New Phage-based Diagnostic Offers Hope for Improved Bovine TB Strategy

Introduction
Despite years of testing and culling, the numbers of cattle infected with bovine tuberculosis (bTB) do not appear to be reducing significantly. One of the issues behind this failing is that the testing uses a proxy – immune response – that does not directly detect the presence of the pathogen *Mycobacterium bovis (M. bovis)*. This can miss animals with low levels of infection, causing a reservoir of disease to remain in the herd and the immediate environment.

Detection is further hindered by the biology of Mycobacterium – which are slow growing and protected by a resilient outer cell wall – and also by its pathophysiology: the bacteria can enter a latent phase, avoiding the host's immune system, before symptoms are evident.

In March 2020 the UK Government issued its response to the 2018 independent review into bovine TB in the UK, led by Prof Sir Charles Godfray. The response from the Department for Environment, Farming and Rural Affairs (DEFRA) highlights the need for improved cattle testing and the development of cattle vaccination.

However, if this strategy is to be successful then a different approach to testing is required that can accurately and sensitively identify the presence of *Mycobacterium bovis* in infected cattle.

This article considers how phage-based detection can address both these requirements. In addition to providing an improved diagnostic of *M. bovis* infections in cattle, it also offers the promise of an accurate test for TB in humans and other species.

Bovine TB and Johne's Disease are Economically Devastating
Tuberculosis in livestock has a devastating economic impact. In the UK alone, 44,115 cattle were culled in the year to November 2019[1] with a cost of £150 million. Although the majority of cattle culled are pre-symptomatic, bTB can cause weakness, fluctuating fever, dyspnoea, an intermittent hacking cough and diarrhoea.

The disease is caused by members of the genus Mycobacterium. This includes: *Mycobacterium bovis (M. bovis*), which causes bTB, and *Mycobacterium avium* sub-species *paratuberculosis* (MAP), which presents as Johne's disease.

Johne's disease is a wasting disease that adds a substantial welfare and economic burden, conferring thousands of pounds in treatment costs for an infected herd[2].

In addition to the huge economic loss, both diseases also have the potential to harm humans. More than 100,000 people worldwide are infected by *M. bovis* each year, where the route of transmission is thought to be primarily by drinking infected milk; this figure is thought to be an underestimate due to lack of surveillance or accurate diagnosis from many countries[3]. MAP has also been associated with development of Crohn's disease in humans[4], a chronic and life-altering inflammatory bowel disease.

Mycobacteria Elude Detection and Identification
Mycobacteria have a thick waxy cell wall that makes them resilient to environmental stresses. They can also be very slow growing, where *M. bovis* and MAP take many weeks to culture. Mycobacteria have also evolved the ability to evade their host's immune system, enabling it to avoid detection and complicating vaccine development efforts.

Although the progression of the disease in animals is less well known, it is thought that its pathophysiology is similar to that of human TB, where there is a subclinical phase of infection in the body prior to clinical symptoms manifesting.

Mycobacteria also have the ability to avoid the killing action of the host's immune system by residing within macrophages and persisting there avoiding the host's immune system.

Clinical diagnosis during this subclinical infection – latent stage of infection is crucial to enable an intervention before the disease progresses further.

Once active tuberculosis infection takes hold, the animal begins shedding mycobacteria primarily via microscopic airborne particles. In the case of MAP, mycobacteria are shed in faeces and through the milk of infected animals, creating the risk of infection to calves.

Current Bovine TB tests lack sensitivity

In the UK cattle are screened in order to mitigate disease spread. However, this has proven to be ineffective, as bTB has remained endemic in many herds for years.

The primary official test to confirm the herd has an officially tuberculosis free status (OFT) is the single intradermal comparative cervical tuberculin test (SICCT). The test involves an intradermal injection of tuberculin, or parts of killed M. bovis, and observation of the resulting local immune response, as measured by an increase in thickness of the surrounding skin area. This immune response takes three days to develop.

The UK Government estimates the accuracy of the SICTT skin test to be:

- **Specificity** – the ability of the SICCT test to correctly reject healthy animals that don't have bovine tuberculosis is 99.98%, although in some settings the presence of related mycobacterial species may reduce this figure.
- **Sensitivity** – refers to the ability of the test to correctly identify those animals with the disease. For the SICCT test this varies between 50 and 90%[5] depending on the conditions under which it is carried out.

Therefore, based on these figures, in one hundred bTB-infected cows, the SICTT test could miss up to 50 of them; leaving them behind in the herd to infect others.

Another problem of the skin test is that it measures the immune response of the animal, rather than detecting the presence of the mycobacteria and actual infection.

Infection with other mycobacteria, such as MAP, may interfere with the SICCT test, producing false positive results, due to the close relationship between the mycobacteria that can infect cattle. Indeed, this is the same problem with vaccinating cattle, as vaccine strains of mycobacteria can cause the false positives using the SICCT test, as this cannot differentiate between vaccinated and infected cattle. Conversely, animals in an early stage of infection which have not yet developed a strong immune response will be missed. The limitations of the SICCT test were acknowledged in the Godfray Review[6], which observed that reliance on the SICCT alone is not advisable, with the recommendation to combine it with others in order to cover the gaps.

Other tests, validated for usage by the World Organisation for Animal Health (OIE), are the interferon gamma test and the IDEXX ELISA serological test.

The government in its review is committing to improving diagnostic testing to root out bTB more effectively. It proposes deployment of more sensitive tests for surveillance supported by greater use of on-farm restriction of cattle with inconclusive test results.

Although the policy paper is not specific about the approaches it will be taking to testing, a promising technique for reducing the time for diagnosis and improving the accuracy of identification even at low levels of infection is through the use of a bacteriophage assay.

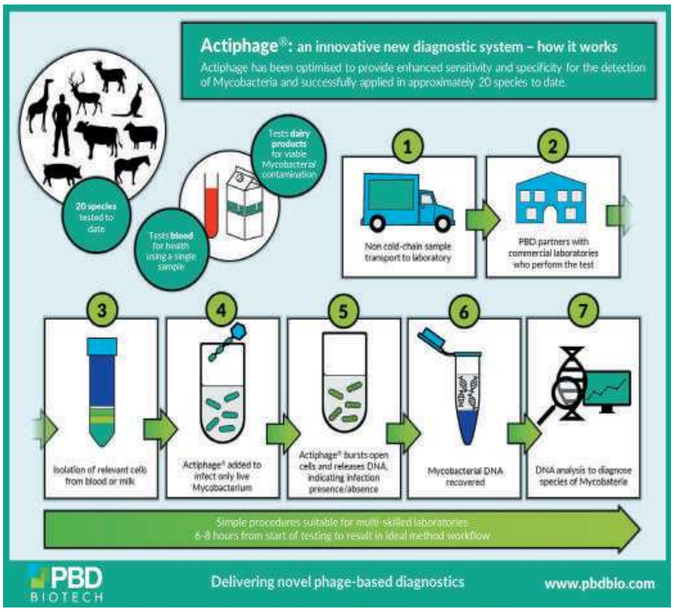
Direct detection of live mycobacteria with a phage test offers an alternative

Bacteriophages are viruses that infect bacteria, replicating inside them and then breaking them open, to release their progeny. This lysis also releases host genomic DNA.

Although direct detection methods of mycobacteria through polymerase chain reaction (PCR), for example, can be accurate, there are difficulties in getting efficient lysis of mycobacteria (due to their thick cell wall), reducing the sensitivity of methods such as PCR.

The solution to this extraction issue lies with phage, the natural enemy of the bacteria. Bacteriophage D29 has been found[7] to target a wide range of mycobacteria and can be used to efficiently break open mycobacteria, releasing its DNA for detection using PCR. This lysis is so efficient that it makes it possible to detect extremely low levels of mycobacteria from clinical blood or milk samples.

The Actiphage® method[8] uses this bacteriophage to lyse the mycobacteria cells in a liquid system. It has been shown to offer the potential for rapid, inexpensive, sensitive detection of live mycobacteria in high-throughput format, reducing the time to detection of the slow growing mycobacteria from months to hours.



The Actiphage test, first developed by scientists at the University of Nottingham[9] and now commercialised, has been evaluated recently against bovine Skin Test status as a comparator[10].

The survey, using Actiphage on 41 SCCIT-positive UK cattle tested at slaughter, found live M. bovis in the blood of all animals with visible lesions 13/13 and in 93% (26/28) of animals with no visible lesions (NVL) following post-mortem examination.

In contrast, no mycobacteria were detected in blood samples from 45 control animals; a high-biosecurity status herd known to be disease-free.

This study confirms that relative to the skin test Actiphage has a sensitivity of 93% and a specificity of 100%. Further trials are required to fully validate the test.

APHA has developed new protocol to manage bTB
The new diagnostic has recently been included within the Animal and Plant Health Agency (APHA) protocol, which allows "exceptional private use of non-validated tests for TB on cattle in England"; this has since been extended also to Wales.

Within this protocol, APHA has outlined a number of non-validated tests that may be proposed for exceptional use in cattle herds with chronic breakdowns, including:

the Actiphage assay, developed by PBD Biotech and the University of Nottingham; the Enferplex TB serological test, produced by Enfer Laboratories Ireland and approved in GB for use in camelids; and the Polymerase Chain Reaction (PCR) testing of faecal samples.

This enables any private veterinary surgeon to request APHA permission for exceptional use of non-validated tests, subject to certain criteria.

Successful disease control using phage test
This new APHA protocol was released after a successful trial of the phage test as part of a private TB eradication plan by vet Dick Sibley on Gatcombe Farm in Devon, UK. Using Actiphage as part of a disease management strategy, he discovered that infection was being maintained in the herd and not identified by the statutory testing.

In addition, there was potential for reinfection from environmental sources such as faecal shedding from cattle.

Extending the test to humans and other animals
A number of trials are currently in progress to evaluate the effectiveness of the Actiphage method for other mycobacterial diseases, such as Johne's Disease or MAP and also its potential for other species including in bison[11], other animals[12], and also people[13].

Combined forms of TB killed 1.5million people last year, and it is estimated that around a third of the world's population is latently infected[14].

Actiphage provides the capacity to detect the pathogen in a blood sample, which potentially allows the disease to be picked up as part of routine blood testing.

A recently published study looked at TB blood testing evaluated the Phage blood test across 66 participants in four groups: active pulmonary TB sufferers; those with latent TB; a control group of patients referred for suspected TB but found not to have the disease; and a control group of healthy individuals.

Each subject was tested twice, 12 months apart. Actiphage tested positive in 73% of people that were subsequently diagnosed with TB – for an experimental study, this was a much higher level than expected. None of the participants in the control groups tested positive with Actiphage and none of the patients with latent TB who tested negative went on to develop active TB.

Intriguingly, two of the three participants with latent TB infection who tested positive with Actiphage went on to develop the disease more than six months later, suggesting the test may have a predictive role in identifying people with the infection at risk of developing the disease.

Way forward

Tuberculosis is truly a cross-species disease and mitigating its impact requires cross-species solutions and engagement from the veterinary and medical communities, hand-in-hand.

The World Health Organisation has recently published its roadmap for dealing with zoonotic TB transmission, recognising that better surveillance and diagnosis are key steps to mitigating the disease.

The phage-based TB test promises decreases in testing time, cost and complexity over current TB tests. A major benefit is that the same technology can be applied to the disease in humans and animals equally easily. It is hoped this will allow the technology to scale more effectively, decreasing costs and improving disease management across the board.

REFERENCES

1. https://www.gov.uk/government/statistics/latest-official-statistics-on-tuberculosis-tb-in-cattle-in-great-britain-monthly
2. D. Radia, K. Bond, G. Limon, S. van Winden and J. Guitian. 2013. Relationship between periparturient management, prevalence of MAP and preventable economic losses in UK dairy herds. Vet Record; 173(14). http://dx.doi.org/10.1136/vr.101408
3. https://www.who.int/tb/zoonoticTB.pdf
4. Naser, S.A., Sagramsingh, S.R., Naser, A.S., and Thanigachalam, S. (2014) Mycobacterium avium subspecies paratuberculosis causes Crohn's disease in some inflammatory bowel disease patients. World J Gastroenterol 20: 7403–7415.
5. https://www.daera-ni.gov.uk/articles/bovine-tuberculosis-tb-testing
6. Bovine TB Strategy Review, March 2020: https://www.gov.uk/government/publications/a-strategy-for-achieving-bovine-tuberculosis-free-status-for-england-2018-review/bovine-tb-strategy-review-summary-and-conclusions
7. Benjamin M. C. Swift, Thomas W. Convery & Catherine E. D. Rees (2016): Evidence of Mycobacterium tuberculosis complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA, Virulence, DOI: 10.1080/21505594.2016.1191729
8. B. M. C. Swift et al, 'The development and use of Actiphage® to detect viable mycobacteria from bovine tuberculosis and Johne's disease-infected animals', Microbial Biotechnology (2019)
9. Benjamin M. C. Swift, Thomas W. Convery & Catherine E. D. Rees (2016): Evidence of Mycobacterium tuberculosis complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA, Virulence, DOI: 10.1080/21505594.2016.1191729
10. B. M. C. Swift et al, 'The development and use of Actiphage® to detect viable mycobacteria from bovine tuberculosis and Johne's disease-infected animals', Microbial Biotechnology (2019)
11. https://news.usask.ca/articles/research/2020/canadian-scientists-first-to-test-novel-diagnostic-tool-for-tb-in-bison.php
12. https://www.pbdbio.com/news/new-test-delivers-early-identification-tb-exotics/
13. Verma R et al. 2020. A Novel, High-sensitivity, Bacteriophage-based Assay Identifies Low-level Mycobacterium tuberculosis Bacteremia in Immunocompetent Patients With Active and Incipient Tuberculosis. Clinical Infectious Diseases, Volume 70, Issue 5, 1 March 2020, Pages 933–936, https://doi.org/10.1093/cid/ciz548
14. World Health Organization. Global tuberculosis report 2015. World Health Organization; 2015

Dr. Ben Swift

Dr. Ben Swift BSc (Hons), MRes, PhD is R&D Director for PBD Biotech and a Research Fellow in Antimicrobial Resistance at the Royal Veterinary College. During his PhD at the University of Nottingham, Ben developed novel diagnostics for mycobacterial diseases, based on using bacteriophage. This technology was successfully patented and later commercialised through PBD Biotech. He continues this work developing novel technology to further to aid the diagnosis of mycobacterial diseases in human and veterinary settings. At RVC Ben is involved in researching drivers of antimicrobial resistance (AMR) in the environment, to address the potential risks in the spread of AMR, both in the UK and in low-middle income countries.