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ABSTRACT

In cattle herds in France, cervical skin tests (STs) using simple intradermal tuberculin (SIT) are performed to detect bovine tuberculosis (bTB). When positive results are found on ST screening, the herd is considered to be 'under suspicion' and confined, raising economic issues. The suspicion can be lifted by carrying out a single intradermal cervical comparative test (SICCT) at least six weeks later. The authors conducted an experimental study in France between 2013 and 2015 to assess the accuracy of the gamma-interferon test (IFN-γ), used in series after a non-negative result to ST screening, and to study the possibility of replacing the SICCT performed six weeks later by an IFN performed within a few days. Data were collected concerning 40 infected and 1825 bTB-free animals from herds with non-negative results to ST screening. This study showed that the IFN-γ test based on specific antigens and performed within a few days of a non-negative result to the ST has higher sensitivity than the SICCT performed six weeks later and equal specificity. The IFN test is more convenient to perform; however, it is more expensive. The IFN-γ test based on MIX antigens may be a useful alternative to the SICCT, to shorten the confinement period of suspect herds without underdetecting bTB.

INTRODUCTION

Bovine tuberculosis (bTB) is an infectious and zoonotic disease mainly due to *Mycobacterium bovis*. According to the EU, France has been officially bTB-free since December 2000. Nevertheless, eradication of bTB is not complete and about 100 outbreaks are notified each year.

In cattle, cervical skin tests (STs) are performed regularly (annually in most infected areas) to detect the infection. Nevertheless, the single intradermal test (SIT) and the single intradermal cervical comparative test (SICCT) are known to be cumbersome to perform and have low specificity due to cross-reactions with non-pathogenic mycobacteria.[1 2] When positive results to ST screening are found in a herd, this herd is considered to be under bTB suspicion. The suspicion can be confirmed by post-mortem histological and bacterial analyses, or ruled out if the results of another skin test (SICCT) are negative. This test must be performed at least six weeks after the first, to avoid a desensitization phenomenon (Council Directive 64/432/EEC): during this period, the herd remains confined (animals and products cannot be sold and new animals cannot be introduced), leading to significant economic issues. False-positive results due to non-specific reactions to ST are frequent, up to 50 per cent in some areas (personal communication with local veterinary officers) and lead to demotivation of breeders, veterinarians and veterinary officers.

Another test, performed on blood samples, is available to detect bTB in cattle: the gamma-interferon test (IFN-γ). This test is performed using two types of antigens: PPDs (bovine and avian purified protein derivatives) and specific antigens ESAT6 and CFP10. This test has been used in other European countries for more than a decade to speed up the eradication of infected animals in bTB outbreaks,[1] but its use as a screening test (ie, for establishing and maintaining an officially TB-free herd status and for certification of intra-EU trade in bovine animals) has not been studied extensively. The use of IFN-γ to replace ST has not yet been authorised in the EU (Council Directive 64/432/EEC)[3].

The authors conducted an experimental study in France between 2013 and 2015 to assess the sensitivity (already partially described by Praud and others[4]) and the specificity of the IFN-γ test, used in series after a non-negative result to a screening skin test. The aim of this experimental study was to examine the possibility of replacing the

SICCT performed six weeks after a screening skin test non-negative result by the IFN-γ test performed a few days after this first non-negative result to the skin test, without underdetecting infected cattle, nor increasing false-positive rates. Here, the authors sum up the sensitivity and specificity results of the complete experimental study.

Materials and methods

The following experimental protocol was designed by ANSES (French Agency for Food, Environmental and Occupational Health and Safety) and the DGAL (French Directorate General for Food), and funded by the French Ministry of Agriculture. It was authorised by the European Commission. Data were issued from French herds in which non-negative results to skin test screening (SIT or SICCT) were observed between 2013 and 2015. These herds were included in the study on farmer's voluntary basis. The skin tests were performed on cattle by veterinarians, as part of the usual official screening programme (in compliance with Council Directive 64/432/EEC). In the case of SICTT, non-negative were only those with doubtful results, positive SICTT animal at screening were considered infected and thus not included in the study. When non-negative results to skin tests were observed in a herd, the farmer and the veterinarian were asked to participate in the study.

Animals with non-negative results to the skin test on day 0 (ST_{D0}), were tested using IFN-γ between days 3 and 8 after the injection of tuberculin (IFN_{D3}) and retested with SICCT and IFN-γ on day 42 ($SICCT_{D42}$, IFN_{D42}). Animals with non-negative results to ST_{D0}, IFN_{D3}, $SICCT_{D42}$ or IFN_{D42} were slaughtered. Samples of lesions (if observed) and respiratory lymph nodes (tracheobronchial, retropharyngeal and mediastinal) were analysed (culture and PCR) for the totality of these animals.

Animals with positive results to PCR and/or culture for *M bovis* were considered infected. Animals from officially bTB-free herds were considered bTB free, that is, herds where the suspicion was ruled out through negative results to the SICCT and direct analyses performed on culled animals.

In regions where the SICCT test was used as a screening test, cattle with a positive result to the $SICCT_{D0}$ were immediately slaughtered and not included in the study.

To encourage farmers to participate in the study, movements of cattle with negative results in a herd with a non-negative result to the tests were allowed in France, when the suspicion was weak (ie, if the first non-negative ST was an SIT or an SICCT with a doubtful result, and if the IFN-γ test performed 3–8 days later yielded negative or inconclusive results for all animals. However, herds under suspicion were usually completely confined.

To perform the skin tests, doses of 0.1 ml of bovine (for SITs) and bovine and avian (for SICCTs) purified protein derivatives (PPD Bovituber and Avituber, Synbiotics, Lyon, France) were injected intradermally. Skinfold thickness was measured before injection (on day 0) and 72 hours later (on day 3) to calculate any increase in skinfold thickness. The tests were performed and interpreted as recommended by Council Directive 64/432/EEC. The results of SIT were positive when the increase in skinfold thickness was at least 4 mm, negative when it was up to 2 mm and doubtful between 2 and 4 mm. The results of the SICCT were negative when the increase in skinfold thickness at the point of bovine PPD injection was up to 2 mm, or when the difference in the increase in skinfold thickness at the points of bovine PPD injection (DB) and avian PPD injection (DA) was lower than 1 mm, doubtful when DB–DA was between 1 and 4 mm, and positive when DB–DA was higher than 4 mm.

To perform the IFN-γ test, whole blood was incubated with different mycobacterial antigens: Bovine and Avian Lelystad PPD (BOVIGAM Tuberculin PPDs, ThermoFisher) and a specific antigen MIX (Peptide Cocktail Prionics PC-EC, ThermoFisher). Released IFN-γ was measured using an ELISA method (BOVIGAM kit, ThermoFisher). IFN was performed according to the manufacturer's recommendations and as described by Faye and others.[5] Optical densities (ODs) were transformed into percentage values by comparing test-sample ODs with control ODs. IFN tests were performed by local laboratories, trained and accredited by the French National Reference Laboratory for bovine tuberculosis (NRL, ANSES, Maisons-Alfort, France), and having successfully completed an interlaboratory proficiency ring trial. Results were interpreted according to table 1. The thresholds were set by the French National Reference Laboratory for bovine tuberculosis, according to the results of previous studies performed in the *Départements* Côte-d'Or and Dordogne (Faye and others[5]; NRL, personal communication). Results to MIX (interpreted separately from PPD) were considered positive when the per cent OD was ≥0.03, and results to PPD (interpreted separately from MIX) were considered positive when OD was ≥0.05.

Data were processed using Excel and Access (Microsoft). McNemar's tests for paired data were performed using R, and considered significant when $P<0.05$.

In the database, 2851 animals had non-negative results to ST screening. At the end of the study, detailed results were available in the database for 40 infected animals from 29 farms, and 1825 bTB-free animals from 744 farms (figure 1). Sensitivity and specificity values were estimated in herds where non-negative results to preliminary ST screening were observed: they were thus conditional to a non-negative result to ST_{D0}.

RESULTS

The cattle herds included in the sample were located in most of the bTB-infected areas in France. In all, 87.4 per cent of bTB-free animals tested in the sample were from Burgundy, and 10.2 per cent were from the south-west, whereas 68 per cent of infected animals were from Burgundy, and 33 per cent were from the south-west. Most infected herds were beef herds (23 out of 29);

Table 1 Interpretation of IFN results according to the OD ratios obtained with PPD and MIX antigens

	PPD		
	PPD<0.05	0.05≤PPD<0.3	PPD≥0.3
MIX: specific antigens			
MIX<0.03	Negative	Inconclusive	Positive
0.03≤MIX<0.1	Inconclusive	Positive	
MIX≥0.1	IF bovine PPD>0.7: positive		

Calculation of ratios: ratio PBS=OD_{PBS}/[3×(OD_{PC}–OD_{NC})], ratio PWM=(OD_{PWM}–OD_{PBS})/[3×(OD_{PC}–OD_{NC})], ratio PPD=(OD_{PPDB}–OD_{PPDA})/[3×(OD_{PC}–OD_{NC})], ratio PPDB=(OD_{PPDB}–OD_{PBS})/[3×(OD_{PC}–OD_{NC})] and ratio MIX=(OD_{MIX}–OD_{PBS})/[3×(OD_{PC}–OD_{NC})]. Results were interpreted when ratio PBS<0.125 and ratio PWM >0.2.
NC, negative control; OD, optical density; PBS, phosphate-buffered saline; PC, positive control; PPDA, avian PPD; PPDB, bovine PPD; PWM, pokeweed mitogen; with MIX, specific antigens ESAT-6 and CFP-10.

five of them were dairy herds and one produced both milk and beef.

The conditional sensitivities assessed in infected cattle are shown in table 2. The conditional sensitivity of IFN_{D3} (positive and inconclusive results, interpreted as recommended by French legislation) was significantly higher than that of the $SICCT_{D42}$ (McNemar's test, $P=1.1\times10^{-4}$). The conditional sensitivities of PPD_{D3} and MIX_{D3} were both significantly higher than those of the $SICCT_{D42}$ (McNemar's test, $P=3.6\times10^{-3}$ and $P=2.3\times10^{-3}$, respectively). No significant differences could be shown between the conditional sensitivities of MIX ($SeMIX_{D3}$ and $SeMIX_{D42}$) and those of IFN-γ and PPD ($SeIFN_{D3}$, $SeIFN_{D42}$, $SePPD_{D3}$ and $SePPD_{D42}$), even though the mean conditional sensitivities of MIX were somewhat lower. The correlations of results in infected animals are described in detail in the study by Praud and others.[4]

The conditional specificities assessed in bTB-free cattle are presented in table 3. These animals were living in bTB-free herds for at least three years. The conditional specificity of IFN_{D3} (negative results only, interpreted as recommended by French legislation) was significantly lower than that of the $SICCT_{D42}$ (McNemar's test, $P=8.7\times10^{-143}$). The results of these two tests were consistent in 47.8 per cent of the cattle. Overall, 46.59 per cent of animals with negative results to the $SICCT_{D42}$ (758 out of 1627) also had negative results to IFN_{D3}. Additionally, 9.9 per cent of animals with negative results to IFN_{D3} (83 out of 841) showed false-positive results to the $SICCT_{D42}$. For 258 cattle, results of PPD on day 3 were inconclusive and could be interpreted only using MIX. The conditional specificity of MIX_{D3} was significantly higher than that of PPD_{D3} (McNemar's test, $P=8.4\times10^{-127}$) and that of IFN_{D3} (McNemar's test, $P=2.4\times10^{-160}$), whereas the conditional specificity of MIX_{D3} was slightly lower than that of the $SICCT_{D42}$ (McNemar's test, $P=5.6\times10^{-3}$).

DISCUSSION

On the basis of the results, when interpreted using both PPD and MIX, the IFN-γ test performed between three and eight days after a non-negative result to ST screening is significantly more sensitive, and significantly less specific, than the recommended test, that is, the SICCT performed 6 weeks after the first skin test. The serial association of screening ST with IFN_{D3} could thus be used to shorten the confinement of suspect herds without underdetecting infected cattle. However, the far lower specificity of this approach would lead to false-positive results and result in culling of uninfected animals. This type of testing would therefore not be well accepted by breeders and would cause economic issues. On the contrary, the MIX_{D3} test appears to be both more sensitive than the $SICCT_{D42}$ and almost as specific as this test. A screening programme combining in series ST on day 0 and MIX on day 3 (on cattle with non-negative results to ST_{D0}) could shorten the confinement period of suspect herds, without underdetecting bTB or culling too many uninfected animals.

The studied sample was built up on a voluntary basis: when non-negative results to ST_{D0} were observed in a herd, the local veterinary services asked the farmer whether he or she wanted to enter the herd into the study. Inclusion was only possible if the farm veterinarian agreed to

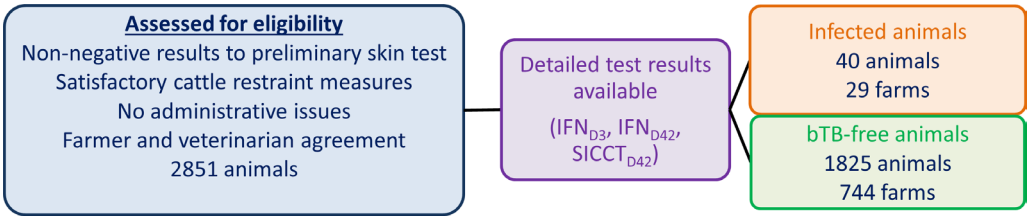

Figure 1 Sampling scheme. bTB, bovine tuberculosis; D3, day 3; D42, day 42; IFN, gamma-interferon test; SICCT, single intradermal cervical comparative test.

Table 2 Conditional sensitivities of the tests, assessed in 40 infected cattle

Test	Proportion of infected cattle with a non-negative result to the test	Conditional sensitivity: mean and 95% CI
$SICCT_{D42}$*	18/40	45% (30% to 60%)
IFN_{D3}*	35/40 (positive results only)	88% (77% to 98%) (positive results only)
	38/40 (positive and inconclusive results)	95% (88% to 100%) (positive and inconclusive results)
IFN_{D42}*	34/40 (positive results only)	85% (74% to 96%) (positive results only)
	39/40 (positive and inconclusive results)	98% (93% to 100%) (positive and inconclusive results)
PPD_{D3}*	25/28	89% (78% to 100%)
PPD_{D42}*	37/38	97% (92% to 100%)
MIX_{D3}	33/40	83% (71% to 94%)
MIX_{D42}	33/40	83% (71% to 94%)

CIs were calculated with Excel.
*Praud and others.[4]
D3, day 3; D42, day 42; IFN, gamma-interferon test with results interpreted using both PPD and MIX (see table 1); MIX, specific antigens ESAT-6 and CFP-10; PPD, purified protein derivatives; SICCT, single intradermal cervical comparative test.

Table 3 Conditional specificities of the tests, assessed in 1825 bTB-free cattle

Test	Proportion of bTB-free cattle with a negative result to the test	Conditional specificity: mean and 95% CI
$SICCT_{D42}$	1627/1825	89.15% (87.72% to 90.58%)
IFN_{D3}	841/1825 (negative results only)	46.08% (43.80% to 48.37%) (negative results only)
	1505/1825 (negative and inconclusive results)	82.47% (80.72% to 84.21%) (negative and inconclusive results)
IFN_{D42}	1108/1825 (negative results only)	60.71% (58.47% to 62.95%) (negative results only)
	1707/1825 (negative and inconclusive results)	93.53% (92.41% to 94.66%) (negative and inconclusive results)
PPD_{D3}	707/1567*	45.12% (42.65% to 47.58%)
PPD_{D42}	1116/1775†	62.87% (60.63% to 65.12%)
MIX_{D3}	1571/1825	86.08% (84.49% to 87.67%)
MIX_{D42}	1719/1825	94.19% (93.12% to 95.26%)

CIs were calculated with Excel.
*On day 3, 258 results were not interpretable on the PPD basis only.
†On day 42, 50 results were not interpretable on the PPD basis only.
D3, day 3; D42, day 42; IFN, gamma-interferon test with results interpreted using both PPD and MIX (see table 1); MIX, specific antigens ESAT-6 and CFP-10; PPD, purified protein derivatives; SICCT, single intradermal cervical comparative test.

participate and if the local official laboratory was accredited to perform IFN-γ tests. Farms where cattle restraint was not satisfactory and herds with administrative issues (non-compliance with health and safety legislation) were not allowed to enter the study. Highly motivated farmers, with well-run farms and motivated veterinarians were most likely to be involved. Most farms were located in the Côte-d'Or *département* (eastern-central France), and Dordogne and Charente (south-west), which are among the areas most affected by bTB in France.[6] The sample can be considered representative of cattle in the country with non-negative results to ST screening, from most infected areas, even though selection bias associated with voluntary participation of farms could not be avoided. The number of beef herds among infected herds in the sample is consistent with their proportion among bTB outbreaks that occurred in France between 2013 and 2015 (76 per cent).

The fact that animals with positive results to the $SICCT_{D0}$ (which reacted most strongly to skin tests) were culled, and are thus not represented in this sample, probably leads to an underestimation of sensitivities and an overestimation of specificities. This could be counterbalanced by the fact that most studied farms were from areas where bTB outbreaks are frequent, especially Burgundy, where cross-reactions of tuberculosis screening tests, due to non-pathogenic mycobacteria, are also known to be frequent, leading to an overestimation of sensitivities and an underestimation of specificities.[7] The prevalence of paratuberculosis among the sampled herds is unknown.

However, the aim of this study was to assess the accuracy of a serial combination of ST and IFN in the field in France: since most non-negative results to ST screening were found in Burgundy, the over-representation of this area in the sample does not appear to be problematic.

The isolation of *M bovis* from lesions or lymph nodes is known to have less than perfect sensitivity, but PCR has higher sensitivity and both methods are highly specific[1 8]: the positive reference chosen to define infected cattle, consisting of a positive result to culture and/or PCR performed on lesions—if observed—or lymph nodes, thus seems to be appropriate. Nevertheless, when no lesion was observed, only thoracic lymph nodes were analysed. Even though only few animals usually develop extrathoracic localisation of bTB (around 16 per cent, according to Corner and others[9]), cattle with latent infection of other lymph nodes could not be detected by the method adopted by the authors of this study. The main limitation due to the negative reference chosen to define bTB-free cattle is the fact that sampled herds were located in highly infected areas. Nevertheless, their recent history was known, and no bTB outbreak was detected among them in the three previous years.

Implementing IFN-γ testing is known to be less cumbersome than SICCT, since it requires only one blood sample from cattle, whereas the SICCT requires excellent restraint of animals, twice in three days. Interpretation of the IFN-γ result relies on the automatic measurement

of ODs, in an officially accredited veterinary laboratory, whereas the interpretation of the ST is more subjective since it depends on the manual measurement of the increase in skinfold thickness by the veterinarian. Furthermore, the results of IFN-γ are recorded automatically, while the results of the SICCT are handwritten and forwarded by the veterinarian to the local veterinary services. IFN-γ is known to detect bTB earlier than ST, even at relatively low infectious doses.[10 11]

Most studies show that a single cervical injection of tuberculin has no effect on the results of IFN-γ, whereas a caudal-fold injection stimulates the production of IFN-γ in infected animals.[1 12–14] The results of studies examining the effect of SICCT on IFN are contradictory. Some authors describe no effect of SICCT,[13 15] while others report a decrease in IFN-γ response when the test is based on specific antigens.[16 17] Regarding the ST, the results of I IFN-γ FN can be influenced by factors such as administration of corticosteroids[18] or infestation by *Fasciola hepatica*.[19] Finally, non-specific reactions can occur in young animals.[20] For this reason, IFN-γ should not be used in calves and is usually performed on animals older than six months.

The most significant limitation of IFN-γ is probably its cost (in France, between €40 and €60 per animal), and the fact that the blood sample must be transported to an officially accredited laboratory within six to eight hours because the IFN-γ response decreases if the blood is not processed within this timeframe.[14] This type of laboratory must therefore be located in the area. During the experiment, in some areas of France, blood samples were collected by local veterinary services to be brought to an accredited laboratory in order to help the veterinarians.

In many countries, ST and IFN-γ are mostly used in parallel to speed up the eradication of bTB during outbreaks.[1 2] The serial use of ST and IFN is not very common and its accuracy has not been studied extensively since it is not authorised by the EU. The IFN test is often performed using the Bovigam kit (PPD), but specific antigens are not systematically used. Nonetheless, some authors recommend their use in areas with low prevalence of bTB.[21]

In 2001, Buddle and others[22] assessed the characteristics of PPD and ESAT-6, when the IFN-γ was performed 8–28 days after a positive skin test. The sensitivity and specificity of PPD were 98 per cent and 85 per cent, respectively. The sensitivity and specificity of ESAT-6 were 88 per cent and 99 per cent, respectively. According to Ryan and others,[23] who assessed the characteristics of the IFN-γ test (PPD) used in series in New Zealand, the sensitivity of IFN-γ was 85 per cent and its specificity was 93 per cent. In France, a previous study conducted between 2009 and 2012,[24] in Côte-d'Or found the sensitivity of IFN-γ to be 88.1 per cent (95 per cent CI 72.8 per cent to 97.5 per cent) and its specificity 62.3 per cent (95 per cent CI 60.2 per cent to 64.5 per cent). In this last study, PPD and MIX were interpreted together and cut-offs were 0.04 OD for both types of antigens. The differences with the results may be due to several factors: the epidemiological context and the sample selection methods (sensitivities and specificities assessed in this study are dependent on a non-negative result to a previous skin test), commercial kits and antigens used, choice of thresholds and interpretation of the results using per cent OD.

In conclusion, this study showed that the IFN-γ test, when including specific antigens (MIX: ESAT-6 and CFP-10) and performed a few days after a non-negative result to a screening skin test, is more sensitive than an SICCT performed six weeks later (42 days) and almost as specific as this test. The IFN-γ test is also easier to perform in the field, even though blood analysis must be performed by an accredited laboratory and it is more expensive than an SICCT. The IFN-γ test based on MIX antigens appears to be a useful alternative to the SICCT, and could be used to shorten the confinement period of suspect herds, without underdetecting infected cattle, and without culling large numbers of uninfected animals.

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REFERENCES

1. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, *et al*. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci* 2006;81:190–210.
2. Aagaard C, Govaerts M, Meikle V, *et al*. Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity. *Prev Vet Med* 2010;96:161–9.
3 European Community. Consolidated (English) version of Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine. *Official Journal of European Community* 1964:P1211977. 29.07.1964.
4 Praud A, Boireau C, Dufour B. Sensitivity of γ-interferon test used in series after tuberculin test to detect bovine tuberculosis. *Vet Rec* 2016;179:174.2–174.
5 Faye S, Moyen JL, Gares H, *et al*. Determination of decisional cut-off values for the optimal diagnosis of bovine tuberculosis with a modified IFNγ assay (Bovigam®) in a low prevalence area in France. *Vet Microbiol* 2011;151:60–7.
6 Cavalerie L, Courcoul A, Boschiroli ML, *et al*. Tuberculose bovine en 2014: une situation stable. *Bulletin Épidémiologique Santé Animale et Alimentation* 2015;71:4–11.
7 Biet F, Boschiroli ML. Non-tuberculous mycobacterial infections of veterinary relevance. *Res Vet Sci* 2014;97 Suppl:S69–S77.
8 Courcoul A, Moyen JL, Brugère L, *et al*. Estimation of sensitivity and specificity of bacteriology, histopathology and PCR for the confirmatory diagnosis of bovine tuberculosis using latent class analysis. *PLoS One* 2014;9:e90334.
9 Corner L, Melville L, McCubbin K, *et al*. Efficiency of inspection procedures for the detection of tuberculous lesions in cattle. *Aust Vet J* 1990;67:389–92.
10 Buddle BM, de Lisle GW, Pfeffer A, *et al*. Immunological responses and protection against Mycobacterium bovis in calves vaccinated with a low dose of BCG. *Vaccine* 1995;13:1123–30.

11 Dean GS, Rhodes SG, Coad M, *et al*. Minimum infective dose of Mycobacterium bovis in cattle. *Infect Immun* 2005;73:6467–71.
12 Whipple DL, Palmer MV, Slaughter RE, *et al*. Comparison of purified protein derivatives and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. *J Vet Diagn Invest* 2001;13:117–22.
13 Gormley E, Doyle MB, McGill K, *et al*. The effect of the tuberculin test and the consequences of a delay in blood culture on the sensitivity of a gamma-interferon assay for the detection of Mycobacterium bovis infection in cattle. *Vet Immunol Immunopathol* 2004;102:413–20.
14 de Lisle GW, Green RS, Buddle BM. Factors affecting the gamma interferon test in the detection of bovine tuberculosis in cattle. *J Vet Diagn Invest* 2017;29:198–202.
15 Schiller I, Vordermeier HM, Waters WR, *et al*. Comparison of tuberculin activity using the interferon-gamma assay for the diagnosis of bovine tuberculosis. *Vet Rec* 2010;167:322–6.
16 Whelan AO, Coad M, Peck ZA, *et al*. Influence of skin testing and overnight sample storage on blood-based diagnosis of bovine tuberculosis. *Vet Rec* 2004;155:204–6.
17 Marassi CD, Medeiros L, Lilenbaum W. The use of a Gamma-Interferon assay to confirm a diagnosis of bovine tuberculosis in Brazil. *Acta Trop* 2010;113:199–201.
18 Goff BS. Effect of dexamethasone treatment of tuberculous cattle on results of the gamma-interferon test for Mycobacterium bovis. *Vet Immunol Immunopathol* 1996;53:39–47.
19 Claridge J, Diggle P, McCann CM, *et al*. Fasciola hepatica is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nat Commun* 2012;3:853.
20 Gormley E, Doyle M, Duignan A, *et al*. Identification of risk factors associated with disclosure of false positive bovine tuberculosis reactors using the gamma-interferon (IFNγ) assay. *Vet Res* 2013;44:117.
21 Molicotti P, Bua A, Cannas S, *et al*. Preliminary data of different methods for the indirect diagnosis of Mycobacterium bovis infection. *New Microbiol* 2011;34:323–5.
22 Buddle BM, Ryan TJ, Pollock JM, *et al*. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. *Vet Microbiol* 2001;80:37–46.
23 Ryan TJ, Buddle BM, De Lisle GW. An evaluation of the gamma interferon test for detecting bovine tuberculosis in cattle 8 to 28 days after tuberculin skin testing. *Res Vet Sci* 2000;69:57–61.
24 Praud A, Boschiroli ML, Meyer L, *et al*. Assessment of the sensitivity of the gamma-interferon test and the single intradermal comparative cervical test for the diagnosis of bovine tuberculosis under field conditions. *Epidemiol Infect* 2015;143:157–66.