would be the addition of a layer of soft foam to the underneath of the box lid, or including an “upside down” tray on top of the vials, to lock them in position as shown in Figure 3.11.

Figure 3.11: Proposed solution to vial movement during transport



The use of an “Upside down” tray to keep the vials in place – the vials in these two pictures are Ficoll Paque Plus – GE Healthcare which were used as dummy reagents during the road testing.

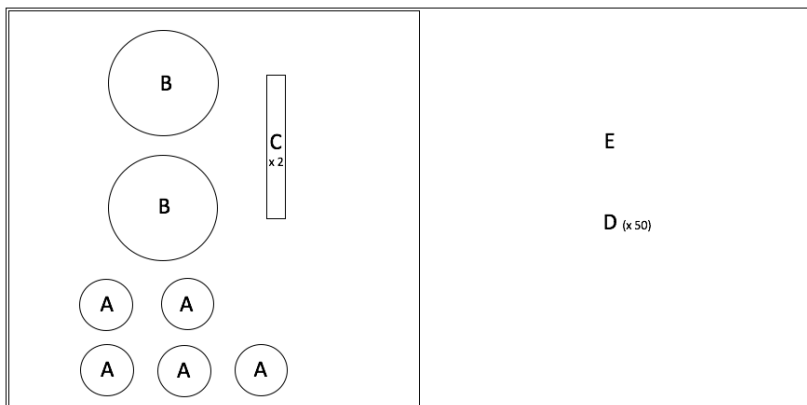
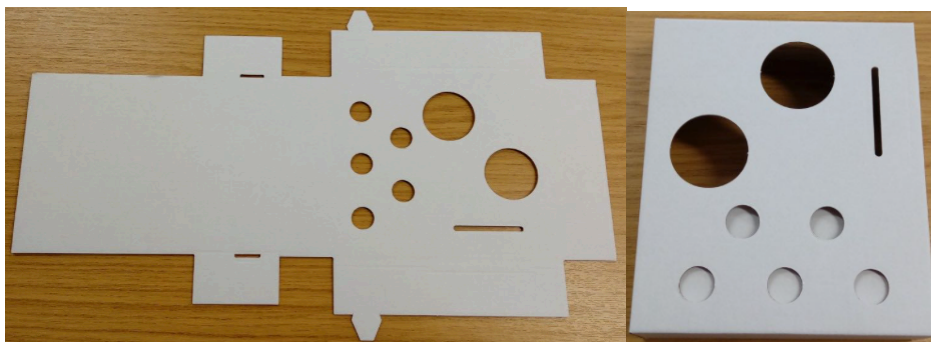
3.4.4 Design of Box and inner trays for ActiphageTM Rapid kits

The inner tray for the ActiphageTM Rapid kit (Fig. 3.12) was designed to accommodate 5 vials of ActiphageTM Reagent (A), 2 vials of ActiphageTM Growth Supplement (B) and 2 ActiphageTM Medium sachets (C). This inner tray was designed to be placed in the same box as before on the left hand side where it occupies only half of the box so that the other half can accommodate a zip-lock bag containing 50 Rapid Tubes (D). In each case these letters correspond to the positions shown in the diagram of the insert

design shown in Figure 3.12. Finally, the instruction for use (E) are placed on top of the other components. The same approach was used to ensure that the vials of different sizes sat at the same height, but in this kit the cardboard supports were part of the insert which was folded into the correct format to support the shorter bottles.

Figure 3.12 Actiphage™ Rapid box and inner tray design

A) Sketches of proposed Actiphage™ Rapid kit layout



B) Flat inner tray

C) Folded inner tray

D) Actiphage™ Rapid box and inner tray (picture on the left) and with kit with contents (picture on the right)



3.4.5 Design of labels

In order to be readily identified, each component of the kit needs to be appropriately labelled. In order to be effective, each label needs to display at least the name of the product or component, lot number, expiry date, storage temperature and any required hazard symbols. In addition, the label should include the PBD Biotech logo, the Company name and address and contact details. To allow all this information to be included some standard symbols and layouts are used. If the product or component is manufactured at PBD Biotech, there is a “Manufacturer” symbol  adjacent to the company name and address, otherwise it is not present. Lot number and Expiry date are positioned in the right-middle part of the label and are indicated by the symbols  and , respectively. Storage conditions are usually indicated by the symbol , or explicitly written. Additional information may include the number of tests the product or component is sufficient for (i.e. on the kits' labels), the weight or the volume of the content (i.e. on the Actiphage™ Agar, Actiphage™ Medium and Actiphage™ Growth Supplement labels), instructions on how to reconstitute the component (i.e. Actiphage™ Agar, Actiphage™ Medium labels).

The font chosen for the name of the product or component was ***Optima Bold Italic*** and the colour was the same shade of **green** as the middle square in the PBD biotech company logo . This colour was chosen to respect the company's colours and it was preferred over the shade of **green** of the bottom square in the logo because of its darker shade, that makes it easier to read the name of the product or component. It was also preferred over the **blue** so that the product or component's name on the label didn't clash with the PBD letters of logo. This shade of **blue** was used, alternated with the same shade of **green** as the product or component name, for the bullet points that separate the different parts of company address and contact details. The font chosen for all the other information is Calibri and the colour is black, with the font size being variable depending on the information and the size of the label. The choice of the label size was based on the size of the kit's component and the labels were chosen among

the ones offered available from a standard supplier (Primera). Images of the final labels produced for each of the kit components is shown in Figure 3.13.

The ActiphageTM Agar and the ActiphageTM Medium labels are printed directly on the sachet by the manufacturer, before filling. An informational label is printed on the back of the sachets (Fig. 3.5). This has the purposes of stimulating the customer to check the website to learn about the product available, and to not to leave one side of sachet blank, making the product more attractive.

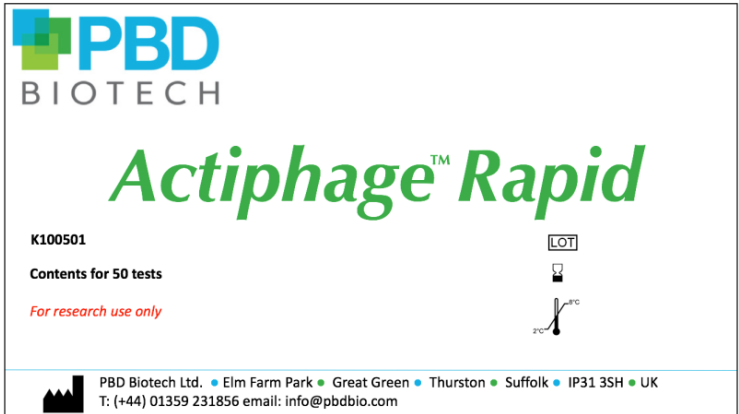
An issue encountered using the types of label chosen is that when storing the labelled products or components in the fridge, they pick humidity and wrinkle, making the look of the product or component less attractive. As this does not happen using gloss labels, these would be used in future production runs as a better option.

Figure 3.13: Examples of label designs

A) Actiphage™ Growth Supplement label (not to scale)



B) Actiphage™ Rapid label (not to scale)



C) Actiphage™ (freeze-dried phage) label (not to scale)



D) Virusol label (not to scale)



Future required development for the kits packaging includes adding additional holes in the insert for additional reagents. For instance, there are plans to include a vial containing a positive control strain to the inner trays for both kits and also adding 5 holes for the Sensor Cells (freeze dried *M. smegmatis* cells) vials to the Actiphage™ kit inner tray as shown in Figure 3.14. As the Sensor Cells vials are taller than the Actiphage™ and Virusol vials, but shorter than the Actiphage™ Growth Supplement vials (Fig. 3.15A), a new system for balancing the height of the different vials will need to be developed. A possible option could be reducing the area of the support obtained folding the foldable board, where the Actiphage™ and Virusol vials stand. This should create some space where to place a shorter support on which the Sensor Cells vials could be placed (Fig. 3.15C). Obviously, with the addition of the positive control the new system, along with the inner tray for Actiphage™ Rapid, will need to be further modified according to number and size of the positive control vials. Moreover, the insert needs to be amended if for some reason the number of one or more vials changes.

Figure 3.14 Draft plan for Actiphage™ kit inner tray with holes for Sensor Cells

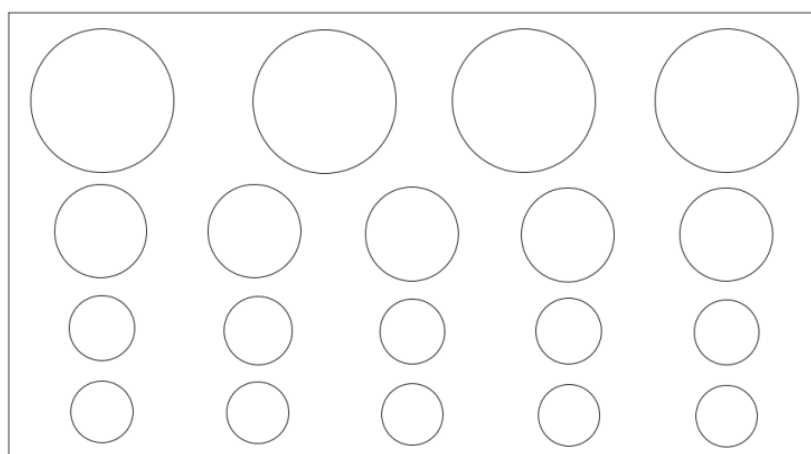
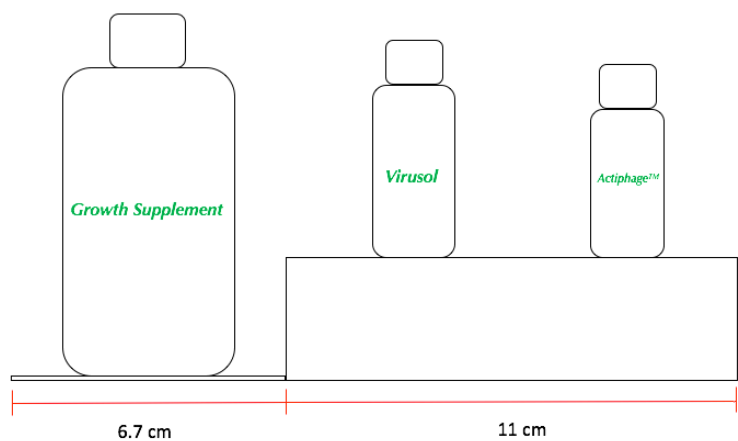


Figure 3.15 Details of proposed system to accommodate Sensor Cells vials

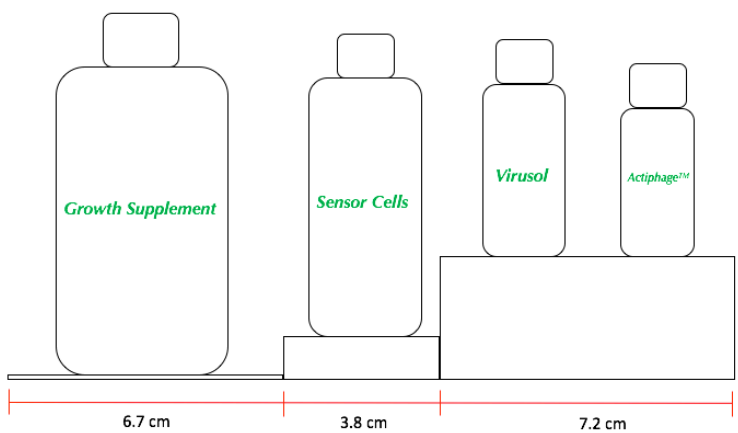
A) Image showing relative size of the proposed Sensor Cells vial



B) Current system for balancing the height of the different vials of the Actiphage™ kit



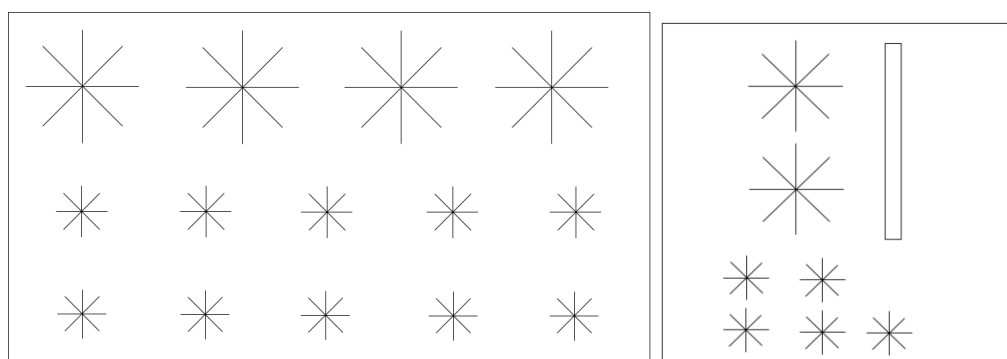
C) Draft Hypothetical new system of balancing the eight of the different vials of the Actiphage™ kit



Another issue that could occur is that, for some reason (i.e. product discontinuation), the size of one type of vial slightly changes (i.e. slightly narrower and taller, or slightly wider and shorter). The result of this would be the malpositioning of those vials in the inner tray. In addition, if the slots are too wide the vials will be too loose or, if the slots are too narrow, they would not fit the vial. This could make the inner trays useless, leading to a need to totally redesign the insert which would be a commercial waste.

Considering cardboard inner trays, a possible solution to this problem could be a system illustrated in Figure 3.16 in which the vial is pushed through the star shaped set of cuts, to produce a hole with flaps that is slightly wider (i. e. ≤ 1 mm) than the size of the vial that is normally used. The slightly bigger diameter of the holes would help if the diameter of the vial slightly increases, and the flaps would hold the vial in place in case the diameter of the vial slightly decreases.

Figure 3.16 Hypothetical inserts for Actiphage™ and Actiphage™ Rapid kits



Each star shape indicates where the card insert would be cut.

3.5 Discussion of kit development process

During the development of the kit components a number of technical issues were identified. For instance, the original idea for the packaging for the FAS tablets was to supply the tablets in a blister pack which would help address the fact that the FAS is sensitive to oxidation on storage. However, it was not possible to find a supplier who could make the tablets and supply them packaged in this way, and therefore they were packaged loose in packs but the tablets were found to be too soft to do this without them breaking down. It is known that fragile granulations may be due to an insufficient quantity of binder in which particles segregate easily. Including larger quantities of the granulating substance tend so to increase the hardness of the tablets it would be necessary to optimise the amount of the PEG 6000 added to the mixture. If this was done, a cost effective method would be needed to measure the hardness of the tablets produced, as this would provide another useful parameter that could be used as part of the quality assurance process, but at present a suitable apparatus to do this is not been identified.

However, an unexpected advantage of producing a softer tablet was that they dissolved very rapidly (less than 5 min) whereas the original FPTB Virusol tablets took more than 30 min to completely dissolve. It is possible that this softer tablet may affect the rate of oxidation of the tablets and therefore shelf life studies are in the process of being performed. To date the tablets have been shown to retain activity for more than 30 months.

One limiting factor that was discovered was that packaging companies have a limit on the lower amounts of powder that can be accurately dispensed into sachets. The volume of reconstituted ActiphageTM Agar that is necessary to perform 50 tests plus one positive and one negative control for each set of ten tests using the ActiphageTM kit is 360 ml (6 ml required per test). To achieve this volume, the content of one ActiphageTM Agar sachet, 2.6 g, needs to be reconstituted in 120 RO or distilled water, and therefore three sachets are provided with the kit. Ideally this would be presented as five ActiphageTM Agar sachets containing approximately 1.6 g of powder each, which would provide one sachet for each set of 10 reactions but this was not possible due to the fact

that the smallest fill weight that can be packaged by the current sachets manufacturer is 2 g. Therefore, in the future it would be worth trying to find a manufacturer that could package smaller fill weights. Further work also needs to be done on establishing the shelf life of Actiphage™ Medium sachets as at present this is determined by those given by the suppliers of the Middlebrook 7H9 Broth Base.

This same problem applied to the sachets of Actiphage™ Medium base, and this could also be addressed by identifying a sachet manufacturer that can make sachets containing less than 2 g of powder, and then adjusting the volume dispensed into the Actiphage™ Growth Supplement vials accordingly. While the cost of the agar and Actiphage™ Medium base are relatively small, the Actiphage™ Growth Supplement represents a major cost component of the kit and therefore it would be more economic to include smaller amounts of this in the kit. However, although the methods used to prepare the media are very standard for microbiological labs, other potential users of these kits may find that the media preparation steps are overly complicated. To address this problem another option could be to freeze dry the required volume of Actiphage™ Medium Plus, pre-aliquoted into glass vials which could then be reconstituted with the appropriate volume of sterile distilled water, and Actiphage™ Medium Plus would then be instantly ready for use. This would both simplify the steps required to use of the kits, as it would eliminate the needs of preparing and sterilising the medium base, with obvious saving of time and resources for the customer and would also help reduce overall costs as only the exact amount of reagent would need to be provided. A test was carried out to see if this approach was feasible, by freeze drying one 5 ml vial of Actiphage™ Medium Plus using the same parameters used to dry the Actiphage™ Reagent which is suspended in Actiphage™ Medium Plus before drying. The drying process was successful but there was excessing boiling of the liquid as the drying process was initiated. This was solved by degassing the liquid under vacuum until it stopped bubbling, before freeze drying. This produced a freeze-dried sample of Actiphage™ Medium Plus that was able to support good growth of *M. smegmatis*. This approach needs full evaluation but could really solve the problem of having to provide excess volumes of reagents. However, as this media would not be sterilised before used it would have to be aliquoted under very strict containment to ensure it is not

contaminated before freeze drying, and manually aliquoting small amounts into vials would be very labour intensive and therefore may require investment in robotics. Similarly, the current capacity of the freeze-drier is only approx. 150 x 3 ml vials per batch and therefore big batches of product cannot be made in one run and therefore further investment in new machinery would be required to make this a practical approach. However, as it is proposed to also provide vials of Actiphage™ positive controls and Sensor Cells in the future, the capacity of the freeze drier will have to be increased. Alternatively, the production of the freeze dried Actiphage™ Medium Plus could be subcontracted to a media manufacturer and since there are no viable microbes in this product it is easier to find suitable manufacturers.

It was pointed out by a customer that when the product is shipped, the shipping documents are attached to the top of the box, covering the product label. A solution to this problem could be packing the boxed product in a box provided by the shipping company. This could be also useful when sending multiple kits to a single customer.

The same customer also pointed out that the box could easily open during transit. If multiple boxes are sent within a box provided by the shipping company, this is probably not an issue. However, if sending single boxes this is something this needs to be addressed. This could be done for example by adding an adhesive and a tear off strip to the lid (Fig. 3.17) to both properly seal the box and to allow the customer to know when it has been opened.

Figure 3.17 Examples of adhesive and tear off strips for kit boxes



3.6 Price analysis

When developing new diagnostic tests, it is also important to understand the cost of market competitors. Currently the price of both ActiphageTM and ActiphageTM Rapid tests are £15.00 per test and the time to test result is 48 h and 6 h, respectively. To try and identify how this cost compared with competitive assays, a survey was carried out on all the different types of test for bTB infection that are currently available. This survey used information either freely available from websites or from information provided by the companies on request.

The series of tables presented below provides a list of international suppliers of tests for the detection of mycobacteria either by culture (Table 3.13), serology (Table 3.14), PCR (Table 3.15) and immunology (Table 3.16). As the number of tests for cattle is limited, the search was extended to include bTB tests for all species of animals (apart from humans) and also to include tests for other animal mycobacterial diseases such as Johne's disease. Since the ActiphageTM and ActiphageTM Rapid tests can be applied for the detection of Johne's disease as well as bTB infection (Swift *et al.*, 2013), and the types of tests are the same for both diseases, then it was also relevant to establish the costs of these tests as well and it also increased the number of individual tests that could be added to the analysis.

From this analysis, it was deduced that the average price reported in this price analysis for the mycobacterial culture is £33.43 and the average turnaround time is about 2 months. In contrast, the average price of a serology test from this price analysis is £7.65. Although considerably lower cost than the price of the kits for the phage assays, results can take up to 7 days and – as discussed in Chapter 1 – not all animals will give a positive test result at all stages of the disease. Indeed, for the Johne's disease milk ELISA tests it is generally accepted that 3 consecutive positive tests results are required before infection is identified, therefore the actual cost of diagnosis is £22.95, and if a blood ELISA is used there are additional costs associated with the veterinary visit to take the blood sample.

Regarding PCR, the average price according to this price analysis is £33.52 and the time to get a result could be up to 10 days and therefore these tests are both more expensive and take longer than the ActiphageTM and ActiphageTM Rapid tests. In addition, the test sample used for direct PCR is often faeces and these tests have a very high LOD due to the presence of inhibitors and dilution of the bacteria into a large sample volume. Finally, the immunological tests, such as γ -IFN, have an average price of £50.60 and the turnaround time according to this analysis of 3-4 days. Again, as discussed in Chapter 1, these tests are also affected by the disease progression and by other factors such as exposure to other mycobacterial infections.

From this analysis it seems that the test price of £15, the short time to test result and the fact that the test directly detects mycobacteria in either blood or milk rather than monitoring the immune response of the organism, means that the ActiphageTM and ActiphageTM Rapid tests are well placed in the market to provide a positive contribution to the diagnosis of bTB infections.

Table 3.13 Mycobacterial culture

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Biobest laboratories Ltd.	UK	Farm Animals			Routine bacterial culture & sensitivity (excluding faeces)	£30.00	
Biobest laboratories Ltd.	UK	Farm Animals		Milk	Milk bacteriology culture & sensitivity (milk 5+ samples)	£22.00	
Biobest laboratories Ltd.	UK	Farm Animals		Milk	Milk bacterial culture without sensitivity	£16.00	
Biobest laboratories Ltd.	UK	Farm Animals		Milk	Milk bacterial culture without sensitivity (5+ samples)	£10.00	
Biobest laboratories Ltd.	UK	Farm Animals		Faeces	Faecal bacterial culture & sensitivity	£32.00	
Animal & Plant Health Agency	UK	Various animal species	MAP		Liquid culture	£45.40	78 working days
Scotland's Rural College	UK	Cattle, sheep	MAP	Faeces/Tissues	Johne's MAP culture + PCR	£44.00	Up to 2 months
Scotland's Rural College	UK	Sheep	MAP	Faeces	Johne's MAP culture + PCR (pool of 10 faeces sample)	£75.40	Up to 2 months

Table 3.13 Mycobacterial culture continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Nation Wide Laboratories	UK	Cattle	<i>M. paratuberculosis</i>	Faeces	M. paratuberculosis culture		45+ working days
Diagnostic Laboratories Royal Veterinary College	UK		Mycobacteria		Mycobacteria culture	£90.50	
Animal Health Trust Diagnostic Laboratory Services	UK	Companion animals	Mycobacteria	Swab/Tissue	Mycobacteria culture and stain	£120.00	Up to 12 weeks
Animal Health Trust Diagnostic Laboratory Services	UK	Companion animals	Mycobacteria	Swab/Tissue	Mycobacteria fast track PCR, culture and stain	£160.00	Culture: up to 12 weeks PCR: up to 10 working days
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Clinical samples, fresh or fixed tissue			Contact
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	Johne's culture bovine - JCB	£30.16	35+ working days

Table 3.13 Mycobacterial culture continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Cornell University - College of Veterinary Medicine	USA	Non bovine	MAP	Faeces	Johne's culture non-bovine - JCNB	£30.16	12-16 weeks
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	Johne's culture pooled - JPCI	£22.10	35+ working days
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	Johne's culture pooled (non-NY)- JPCO	£35.22	35+ working days
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	Johne's culture pooled NYSCHAP - JPCN	£17.23	35+ working days
Cornell University - College of Veterinary Medicine	USA	Environment	MAP	Environmental samples	Johne's Environmental culture - JECI	£14.98	35+ working days
Cornell University - College of Veterinary Medicine	USA	Environment	MAP	Environmental samples	Johne's environmental culture non-NY - JECO	£32.21	35+ working days
Cornell University - College of Veterinary Medicine	USA	Environment	MAP	Environmental samples	Johne's Environmental culture, NYSCHAP - JECN	£8.62	35+ working days

Table 3.13 Mycobacterial culture continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Colorado State University	USA		<i>Mycobacterium spp.</i>	Faeces, intestine, lymph node, rectal biopsy, skin biopsy	<i>Mycobacterium species</i> solid culture	£26.22	Up to 16 weeks
CVR Laboratory	UAE		Mycobacteria		Mycobacterium culture only	£40.27	
Agri Food and Biosciences Institute	Northern Ireland		MAP	Faeces	Johne's faecal culture	£33.00	
Michigan State University	USA	Most species	Mycobacteria	Environmental, lung, lymph nodes	Mycobacterium culture	£54.43	60 days
Michigan State University	USA	Most species	Mycobacteria	Fluid	Mycobacterium culture - sterile	£22.47	7-56 days
Michigan State University	USA	Bovine, caprine, cervidae	<i>M. paratuberculosis</i>	Feces	<i>M. paratuberculosis</i> culture accelerated	£26.22	10-38 days

Table 3.13 Mycobacterial culture continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Michigan State University	USA	Bovine, caprine, cervidae	<i>M. paratuberculosis</i>	Faeces	<i>M. paratuberculosis</i> culture pooled fecal	£22.47	10-45 days
Michigan State University	USA	Bovine, caprine, cervidae	<i>M. paratuberculosis</i>	Faeces	<i>M. paratuberculosis</i> culture pooled followup	£17.23	10-45 days
Michigan State University	USA	Bovine, caprine, cervidae, ovine	<i>M. paratuberculosis</i>	Faeces	<i>M. paratuberculosis</i> culture routine	£20.97	10-45 days
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Faeces	Fecal culture	£12.73	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Faeces	Fecal culture (program testing)	£9.74	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Faeces	Pooled fecal culture	£14.98	

Table 3.13 Mycobacterial culture continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP		Culture/sample from positive pool	£12.73	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP		Culture/sample from positive pool (program testing)	£9.74	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP		Culture/sample from positive pool	£4.50	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP		Environmental culture	£12.73	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP		Environmental culture (program testing)	£9.74	
Average price per test						£33.43	

Table 3.14 Serology

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Biobest Laboratories Ltd.	UK	Cattle	MAP	Serum, plasma, whole blood, milk	Johne's ELISA	£4.80	3 working days
Biobest Laboratories Ltd.	UK	Cattle	MAP	Serum, plasma, whole blood, milk	Johne's ELISA (50+ samples)	£4.20	
Biobest Laboratories Ltd.	UK	Sheep	MAP		Johne's disease ELISA	£4.80	3 working days
Biobest Laboratories Ltd.	UK	Sheep (HiHealth Flockcare)	MAP		Johne's disease Ab ELISA	£4.00	
APHA scientific	UK	Cattle	<i>M. bovis</i>		Cattle TB antibody ELISA (IDEXX)	£9.30	10 working days
APHA scientific	UK	Camelid	<i>M. bovis</i>		Camelid TB antibody ELISA	£8.00	10 working days
APHA scientific	UK	Camelid	<i>M. bovis</i>		Camelid TB Vet DPP antibody test (Test type: Lateral Flow)	£16.60	2 working days
APHA scientific	UK	Badger	<i>M. bovis</i>		DDP Vt TB antibody test for badgers TB (Test type: Lateral Flow)	£16.60	2 working days

Table 3.14 Serology continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
APHA scientific	UK	Badger	<i>M. bovis</i>		DDP Vt TB antibody test for badgers TB (Test type: Lateral Flow)(5+ samples)	£13.60	
APHA scientific	UK	Cattle, sheep, goat	MAP	Serum	Johne's (<i>M. avium</i> subsp. <i>paratuberculosis</i>) serum antibody detection ELISA	£5.10	7 working days
APHA scientific	UK	Cattle, sheep, goat	MAP	Serum	Johne's (<i>M. avium</i> subsp. <i>paratuberculosis</i>) serum antibody detection ELISA (5+ samples)	£4.50	
APHA scientific	UK		MAP	Milk	<i>M. paratuberculosis</i> (Johne's disease) in individual milk (Ab ELISA)	£6.10	7 working days
Animal & Plant Health Agency	UK	Camelid	<i>M. bovis</i>		Camelid TB Vet DPP antibody test (Test type: Lateral Flow)	£16.00	2 working days
Animal & Plant Health Agency	UK	Camelid	<i>M. bovis</i>		Camelid TB Vet DPP antibody test (Test type: Lateral Flow) (5+ samples)	£13.10	
Animal & Plant Health Agency	UK	Badger	<i>M. bovis</i>		DDP Vt TB antibody test for badgers TB (Test type: Lateral Flow)	£16.00	2 working days

Table 3.14 Serology continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Animal & Plant Health Agency	UK	Badger	<i>M. bovis</i>		DDP Vt TB antibody test for badgers TB (Test type: Lateral Flow) (5+ samples)	£13.10	
Animal & Plant Health Agency	UK	Cattle, sheep, goat	MAP	Serum	Johne's (<i>M. avium</i> subsp. <i>paratuberculosis</i>) serum antibody detection ELISA	£5.00	7 working days
Animal & Plant Health Agency	UK	Cattle, sheep, goat	MAP	Serum	Johne's (<i>M. avium</i> subsp. <i>paratuberculosis</i>) serum antibody detection ELISA (10+ samples)	£4.50	
Animal & Plant Health Agency	UK	Cattle	MAP	Milk	<i>M. paratuberculosis</i> (Johne's disease) in individual milk (Ab ELISA)	£5.50	7 working days
Scotland's Rural College	UK	Cattle	MAP	Serum	(1-39 samples)	£4.80	Day after test
Scotland's Rural College	UK	Cattle	MAP	Serum	(40+ samples)	£4.20	
Scotland's Rural College	UK	Cattle	MAP	Milk		£5.00	Day after test
Pinmoore Animal Laboratory Services Ltd	UK		MAP	Serum		£11.00	
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Serum, Milk	Serum and Milk Antibody Assay (ELISA) 1-10 samples	£5.99	3-5 working days

Table 3.14 Serology continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Serum, Milk	Serum and Milk Antibody Assay (ELISA) 11-99 samples	£4.50	
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Serum, Milk	Serum and Milk Antibody Assay (ELISA) >100 samples	£3.75	
Washington State University	USA	Cow, Cattle, Sheep, Caprine, Goat Ovine	MAP	Blood, Serum, Milk	Johne's disease ELISA (Washington Clinets)	£3.75	2-7 days
Washington State University	USA	Cow, Cattle, Sheep, Caprine, Goat Ovine	MAP	Blood, Serum, Milk	Johne's disease ELISA (Non- Washington Clients)	£5.62	
Indipendent Milk Laboratories	Ireland		MAP	Milk, Serum	ELISA (Bulk Milk)	£6.67	Contact
Indipendent Milk Laboratories	Ireland		MAP	Milk, Serum	ELISA (Individual Milk)	£4.45	
Indipendent Milk Laboratories	Ireland		MAP	Milk, Serum	ELISAS (serum)	£4.45	
Irish Equine Centre	Ireland	Bovine	MAP	Blood, individual milk	ELISA	£3.20	
Cornell University - College of Veterinary Medicine	USA	Bovine, caprine, ovine	MAP	Serum, plasma	Johne's MAP commercial ELISA - JCOMZ	£3.75	3-4 working days

Table 3.14 Serology continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Cornell University - College of Veterinary Medicine	USA	Bovine, caprine, camelids, cervidae	MAP	Serum	Johne's AGID - JAGID	£8.87	2-4 working days
Cornell University - College of Veterinary Medicine	USA	Bovine, caprine, camelids, cervidae	MAP	Serum	Johne's AGID - JAGID (cervidae only)	£17.75	
Colorado State University	USA	Caprine, Ovine, Camelid	MAP	Serum	<i>Mycobacterium avium</i> spp <i>paratuberculosis</i> (Johne's) antibody AGID	£5.92	48 hours
Colorado State University	USA	Bovine	MAP	Plasma, serum	<i>Mycobacterium avium</i> spp <i>paratuberculosis</i> (Johne's) antibody ELISA (1-20 tests)	£4.07	Same or next day
Colorado State University	USA	Bovine	MAP	Plasma, serum	<i>Mycobacterium avium</i> spp <i>paratuberculosis</i> (Johne's) antibody ELISA (21-99 tests)	£2.96	
Colorado State University	USA	Bovine	MAP	Plasma, serum	<i>Mycobacterium avium</i> spp <i>paratuberculosis</i> (Johne's) antibody ELISA (100+ tests)	£2.59	
CVR Laboratory	UAE		MAP		<i>Mycobacterium paratuberculosis</i> (Johne's disease) for animals including camelids - Ab ELISA	£30.20	

Table 3.14 Serology continued

Service Provider	Country	Species	Organism	Sample	Test	Price per test	Turnaround Time
Agri Food and Biosciences Institute	Northern Ireland		MAP		Johne's (<i>M. avium</i> subsp. <i>paratuberculosis</i>) ELISA	£4.70	
Agri Food and Biosciences Institute	Northern Ireland		MAP	Milk	Johne's disease ELISA	£4.30	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Serum	Johne's disease (serum) ELISA (program)	£2.22	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Serum	Johne's disease (serum) ELISA (non-program)	£2.59	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Milk	Johne's disease (milk) ELISA (program)	£3.70	
Pennsylvania Animal Diagnostic Laboratory System	USA		MAP	Milk	Johne's disease (milk) ELISA (non-program)	£4.07	
Average price per test						£7.65	

Table 3.15 PCR tests

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Biobest Laboratories Ltd.	UK	Cattle	MAP	Faeces	£30.50	7 working days
Biobest Laboratories Ltd.	UK	Cattle	MAP	Faeces (5 faecal samples)	£36.50	7 working days
Biobest Laboratories Ltd.	UK	Cattle (health scheme)	MAP	Faeces (individual faecal sample)	£27.00	7 working days
Biobest Laboratories Ltd.	UK	Cattle (health scheme)	MAP	Faeces pooled faecal up to 5 samples)	£34.00	7 working days
Biobest Laboratories Ltd.	UK	Sheep	MAP	Faeces	£30.50	7 working days
Biobest Laboratories Ltd.	UK	Sheep	MAP	Faeces (5 faecal samples)	£36.50	7 working days
Biobest Laboratories Ltd.	UK	Sheep (HiHealth Flockcare)	MAP	Faeces (individual faecal sample)	£27.00	7 working days
Biobest Laboratories Ltd.	UK	Sheep (HiHealth Flockcare)	MAP	Faeces (pooled faece up to 5 samples)	£34.00	

Table 3.15 PCR tests continued

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Animal & Plant Health Agency	UK	Various animal species	MAP	(pool of up to 5 samples)	£31.60	10 working days
Scotland's Rural College	UK	Cattle	MAP	Faeces	£29.50	Day after test
Pinmoore Animal Laboratory Services Ltd	UK		MAP	Faeces	£42.00	
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Faeces	£23.67	5-7 working days
Johne's Information Center, University of Wisconsin - School of Veterinary Medicine	USA	Cattle	MAP	Faeces	£29.58	5-7 working days
Washington State University	USA	Cow, Cattle, Deer, Elk, Sheep, Caprine, Goat, Ovine, Camelid	MAP	Feces, Intestine (1-3 samples, Washington clients)	£33.28	1-7 days (average 4 days)
Washington State University	USA	Cow, Cattle, Deer, Elk, Sheep, Caprine, Goat, Ovine, Camelid	MAP	Feces, Intestine (1-3 samples, non-Washington clients)	£49.92	

Table 3.15 PCR tests continued

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Washington State University	USA	Cow, Cattle, Deer, Elk, Sheep Caprine, Goat, Ovine, Camelid	MAP	Feces, Intestine (4+ samples, Washington clients)	£25.88	
Washington State University	USA	Cow, Cattle, Deer, Elk, Sheep Caprine, Goat, Ovine, Camelid	MAP	Feces, Intestine (4+ samples, non- Washington clients)	£38.83	
Irish equine centre	Ireland	Bovine	MAP	Faeces	£50.00	
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	£27.18	7-10 working days
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	£14.05	7-10 working days
Cornell University - College of Veterinary Medicine	USA	Bovine	MAP	Faeces	£10.35	7-10 working days
Cornell University - College of Veterinary Medicine	USA	Bovine	<i>M. bovis</i>		£27.18	
Cornell University - College of Veterinary Medicine	USA		<i>M. avium</i> complex	Culture (solid or liquid) or isolate, tissue, swab	£27.18	3-5 working days

Table 3.15 PCR tests continued

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Colorado State University	USA	Cattle	MAP	Faeces	£22.18	Reported on Wednesday
MBG Lab	UAE		MAP	EDTA blood, tissue, stool, nasal/oropharyngeal swab, culture	£50.34	Within 5 working days
MBG Lab	UAE		<i>Mycobacterium species</i>	EDTA blood, tissue, stool, nasal/oropharyngeal swab, culture	£40.27	Within 5 working days
MBG Lab	UAE		MAP	EDTA blood, tissue, stool, culture	£50.34	Within 5 working days
MBG Lab	UAE		<i>Mycobacterium tuberculosis</i>	EDTA blood, Nasal swab, body fluids	£50.34	Within 5 working days
Agri Food and Biosciences Institute	Norther Ireland		MAP	Faeces	£28.00	
UC Davis Veterinary Medicine	USA	Bovine, Caprine, Ovine	MAP	Faeces (In state)	£18.71	2 weeks
UC Davis Veterinary Medicine	USA	Bovine, Caprine, Ovine	MAP	Faeces (Out of state)	£37.42	2 weeks

Table 3.15 PCR tests continued

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Pennsylvania Animal Diagnostic Laboratory System	USA		<i>M. paratuberculosis</i>	Faeces (individual PCR)	£12.57	
Pennsylvania Animal Diagnostic Laboratory System	USA		<i>M. paratuberculosis</i>	Faeces (pooled PCR)	£14.79	
Average price per test					£33.52	

Table 3.13 Immunology

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
Biobests	UK	Dogs	Canine TB	2 ml heparinised blood	£200	
Biobests	UK	Cats	Feline TB	2 ml heparinised blood	£200	Call for advice
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High specificity test, 1 - 5 samples)	£22.20	
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High specificity test, 6 - 9 samples)	£18.00	
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High specificity test, 10+ samples)	£17.00	
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High sensitivity test, 1- 5 samples)	£16.20	
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High sensitivity test, 6 -9 samples)	£13.00	

Service Provider	Country	Species	Organism	Sample	Price per test	Turnaround Time
APHA	UK	Cattle	<i>M. bovis</i>	Blood (High sensitivity test, 10+ samples)	£12.00	
UC Davis Veterinary Medicine	USA	Bovine	<i>M. bovis</i>	Blood	£36.97	
Michigan State University	USA	Bovine	<i>M. bovis</i>	Blood	£36.97	3-4 days
Nevada Department of Agriculture	USA	Bovine	<i>M. bovis</i>	Blood	£29.58	Next day
Fisher Scientific (IFN gamma Bovine ELISA kit; Research use only)	UK	Bovine			£5.31	
Average price per test					£50.60	

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 General discussion

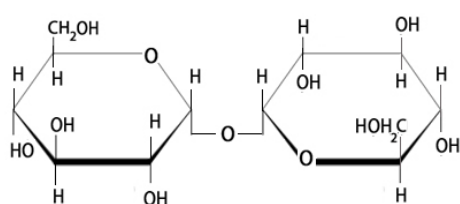
The aim of the work presented in this thesis was to investigate the practical challenges associated with developing a new test for bovine tuberculosis. This has included both practical work to establish new manufacturing processes and also research into the design and labelling of products and an investigation of appropriate pricing of product. Therefore, this MRes study has required research into a wide range of topics, from the theory of freeze drying to the legal requirements surrounding labelling of products.

However, the biggest practical challenge was to establish a method to prepare and freeze dry the D29 phage itself. Since phage are primarily composed of protein and nucleic acid, and are metabolically inert, they cannot repair themselves if damaged by physical processes. The method for production was based and tried and tested phage growth methods, but was optimised to allow high phage titres to be reproducibly achieved. Although the freeze drying process was successful, stability studies have shown that the product is not stable for long periods of time. To increase the shelf life of the freeze-dried phage, cryoprotectants such as trehalose could be added to the phage stocks before freeze drying.

Trehalose is a disaccharide composed of two glucose molecules bound by an alpha, alpha-1,1 linkage (Fig. 4.1). Since the reducing end of a glucosyl residue is connected with the other, trehalose has no reducing power. Trehalose is widely distributed in nature. It is known to be one of the sources of energy in most living organisms and can be found in many organisms, including bacteria, fungi, insects, plants, and invertebrates. Furthermore, trehalose protects organisms against various stresses, such as dryness, freezing, and osmotic pressure. In the case of organisms that can survive in a dry state, when the water evaporates, the organisms dehydrate as well. However, these dried organisms can successfully revive when placed in water or, as in this case, in an aqueous medium. The anhydrobiotic organisms are able to tolerate the lack of water owing to their ability to synthesize large quantities of trehalose, and the trehalose plays a key role in stabilizing membranes and other macromolecular assemblies under extreme environmental conditions (Higashiyama, 2002). Tests are currently underway to

determine if the addition of trehalose to the D29 phage stocks before it is freeze dried will extend the shelf life of the Actiphage™ reagent and also – more importantly – produce a room temperature stable reagent. As this is the only temperature sensitive reagent in the current kits, this is particularly important if the kits need to be transported over long distances for international sales or if they are to be used in laboratories in parts of the world where there is a lack of available cold chain for transport or storage.

Figure 4.1 Chemical structure of Trehalose



4.2 Conclusions

Having successfully developed the methods to produce the kit components, and also designed suitable packaging, the value of beta testing became apparent. Not only was it found that during transport the kit contents could become dislodged, feedback from customers also indicated that improvements could be made to the way that the kits were delivered. It is almost impossible to foresee all of these problems but generally the design of the kit boxes proved to be very flexible and adaptable to allow the different kits to be accommodated in one packaging system. In terms of business development this is important so that costs are kept to a minimum. However, this need for flexibility also led to a new idea for the packaging insert which will both be more flexible and also less vulnerable to changes in the specification of the vials available for freeze drying the reagents and therefore makes the packaging more resilient for future changes in kit content.

Similarly, once all of the kit components had been sourced and prepared, it was also clear which were the most costly elements. This led to a re-think about the original method chosen to deliver the media (sachets and liquid supplement) and has led to a

new idea about how best to provide the growth media provided in the kits that will simplify the preparation of media and make the kits accessible to a wider number of laboratories (i.e. not only laboratories that have the facilities for media preparation). Initial trials to freeze dry the ActiphageTM Media Plus were successful and this will also be a focus of future development work.

Finally, an analysis of the competitive tests revealed that the ActiphageTM tests are competitively priced, but the biggest advantage is the shorter time to test results and the fact that these tests can be applied for detection of a range of mycobacterial diseases, extending the market beyond just the diagnosis of bTB infections. This versatility, and the ability to use the test both for diagnosis of infection and food quality assurance analysis, gives the ActiphageTM system a real advantage over other more specialist tests.

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