

# **What is the scope for existing (including recently developed) diagnostic methods to detect infected cattle which are not currently detected by the existing programme?**

## **TB Scientific Working Group**

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### **1 Background information on the bovine tuberculosis testing programme in Ireland and the diagnostic tests currently available**

1. Diagnostic tests have both biological and operational considerations. The tests must be highly effective in detecting infected animals and in differentiating infected from non-infected animals. They must also be sufficiently simple and cost-effective to be applied in the field or laboratory. The primary aim of the Bovine TB eradication programme in Ireland is to identify animals infected with any member of the *Mycobacterium tuberculosis* Complex (MTBC), (primarily *Mycobacterium bovis*, *Mycobacterium caprae* and *Mycobacterium tuberculosis*) followed by isolation and removal of those animals. Identification of infected animals involves an annual screening of all cattle herds and prompt removal of test positive animals (reactors) [1]. Following the detection of a positive reactor animal, the herd is restricted from trading, except under permit (and generally then only direct to slaughter). Movement restrictions continue until two consecutive negative herd tests are achieved, approximately 60 days apart. Following derestriction, a herd test is repeated after 6 months.

#### **1.1 Primary Diagnostic Tests used in Ireland**

2. Screening, of the cattle population, in Ireland is carried out using the Comparative Intradermal Tuberculin Test (CITT<sup>1</sup>) in compliance with Commission Delegated Regulation (EU) 2020/689. Intradermal injections of 0.1 mL of avian tuberculin and 0.1 mL of bovine tuberculin are administered, each at an unblemished site, in the mid-third of the neck using a McLintock tuberculin syringe; the skin thickness at the site of the injection is recorded at the time and at test reading 72 hours [ $\pm 4$  hours] later. The nature of any reaction and the relative increase (measured in millimetres) in skin fold thickness at each injection site is evaluated at test reading. The basis for the CITT is that the injection of tuberculin purified protein derivative (tuberculin PPD, bovine or avian), into the skin of an animal infected

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<sup>1</sup> A number of different abbreviations are used in the scientific literature for the Comparative Intradermal Tuberculin Test, including SICTT, SICCT and SCITT. In this document, the abbreviation CITT will be used in line with Commission Delegated Regulation (EU) 2020/689. Following the same approach, the abbreviation SITT will be used for the Single Intradermal Tuberculin Test.

with MTBC causes an animal to produce a predominantly T-cell-mediated immune reaction known as a delayed-type hypersensitivity (DTH) response.

Two interpretations of the CITT are used in Ireland: an animal is defined as positive under the **standard interpretation** if clinical signs such as oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts are observed or the bovine reaction is positive ( $\geq 4$  mm) and exceeds the avian reaction by more than 4 mm. An animal is positive under the **severe interpretation** if no clinical signs are observed and the bovine reaction is either positive ( $\geq 4$  mm) or inconclusive ( $> 2$  mm and  $< 4$  mm) and exceeds the avian reaction. The standard interpretation is routinely used in herds with a bovine TB-free history. The **severe interpretation** is used in infected herds or herds at higher risk for bovine tuberculosis. Under the testing protocol used in Ireland, the testing veterinarian is advised to change to the severe interpretation when one animal in a herd is positive to the standard interpretation in a previously clear herd. The CITT is also the main *ante-mortem* test in Northern Ireland and in other parts of the UK [2].

3. In many countries in Europe and elsewhere, a Single Intradermal Tuberculin Test (SITT) is used. This involves a single injection with bovine tuberculin in the neck. In the USA, Australia and New Zealand, a variation of this test, known as the Caudal Fold Test, is used as the screening test where the bovine tuberculin is injected into the caudal fold near the tail base rather than into the neck. In Ireland, a comparative test involving both avian and bovine tuberculin is used because various pathogenic mycobacteria e.g. *Mycobacterium paratuberculosis* subsp. *avium*, non-pathogenic environmental mycobacteria such as *M. hiberniae*, and a variety of other organisms such as *Nocardia* species, which share antigens with members of the MTBC, are abundant in the Irish environment and may cause non-specific sensitization to bovine tuberculin [3,4]. If the test was based only on bovine tuberculin, a considerable number of Irish cattle would be recorded as positive. In one study, tests were undertaken on 7,735 cattle in 236 attested herds in 17 counties; 7.8% of animals gave positive reactions to the single intradermal test. Only 0.34% of animals were positive to the comparative test [5].

In 2014, data on the herds initially selected for the 2008 ‘Greenfield study’, which focussed on TB prevalence in badgers in areas of low TB prevalence in cattle, were re-examined. The ‘Greenfield study’ herds had been selected on the basis that there was a relative absence of evidence of MTBC in the herds and in the broader locality during the five years prior to 2008. The selected herds were then tested by DAFM staff during 2008 and those that were TB-free (no reactors and no inconclusive reactors to the CITT) were the herds included for the ‘Greenfield study’. The re-evaluation of the herds in the ‘Greenfield study’ selected only those that continued to remain TB-free up to the end of 2010. The 127-herds that met this criterion were adjudged to have had the lowest possible risk of having any SITT positive response (a recorded increase of 3 mm or more) in 2008 being actually due to MTBC exposure. When the results from the 2008 DAFM tests in these 127 MTBC-free-herds were examined, they showed that 6.3% of cattle had a positive response to the SITT and 44.5% of herds had at least one positive responder to the SITT (M. Good, personal communication). Data analysis, presented in 2014 at the VI<sup>th</sup> International *M. bovis* Conference on high risk herd-test results collected during “clear” CITT tests from 2009 and 2012 also showed similar findings, i.e. 44% and 47% of herds respectively had at least one animal responsive to the SITT [6]. This shows that the profile of responses, to bovine

tuberculin PPD, in TB-negative animals/herds has not changed in the years between the O'Reilly and McClancy [5] CITTs performed in 1975 and those from CITTs performed in more recent years.

4. The CITT and the SITT are prescribed *ante-mortem* diagnostic tests permissible for use in the EU to determine TB status in cattle (Commission Delegated Regulation (EU) 2020/689). The World Organisation for Animal Health (OIE) designates the CITT and the SITT together with the Caudal Fold Test as the prescribed tests for international trade. To ensure that these tests can perform to their maximum potential, both the OIE and the EU have set standards for how the tests need to be conducted, the interpretation of the tests, and also the tuberculin used, including the setting of minimum tuberculin potency attributes [1,7].

## 1.2 Ancillary Tests Used in Ireland

5. The BOVIGAM® interferon-gamma (IFN- $\gamma$ ) assay, a blood test supplied by Thermo Fisher Scientific, is used as a supplementary test in conjunction with the CITT in severely infected herds or in groups of animals in Ireland and other countries [3,8–12]. As with the aforementioned skin tests, it is also based on the DTH response to infection with MTBC and thus a high correlation is expected with animals responding to the CITT. The test is targeted at herds with a high probability of containing infected animals or at those herds chronically infected over a number of years. In Ireland, the animals that are tested are predominantly those from breeding herds with 5 or more positive animals (or 5% of large herds) (DAFM, personal communication). The IFN- $\gamma$  assay is not suitable as a screening test because it identifies a relatively high number of false positive animals. The OIE includes the IFN- $\gamma$  assay as an alternative test for international trade. This implies that the tuberculin skin tests (CITT, SITT or Caudal Fold Test) are considered optimal and that the IFN- $\gamma$  assay can be used for international trade where there is mutual agreement between the importing and exporting country [12]. The IFN- $\gamma$  assay is also a prescribed test under Commission Delegated Regulation (EU) 2020/689.
6. Serological tests to detect humoral antibody response offer an alternative support test for screening livestock for MTBC infection. The antibody-specific response to TB infection is generally regarded as a 'late stage' response by the host (in this case cattle) meaning that it develops later along the infection process and lasts longer than the DTH-based tests such as the CITT, the SITT and the IFN- $\gamma$  assay. These latter tests are more sensitive at detecting animals at an earlier stage after infection [13]. In Ireland, serological tests are therefore targeted at MTBC chronically infected herds.

Several antigens have been described as potential specific diagnostic targets for MTBC antibodies (e.g. ESAT-6, CFP-10, MPB70 and MBP83), although antibody response is mainly restricted to *M. bovis* antigen MPB83 in cattle [14–17]. The combination of diagnostic tests based on the skin test response against MTBC, together with serological diagnosis potentially increases the level of TB detection [18]. An enhanced sensitivity of antibody detection is evident in cattle when blood samples are taken after performing the CITT or SITT [19–21]. This is known as an anamnestic effect and is due to the fact that prior skin testing of an infected animal sensitises the immune system giving rise to higher MTBC-specific antibody levels in the blood than would be the case where skin testing is

not carried out. The application of an anamnestic antibody test is optimally applied to known infected herds in which the presence of one or more anergic (non-responsive), clinically normal appearing, animals is suspected. This may occur, for example, where there is ongoing disclosure of CITT reactors into or past the third reactor retest despite accurate performance and severe interpretation of the CITT (DAFM, personal communication).

One commercially available antibody test is the IDEXX indirect ELISA test [22,23]. The IDEXX indirect ELISA test is approved by the OIE as an ancillary test but it is not approved by the EU. Consequently, animals removed solely on basis of ELISA testing, under the TB eradication programme, may not be eligible for any co-funding from the EU Commission unless they confirm as MTBC positive.

7. A number of methods are used to diagnose tuberculosis in wildlife in Ireland. *Post-mortem* examination and culture are useful methods for disease surveillance, but immunological diagnostic tests based on cellular and humoral immune response detection are gaining importance in wildlife TB diagnosis. Detailed information on this area can be obtained from a review carried out by Thomas et al. [24].

### **1.3 Tests that are currently not used in Ireland**

8. The Enferplex indirect chemiluminescent multiplex serological test, developed by Enfer Scientific, also detects antibodies to individual defined *M. bovis* antigens in blood samples [25–27]. This test is approved by the OIE as an ancillary test but it is not approved by the EU. Trialing of the test is currently underway in Ireland (DAFM, Personal Communication). It could potentially play a similar role as the IDEXX indirect ELISA test in the TB Eradication Programme.
9. Lateral-flow assay (LFA) rapid tests based on immunochromatographic techniques have also been developed and evaluated for different species and some are commercially available [15, 28–34]. The LFA devices offer many advantages since they are easy to perform, stable at room temperature and are not logistically demanding. The tests are biased towards detecting late infection and for this reason, it is unlikely that they would be suitable for use as a screening test, but they could potentially play a role as ancillary tests. These tests are currently not approved by the OIE or by the EU.
10. The Lymphocyte Proliferation Assay is a blood test that compares the reactivity of peripheral blood lymphocytes to tuberculin [35–37]. This test has scientific merit for research purposes but is not used for routine diagnosis because the test is time-consuming and requires specific laboratory equipment and expertise. The test is also relatively expensive. This test is currently not approved by the OIE or by the EU.
11. The advent of DNA-based tests in the form of the Polymerase Chain Reaction (PCR) has promised to herald a new era in diagnostic technology. The application of PCR for bovine tuberculosis requires a bacterial DNA target sequence to be present at minute or higher concentrations. Such a target might be found in sputum or faeces of heavily infected animals. However, with the current approved surveillance systems in place that facilitate the diagnosis of infected cattle at an early stage of the disease, cattle are rarely clinically diseased to the extent that

they persistently shed *M. bovis* [20]. The low diagnostic sensitivity of the PCR test when used directly on clinical samples limits its usefulness in cattle, as the majority of these infected animals are readily identified by using the skin test and/or the IFN- $\gamma$  assay. Current developments in PCR technology are focused on speeding up the processes for confirmation of *M. bovis* infection in post-mortem samples, as an alternative to microbiological culture which can take up to three months to complete [38–40].

12. The Actiphage test uses a virus that infects bacteria (a bacteriophage) to detect *M. bovis* in the blood and milk of infected animals, allowing the DNA released from the *M. bovis* to be detected [41]. In this test, a phage that is specific for mycobacteria and only replicates in live or viable mycobacteria is used. It was developed by the University of Nottingham and is marketed by PBD BioNTech. It is currently not validated or approved by the OIE or by the EU.

In summary, the Enferplex Bovine TB Antibody test is approved by the OIE and could potentially be used to supplement or replace the IDEXX indirect ELISA test. Some of the other tests such as the LFA rapid tests are easy to perform in the field and could potentially play a role as a diagnostic test while others such as the Lymphocyte Proliferation Assay and the PCR tests are likely to have more limited use. Apart from the Enferplex Bovine TB Antibody test, none of the other tests are fully validated or approved by the OIE and none are approved by the EU. Consequently, they are currently not suitable for use in the TB Eradication Programme in Ireland.

## 2 The accuracy of diagnostic tests

13. When it comes to reporting the accuracy of diagnostic tests, regardless of disease or species, there are two key considerations, namely sensitivity and specificity:

**Sensitivity** refers to the proportion of infected animals that a test correctly identifies. A highly sensitive test will identify the majority of infected animals as positive. With reduced test sensitivity, there is increasing likelihood of false negative test results.

**Specificity** refers to the proportion of non-infected animal that a test correctly identifies. A highly specific test will identify the majority of animals that are not infected as negative. With reduced test specificity, there is increasing likelihood of false positive test results.

14. A number of factors influence test sensitivity [20,42,43]. Stage of infection and disease is particularly important, with test sensitivity generally increasing with increasing time since infection. Other important factors that can influence test sensitivity relate to:
  - a) The animal (test administered too soon after infection, desensitisation because the test has been administered too soon after the previous TB test, anergy as a result of overwhelming infection, concurrent infection, peri-parturient transient-immunosuppression, nutritional and transport stress).
  - b) The tuberculin used, including, in the case of both the SITT and CITT, tuberculin potency, and antigenic formulation, storage conditions and

manufacturing errors and in the case of the CITT alone, the relative potency of both the avian and bovine tuberculin PPDs.

- c) Factors associated with the administration, reading and recording of the test, including the site of injection, palpation of the intended site (blemish/lump free), site identification (clipping), accuracy of delivery of the intradermal injection, the timing of test reading, errors in recording of readings, errors in identifying reactor animals, tester bias.
15. Test specificity is mainly influenced by animal exposure to various species of bacteria that share antigenic proteins with bovine tuberculins, leading to non-specific reactions. The CITT is used in Ireland specifically to distinguish animals infected with TB from non-specific reactors. The testing of cattle vaccinated with BCG vaccine will also result in a false positive CITT result [20].
16. Both sensitivity and specificity are inter-related with specificity being most critical in non-infected herds where false positives are undesirable and sensitivity being more critical in infected herds where failure to detect TB infected animals prolongs and potentially exacerbates a TB problem. In the final stages of an eradication programme, a more sensitive and specific test is required if the campaign depends on only a single test. In practice, a combination of tests are used together with other approaches such as isolation of infected farms and maintenance of disease-free areas [44].
17. In herds where TB has been established, the use of the severe interpretation, which lowers the cut-off points for an animal to be declared a reactor, enhances the sensitivity of the CITT over the normal standard interpretation and can be done immediately a reactor is identified [45]. This is the first step to ensure that the maximum number of infected cattle are detected in the bovine TB eradication programme.
18. Ideally, studies to estimate sensitivity and specificity require the true infection status of an animal to be known. Traditionally, the MTBC status of an animal was based on detailed *post-mortem* examinations and the subsequent culture and histopathological examination of lymph nodes and other tissues [9,46]. However, this approach may bias sensitivity and specificity estimates in favour of tests that identify animals at this (later) stage of infection and against tests that identify infected animals at earlier stages of infection (when for example, visible lesions are less likely) [27]. Modern statistical methods complement these traditional approaches and can indirectly measure test characteristics, such as sensitivity and specificity. The estimates obtained from a particular study will depend on the epidemiological circumstances under which the study is carried out and the study methodology. Variables that must be considered include tuberculin manufacturer, tuberculin concentration and potency, tuberculin antigenic profile, test interpretation, target animal population, interval between successive tests, desensitisation including peri-parturient temporary immunosuppression, deliberate interference, observer variation, rigour of test procedure, disease stage in the animals, cross-reactivity, which may be environment dependent, and concurrent disease [20,42,47–51]. Some studies used to estimate test sensitivity were carried out in herds with chronic TB breakdowns, which is a biased population having been censored by previous CITT testing. In other words, animals that reacted well to the tuberculin test may have been removed at earlier CITT tests leaving poorly reacting animals at the test used to estimate sensitivity. Consequently, the results

from such studies may have underestimated the test sensitivity and caution is required in extrapolating these study results.

In summary, the accuracy of the diagnostic tests that are used in an eradication programme are critical to its success. It is vital that the tests are able to distinguish between infected and non-infected animals to a very high degree. In that context, the test sensitivity (the ability of a test to correctly identify infected animals) and the test specificity (the ability of a test to correctly identify non-infected animals) are key measures of the performance of a test. The sensitivity and specificity of a test are influenced by a number of factors and the estimates of sensitivity and specificity depend on the study conditions under which they are obtained. Consequently, a wide variety of estimates can be obtained for a particular test.

## 2.1 Sensitivity and specificity of the CITT

19. As highlighted previously, the sensitivity of the CITT will differ substantially depending on the context. In detailed reviews conducted across a range of studies, sensitivity estimates under field conditions have been low: 52.9-62.8% in Ireland [27], 50% (with wide credibility intervals) based on data from a number of different countries [52] and 66% in the UK [53]. In single studies conducted on the island of Ireland, estimates have generally been higher, including 88.6-90.6% in a study conducted in Northern Ireland using standard interpretation [54]. This study was conducted using a no-gold-standard method, currently accepted as the most appropriate method to use to obtain these estimates. In earlier work from Ireland, using methodology with greater potential for bias, an estimate of 90.9% using the severe interpretation was determined [46]. In broad terms, an assessment of these studies and reviews of the available literature [20,42,47] indicate that the sensitivity of the CITT at standard interpretation lies between 75% and 95%, although this does depend on the relative potency of the tuberculins used and the other factors that influence test sensitivity.
20. Reactivity to the CITT is dependent on the time from infection [55], termed the occult or pre-allergic phase where animals are infected but not yet detectable. This can last for up to 6 weeks post-infection; some studies have pointed out the pre-allergic phase as the cause of false negative reactions and therefore, the lack of sensitivity in recently infected animals [20,56,57]. As a consequence, the effectiveness of testing within a herd is dependent on the timing and frequency of testing as well as the characteristics of the CITT [20,49,52,58]. Short-interval ( $\geq 60$ -day) repeat testing in infected herds will improve the overall sensitivity of the CITT to remove all infected animals from the herd as animals in the pre-allergic phase of infection at the first test will be identified at the second test. Moreover, anergic (non-responsive) cattle have been reported due to advanced or generalised TB or being temporarily under stress [13].
21. The specificity of the CITT is very high. It has been estimated to be 99.5% (median, ranging from 78.8-100%) based on international studies in cattle populations free of TB [20] and 99.2-99.8% in Ireland using no gold-standard methodology [27]. Based on a study carried out in the UK, Goodchild et al. calculated a specificity of 99.98% for the CITT, using the standard interpretation

[59]. Similar high specificity values were obtained in studies using the no gold-standard method [52,54].

22. It is important to note that there is a difference in the animal-level sensitivity and the herd-level sensitivity (HSe), i.e. how likely a test is to identify infection in a herd. HSe is a function of the animal-level sensitivity but also the within-herd prevalence of infection and herd size [60,61]. HSe rapidly increases to its maximum level (100%) as the number of animals tested in the infected herd increases [20]. The converse is true for herd-level specificity (HSp), i.e. how likely a herd is to be designated as infection-free at a test when it is infection-free. HSp decreases as the herd size increases.

In summary, a wide range of estimates have been obtained for the sensitivity of the CITT, depending on the study conditions in place for obtaining the estimates. In broad terms, the studies indicate that sensitivity of the tuberculin test in Ireland at the standard interpretation lies between 75% and 95%. The studies indicate that the specificity of the CITT under the standard interpretation in Ireland lies between 99.5% and 99.98%.

## 2.2 Sensitivity and specificity of the IFN- $\gamma$ assay

23. In studies conducted in Ireland, the sensitivity and specificity of the IFN- $\gamma$  assay were estimated at 56.2-88% and 88.1-96.6%, respectively, depending on the cut-off values used [3,9,27]. In Northern Ireland, the mean performance estimates were reported as 85.8–93.0% (sensitivity) and 75.6%–96.2 % (specificity) using the local cut-off values [62]. A recent statistical meta-analysis of published results from a large number of studies in different countries provided an estimate of 67% for sensitivity, and 98% for specificity of the test [52]. Data provided to the OIE as part of an approval process indicated that the BOVIGAM® IFN- $\gamma$  assay had a sensitivity of 84.6% and a specificity of 97.4% using classical statistical methods [63]. Given the variation in source, potency, relative potency and time of manufacture of the tuberculins used in the studies under analysis, it is not possible to make robust inferences regarding the outcome characteristics.
24. Buddle et al. concluded that the IFN- $\gamma$  test is more sensitive than the CITT and stated that this is likely to be due to the fact that the IFN- $\gamma$  assay detects TB infected animals as early as 14 days following infection [64]. More importantly, studies in the UK [65] and Ireland [11] have shown that CITT negative but IFN- $\gamma$  positive animals are more likely to be infected with TB than CITT positive and IFN- $\gamma$  negative cattle and that removal of all animals reacting positive to either of the two tests is critical to controlling bovine TB outbreaks. The combined sensitivity of the IFN- $\gamma$  assay and the CITT (applying severe interpretation) has been estimated in one study to be 93% relative to lesion detection [11]. Consequently, the IFN- $\gamma$  assay is used as an ancillary test in Ireland in combination with the CITT with the objective of identifying infected animals in herds with a high probability of containing infected animals.
25. Because the CITT and the IFN- $\gamma$  assay stimulate the same DTH response pathways, there is a high degree of correlation expected between the animals identified by both tests, particularly where the same tuberculin is used for both tests. Where blood samples from CITT reactors are tested one day after blood

collection using a sensitive interpretation to compensate for the 24-hr time lag, the IFN- $\gamma$  assay may also be used as part of a general quality assurance scheme. This approach can be used to monitor the field performance of the CITT to detect and/or minimise the occurrence of deliberate interference, operator variation and ensure rigour of test procedure. All of these have been identified as impacting test sensitivity and specificity [66,67].

26. The relatively low specificity of the IFN- $\gamma$  assay in comparison with the CITT has constrained its usage in TB free herds undergoing surveillance testing, as it is likely that an unacceptable number of false positive reactors would be identified [3]. Consequently, the IFN- $\gamma$  assay is not used as a screening test in Ireland.
27. As with the CITT, both the source and relative potencies of tuberculins used in the IFN- $\gamma$  test have been shown to affect the performance of test [68]. There are known sub-potent tuberculins in circulation and being used worldwide [7]. Since the 1970s as part of the quality control for the national Bovine TB Eradication Programme, Ireland routinely conducts tuberculin potency assays on naturally infected tuberculous cattle to ensure that the tuberculin PPD meets OIE requirements and even exceeds the minimum required by the EU. For Ireland, this is regarded as critical as Ireland is an exporting nation for live cattle, beef, milk and products thereof on international markets [1].

In summary, the evidence from scientific studies indicates that the sensitivity of the IFN- $\gamma$  assay is equal to or greater than the skin tests (CITT and SITT). However, the relatively low specificity of the test precludes its use as a screening test in Ireland.

### **2.3 Sensitivity and specificity of the antibody-based tests**

28. In general, antibody-based tests such as ELISA correlate more with lesion severity rather than early infection and their highest performance is usually reported in cattle with visible gross TB-lesions [13,69–71]. This can have the beneficial result of detecting animals at more advanced stages of infection [72] that otherwise could potentially continue to spread TB. However, the sensitivity of ELISA antibody detection is generally lower than for tests targeting the DTH response [47] although that will also depend on the stage of disease in the cattle population, the type of DTH test that is performed and the tuberculin used for the test [69]. The application of an ELISA test after a skin test increases the sensitivity of the test [19,21]. Data from Northern Ireland supports the contention that the performance of the ELISA tests is significantly improved (with respect to identifying infected animals with confirmed TB lesions) when carried out 15 days after the CITT with a sensitivity of between 66.7% and 85.2%, relative to animals tested before or 72 hours after a CITT [73]. Data provided to the OIE as part of an approval process indicated that the IDEXX indirect ELISA test had a sensitivity of 65% [63].
29. It is claimed that the specificity of the antibody detection techniques is very high, meaning that most non-infected animals, particularly in infection-free populations, will be negative to an ELISA test [18]. Data provided to the OIE as part of an approval process indicated that the IDEXX Elisa test had a specificity of 98% [63].

30. As with all tests for TB in bovines, care must be taken when selecting which ELISA test to use, noting, as stated above, different ELISAs use different tuberculins or different individual TB antigens and thus not all ELISAs will perform the same or even consistently perform across different populations of cattle [22,73].

In summary, the antibody ELISA tests detect infection at a relatively advanced stage in the infection process. Consequently, the sensitivity of these tests is likely to be lower than the skin tests (SITT and CITT) for detection of early infection. Moreover, for optimum sensitivity to be achieved, the ELISA test must be carried out in conjunction with a skin test. The specificity of the ELISA, while high, is likely to be lower than that of the skin tests.

#### 2.4 Other considerations in relation to the performance of diagnostic tests

31. The positive predictive value is a measure of the probability that an animal with a positive test actually is infected. Goodchild et al. estimated that 91.1–93.7% of CITT test reactors are infected with TB; this is over twice as large as the proportion (30–40%) of CITT reactors that have a visible lesion at *post-mortem* examination or are culture positive [59]. They concluded that *post-mortem* and laboratory investigations “profoundly underestimates the proportion of reactors that is truly infected”. DTH tests are early-stage infection tests and animals will be positive to the test, particular the IFN- $\gamma$  assay, long before lesions can develop to the stage where they may be visible, even with detailed *post-mortem* [13,74]. The examination of animals at routine slaughter is neither a detailed necropsy nor a TB diagnostic instrument. The median sensitivity, for visible lesion detection, of routine post-mortem examination at meat inspection is relatively low varying between 54% [49] and 71% [52] but with an agreed high specificity. Multiple studies have shown that a more thorough and detailed examination would confirm far more skin test-positive animals as TB infected [13,45,74]. Nevertheless, abattoir surveillance remains a valuable method for identifying herds infected with tuberculosis in Ireland [75,76].
32. Machine learning, an approach based on artificial intelligence, is now being applied to existing TB data with the objective of maximising identification of TB infected herds and animals. This approach has shown promise through its application to TB data in Great Britain where a herd level model has indicated that the herd-level sensitivity and specificity of predicting outbreaks would both be increased through its use (HSe from 61.3% to 67.6%; HSp from 90.5% to 92.3% [77]).

In summary, all available tests have strengths and weaknesses. This is a normal challenge for disease diagnosis. General population surveillance requires a test sensitivity that will identify TB, especially at herd level allied with maximal specificity to minimize disclosure and removal of false-positive uninfected animals. Once infection in a herd is identified, it is prudent to use tests with high individual animal-level sensitivity to identify and remove infected animals. In managing infected herds, the available diagnostic tools should be applied optimally taking account of local epidemiological conditions.

### **3 Evidence that infected cattle are not being detected by the current testing programme in Ireland and other countries**

33. Residual infection (that is, infected animals that test negative to diagnostic tests by evading the host immune response) is a feature of TB in cattle in Ireland and other countries.
- a) Residual infection is likely to persist despite ongoing skin testing. Using mathematical modelling, Conlan et al. have estimated that at least 10% of breakdowns in Great Britain have at least one infected animal still present at the time of de-restriction [53]. In Ireland, in approximately 80% of breakdowns that were first detected at the factory, no further reactors were subsequently detected at a full herd retest [78]. Many of these animals with TB lesions at the factory were present, and tested negative, at one or multiple earlier CITTs.
  - b) Residual infection is linked with past exposure. In animals where infection is first detected at the factory, Olea-Poppelka et al. found that infection could be linked with past exposure [78]. If the index animal (the animal with gross lesions at the factory) had previously been introduced into a herd, the increased risk was associated with both the index herd (the number of months that the index animal had been present in the herd, the herd size, the number of contiguous herds) and the index animal (whether the animal had been present in a TB episode in a previous herd). If the index animal was homebred, risk increased with a range of herd-level factors (time since last test, herd size, number of contiguous herds) and decreased with animal age. If the animal had been in a previous TB restriction, risk increased with increasing time since this restriction. These results highlight the risk associated with a previous TB episode, with this risk increasing with time and, reasonably, the opportunity for transmission of infection to cohort animals. An age-matched case-control study was conducted on CITT non-reactor animals slaughtered in 2012 with a confirmed TB lesion identified during routine abattoir surveillance and with no evidence of within-herd transmission slaughtered in 2012 from officially TB-free herds but with no lesion found. The three key conclusions from this study were that the main risk factors for detection of a confirmed TB lesion were: previous exposure history, previous inconclusive reactor result at CITT and number of inter-herd movements [79].
  - c) Residual infection poses a future risk to in-contact animals. Many Irish studies have identified herd TB history as a key risk factor for TB presence within a herd [80–89]. Animals moving following derestriction were 1.91 times more likely to test positive in the following 2 years compared with animals that

moved from herds without such a history [90]. Risk increased with increased exposure: in comparison to unexposed animals, cattle moving from herds within 7 months of the date of de-restriction following a breakdown with 1-7 and 8 or more reactors were 1.23 and 1.77 times more likely to be reactors over the following 2 years [89]. In New Zealand, unresolved infection was found to contribute to further TB episodes in the first 2 years after herd clearance [91].

34. A recent UK study traced a new TB outbreak in the UK to a TB strain that had been imported from Northern Ireland a minimum of 6 and possibly as many as 16 years earlier which ended up by the time the first bovine was detected in 2014, spreading to infect multiple herds in multiple counties and into the local badger population [92]. The first case in a seven-month-old home-bred animal was evidently not the index case of the outbreak under investigation, the introduced case of the NI origin TB-strain was never determined. One of the key observations from this study, was the length of time the TB infection in cattle had remained undetected. Undoubtedly, multiple infected animals had evaded detection at slaughter and by the CITT during the period prior to the first detection of this imported *M. bovis* strain.
35. It is important to note that latent TB, i.e. a dormant, symptomless infection, is a very common feature of TB infection in humans, caused by *M. tuberculosis* [93]. The person may or may not be positive to the tuberculin skin test. In this state, the organism uses various mechanisms to evade the host immune response, persist as a latent infection and subsequently emerge from the latent state to reactivate and cause disease and transmission. It has been postulated that a similar phenomenon may occur in cattle [13,94,95]. However, based on a review of the literature, Álvarez et al. [94] concluded that, there is no clear evidence of a different status of active versus latent infection, and the concept of latent infection has remained more as a speculation than a proven fact. Based on a later review, Sabio y García et al. [95] concluded that the genetic factors required for *M. tuberculosis* in humans to remain in a replicative state of latency are conserved or even active in *M. bovis* in cattle. However solid evidence demonstrates that the activation of latency is different quantitatively and perhaps qualitatively in both species (humans, cattle).

#### **4 Potential for improving the performance of current tests or developing new diagnostic tests**

36. Many tests have been developed for the diagnosis of bovine tuberculosis in recent years. Frequently, the performance of these tests under natural field conditions does not match the claims of developers or manufacturers and the initial promise shown at the early research stage. Validation of a new test to the OIE standard is a critical first step in considering a test for field deployment. In that context, the OIE has approved two antibody-based tests (Mycobacterium bovis Antibody Test Kit from IDEXX Laboratories and Enferplex Bovine TB Antibody Tests from Enfer Laboratories and the IFN- $\gamma$  assay (BOVIGAM®) from Thermo Fisher Scientific. Two of these are now being used in the bovine tuberculosis eradication programme in Ireland as ancillary tests.
37. Research to identify DTH responses to defined antigens is ongoing for the skin tests and for IFN- $\gamma$  assay [96,97]. The use of specific antigen cocktails in place of

tuberculin offers the best possibility of improving the performance of the CITT and the IFN- $\gamma$  assay and replacing the cumbersome (i.e. highly variable) production, quality control, standardisation and potency evaluation processes required for the current tuberculin PPD products. Work is also ongoing in further improving the other diagnostic tests mentioned in Section 1.3.

38. For the foreseeable future, it is likely that the CITT will remain the screening test of choice for cattle and other livestock with other tests, i.e. currently approved tests and any new tests that are developed, being used as ancillary tests.

## **5 How to optimise the use of currently available tests to achieve eradication**

39. Regardless of the diagnostic tool being used, it is critical that the test performance is maximised. The following points are critical;

a. For the CITT:

- i. Tuberculin PPDs of optimal relative potency are used and stored appropriately
- ii. Syringes and measuring devices are serviced, calibrated and working properly
- iii. TB testers are trained and perform and record the test correctly
- iv. There is a system of measuring performance and quality outputs in relation to testers
- v. Testing facilities are commensurate with the number, type and nature of the cattle to be tested
- vi. Sufficient time and labour is provided on each day of test, to ensure that the animal is properly presented/restrained so that testing can be performed
- vii. The test result is reported promptly
- viii. A quality assurance system operates to ensure test results are consistent across testers and with any IFN- $\gamma$  assay results.

b. For the IFN- $\gamma$  assay:

- i. The blood sampling is performed and recorded appropriately
- ii. Tuberculin PPDs are of equivalent potency to that used in the CITT
- iii. Samples are delivered to the laboratory in a timely fashion to optimise sensitivity
- iv. A quality assurance system operates in the laboratory to ensure consistent and repeatable results

c. For the serological tests:

- i. The sampling is performed and recorded appropriately
- ii. Blood sampling time is optimal for the detection of anamnestic responses
- iii. The test is validated to at least OIE standards

- iv. Samples are delivered to the laboratory under appropriate conditions to optimise sensitivity
  - v. A quality assurance system operates in the laboratory to ensure consistent and repeatable results
40. A comprehensive set of systems have been put in place by DAFM to monitor these requirements and to ensure that test performance is maximised. The effectiveness of the CITT test is highly reliant on the skill and experience of the individual tester [20,42]. A broad range of factors may adversely affect test accuracy, including the quality of handling facilities, site selection and preparation, and tuberculin injection technique [98]. In the UK and Ireland, there has been an emphasis on the need for a robust assurance system for test delivery to ensure the rigour of the testing procedure [99,100]. Field inspection of testers has been conducted in Ireland for many years. Since 2008, this has been supplemented with quantitative performance reports, enabling the performance of each tester to be evaluated and ranked using a range of performance indicators [100]. The results from a study by Clegg et al. showed that the variation in the performance of testers significantly decreased between 2008 and 2011, suggesting an increase in testing consistency, after accounting for other known risk factors [98].

## **6 Successful eradication can be achieved without perfect tests**

41. Successful eradication can be achieved without perfect tests. Many European countries have achieved eradication primarily using the skin test and abattoir surveillance [101,102]. Similarly, there was total reliance on the skin test and abattoir surveillance to detect infected animals throughout the TB Eradication Programme in Australia [103]. The IFN- $\gamma$  assay was developed by Australian scientists specifically to address the deficit in available tests for diagnosis of residual infection [8]. However, it was not used routinely due to logistical challenges and because of the decline in TB incidence resulting from success in existing areas of the eradication programme. Similarly, rapid progress towards eradication in New Zealand is being made using similar tests that are currently available in Ireland alongside a programme to deal with transmission to cattle from wildlife [104].
42. In the absence of perfect tests, non-testing methods have been used to support national eradication efforts. Specifically, risk-based approaches are the only method available to adequately address the problems caused by residual infection and animal movement, whilst also facilitating ongoing commerce within the farming community. This approach has been central to the national eradication programmes in Australia [103] and New Zealand [104]. The following broad principles were applied:
- a. TB risk was assessed at the level of the herd (not the animal), cognisant of the epidemiology of infection (including the potential for residual infection) and the imperfect sensitivity and specificity of available diagnostic tests
  - b. A dynamic system of risk-based classification was implemented, with herds progressively moving from a *high* TB herd risk score (at the time of derestriction) to a *low* TB herd risk score over a series of years

- c. Risk-based trading allowed ongoing commerce whilst limiting the potential for infection to spread from herds of *higher* to *lower* TB risk through animal movement. This was achieved by allowing farmers to sell cattle *to herds of equivalent or higher* TB herd risk and to source cattle *from herds of equivalent or lower* TB herd risk
- d. A broader range of measures was put in place to assist keepers of *high* TB risk herds to manage risks associated with animals within their herd without placing other herds at risk [105].

Concurrently, effective strategies were put in place in these countries to manage transmission from wildlife to cattle.

- 43. Additional tools which may assist the eradication effort have recently been developed or are in the process of development. These include breeding for resistance, whole genome sequencing, machine learning and advances in the area of epidemiology, including modelling approaches.

## **7 Conclusions on the scope for existing (including recently developed) diagnostic methods to detect infected cattle which are not currently detected by the existing programme**

- 44. The diagnostic testing regime being used in Ireland, i.e. the CITT as a screening test and the IFN- $\gamma$  assay and an antibody test as ancillary tests, along with meat inspection surveillance, is the best that is currently available both to detect infected herds and to minimise the restriction of herds due to the designation of non-infected herds as test-positive. The manner in which the different tests are used strategically takes maximum advantage of the strengths of the individual tests and minimises their limitations. Consequently, the scope for existing diagnostic methods to detect infected cattle which are not currently detected by the existing programme is very limited.
- 45. The CITT has been used in bovine TB eradication programme in Ireland for many decades. While not perfect, it has been shown to be a highly effective test in identifying infected cattle and in discriminating between infected and non-infected cattle. The test is highly reliant on the skill and experience of the individual tester and it is of the utmost importance that it is performed properly. Research work is ongoing to further improve the performance of the test through the use of specific antigens or antigen cocktails in place of tuberculin but experience to-date has shown that this is a slow and challenging process.
- 46. Research work is also ongoing to further improve the performance of the IFN- $\gamma$  assay and the antibody-based tests, again through the use of more specific antigens or antigen cocktails. The main focus in relation to the performance of the IFN- $\gamma$  assay is to improve the test specificity which is relatively low compared with the CITT. If a test specificity of the IFN- $\gamma$  comparable to the CITT is achieved, the IFN- $\gamma$  could potentially be used as a screening test. Despite considerable effort, however, limited progress has been made in this area since the test was first developed in the early 1990s.
- 47. Antibody-based tests such as the IDEXX indirect ELISA and the Enferplex Bovine TB Antibody test play a valuable role as ancillary tests, but they are limited to detecting infection at a relatively late stage in the infection process. It is

likely that any further progress in the development of these tests will enhance their value as ancillary tests rather than lead to their use as screening tests.

48. The Actiphage test presents a new concept for the detection of *M. bovis* and diagnosis of TB infection in cattle. However, the test is currently not validated nor approved by the OIE or EU.
49. In summary, there is little evidence of a silver bullet in the area of bovine tuberculosis diagnostics. For the foreseeable future, it is likely that the CITT, using tuberculin PPD, will remain the screening test of choice for cattle. It will be supplemented by the other currently approved tests and newly developed tests as ancillary tests.
50. Experience from a number of countries has shown that eradication can be achieved in the absence of perfect diagnostic tests. For this to happen, the testing programme must be supplemented by a range of risk-based approaches to herd management and animal movement. Where relevant, eradication is only possible if cattle-based efforts are complemented by an effective programme to manage transmission from wildlife to cattle.

## **8 Recommendation**

- 1) The TB Forum and DAFM should continue to monitor and support the efforts of developers of new diagnostic tools for bovine tuberculosis.
- 2) The TB Forum and DAFM should evaluate and, as appropriate, promote the use of any additional tools and strategies which may assist the eradication effort.

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