Transboundary and Emerging Diseases

REVIEW

Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication

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Summary

Existing strategies for long-term bovine tuberculosis (bTB) control/eradication campaigns are being reconsidered in many countries because of the development of new testing technologies, increased global trade, continued struggle with wildlife reservoirs of bTB, redistribution of international trading partners/agreements, and emerging financial and animal welfare constraints on herd depopulation. Changes under consideration or newly implemented include additional control measures to limit risks with imported animals, enhanced programs to mitigate wildlife reservoir risks, re-evaluation of options to manage bTB-affected herds/regions, modernization of regulatory framework(s) to re-focus control efforts, and consideration of emerging testing technologies (i.e. improved or new tests) for use in bTB control/eradication programs. Traditional slaughter surveillance and test/removal strategies will likely be augmented by incorporation of new technologies and more targeted control efforts. The present review provides an overview of current and emerging bTB testing strategies/tools and a vision for incorporation of emerging technologies into the current control/eradication programs.

Introduction

Bovine tuberculosis (bTB), caused primarily by *Mycobacterium bovis*, is endemic in many countries. This zoonotic disease constitutes a significant economic burden to the agricultural industries (Krebs, 1997). Economic losses worldwide are estimated to account for over $ 3 billion annually (Steele, 1995). In the United States of America (USA) and Great Britain, greater than $ 40 and £ 100 million annually, respectively, have been spent for the eradication of bTB in 2008/09. The USA total includes appropriated and emergency funding (K. Orloski, personal communication, 2010; Anon, 2010).

The control and eradication of bTB is mainly based on a test and slaughter policy and/or abattoir surveillance. Despite intensive eradication efforts over decades, bTB continues to be a problem with global perspective. Various factors have been identified as major constraints to eradication. In some countries, wildlife reservoirs constitute a continuous source for re-infection of cattle (Corner, 1994; Wilson et al., 2009; Table 1). Additional factors contributing to the persistence of bTB are limitations of

Table 1. Brief overview of bTB status and diagnostic strategies in the USA, in various European countries and in New Zealand

Country	Prevalence[1]	Wildlife reservoir[2] Maintenance (spillover/sentinel) hosts	Primary screening TST	Ancillary screening IFN-γ, proportion serial:parallel[3]	Compulsory removal of positives	Criteria for whole herd depopulation	Pre-movement testing
USA	<0.001	Deer	CFT	75:25	Yes (CCT and/or IFN-γ)	Decision made by state, federal, and animal health officials using a veterinary risk assessment and in co-ordination with the herd owner	Yes (CFT, positives confirmed by CCT or IFN-γ)[4]
Ireland	4.37	Badger (deer)	CCT	2:98	Yes (CCT and/or IFN-γ)	Decision of the local veterinarian[5]	Yes (CCT)[6]
United Kingdom	3.27					Decision made by Animal Health in consultation with	
Great Britain[8]	3.45	Badger (deer)	CCT	2:98	Yes (CCT and/or IFN-γ)	bTB policy officials and according to a local veterinary	Yes (CCT)[9]
Northern Ireland	2.67	Badger (deer)	CCT	0.1:99.9	No	risk assessment[7]	Yes (CCT)[9]
Spain	1.17	Wild boar, deer	CIT, CCT[10]	0:100	Yes (CCT and/or IFN-γ)	Decision of the local veterinarian[11]	Yes (CIT)[12]
Italy	0.58	(Wild boar)	CIT, CCT[10]	0:100	Yes (CIT and/or IFN-γ)	Decision of the local veterinarian[13]	Yes (CIT)[12]
France (OTF) overall	0.05	Wild boar, deer			Yes (CCT and/or IFN-γ)	At least 1 confirmed positive animal in a herd	Yes (CIT, IFN-γ)
Dordogne	0.8		CIT, CCT	9:91	Yes (CCT and/or IFN-γ)	At least 1 confirmed positive animal in a herd	Yes (CIT, IFN-γ)
Camargue	10.4		CIT, CCT	0:100	Yes (CCT and/or IFN-γ)	>10% positives in a herd	Yes (CCT, IFN-γ)
Germany (OTF)	0.01	Deer				Decision of the local veterinarian	
Austria (OTF)	<0.1	Deer				>40% positives in a herd	
New Zealand	0.32	Possum (ferrets, feral pigs, deer)	CFT	85:15	Yes (CFT and/or IFN-γ)[14]	Decision of the local veterinarian[15]	Yes (CFT,IFN-γ)[16]

[1]Herd prevalence numbers of bTB in 2007 (Anon, 2009c,d; Wilson et al., 2009; P. Livingstone, Animal Health Board, personal communication, 2009).
[2]Maintenance hosts, ''in brackets'' spillover or sentinel hosts (Corner, 2006).
[3]A serial IFN-γ test is used for re-testing TST-positive cattle, and a parallel test is used for testing TST-negative animals.
[4]Pre-movement testing is required for breeding animals moving out of states or zones that have not a TB-free status.
[5]Any herd or infected group of cattle where the total number of animals removed as reactor approaches 30% should be reviewed with a view to deciding the future strategy, e.g. confining of group to a specific location or possible removal of the whole group. IFN-γ assay and/or antibody-detection assay should be used prior to consideration of depopulation. These tests are of particular relevance in larger herds or those with valuable stock. Where the suitability of these tests has been ruled out or where they have failed to resolve the problem, then depopulation of the herd should be considered (E. Gormley, personal communication, 2009).
[6]Recommended for breeding stock. Compulsory for export.
[7]Whole herd slaughters are carried out occasionally, subject to a risk assessment by Animal Health veterinarians taking into account a range of herd and local factors. Mandatory conditions are culture confirmation of *M. bovis* infection and a high prevalence of test reactors (≥ 25%). Further factors taken into account are the local bTB incidence (breakdowns in low incidence areas, to prevent the development of new hotspots of bTB), the proportion of test reactors showing gross TB pathology at post-mortem examination and the severity of those lesions, the risk of re-infection if the premises are restocked with cattle, the affected herd's size, the risks posed to the local cattle herds and wildlife populations, the bTB history and herd incidence in the locality, the evidence that repeat TST and ancillary IFN-γ (and sometimes antibody) testing have failed to resolve the bTB incident (Great Britain: R. De la Rua-Domenech, personal communication, 2010; Northern Ireland: J. McNair, personal communication, 2010).
[8]Scotland has been an OTF region of GB since October 2009 (Anon, 2009f).
[9]Testing performed for movements from a 1 or 2 yearly tested herd, 60 days prior to movement.
[10]CIT in high-prevalence areas and CCT in low-prevalence areas.
[11]Herd with high number of positives in low-prevalence areas (exception: genetically high value animals).
[12]Testing performed for movements from a 1 or 2 yearly tested herd, 30 days prior to movement.
[13]Herd with high number of positives in low-prevalence areas.
[14]Serial testing: Cattle that are positive to both the CFT and IFN-γ are slaughtered. Parallel testing: Cattle that are negative to the CFT but positive to the IFN-γ are slaughtered.
[15]High number of reactors with extended infection observed at slaughterhouse. Depopulation of age cohorts with high prevalence of infection (>50%).
[16]CFT and in parallel IFN-γ in cases with increased risk of infection.

diagnostic tests (concerning both sensitivity and specificity), larger herd sizes, increase in animal movements and trade, and changing options for control, such as limitations on whole herd depopulation. Countries with successful eradication of bTB, like Australia, focussed their national eradication programs on the herd rather than on the individual animal given the limitations of test accuracy (More, 2009). The tuberculin skin test (TST), which constitutes the primary screening test for bTB, is recognized to be a good herd test but a poor test for identifying individual infected animals (Dawson and Trapp, 2004). As herd size continues to increase in many countries, it becomes ever more difficult to justify whole herd depopulation for both economic and animal welfare concerns. Globally, live animal trade is increasing. In addition, trade agreements increasingly include concepts like regionalization, zoning, and compartmentalization as principles of disease control, as proposed by international organizations (Anon, 2008d). In Europe, the improvement of competitiveness assuring proportionate animal movement is a cornerstone of the European Union (EU) Animal Health Strategy 2007–2013 (Anon, 2006). Similarly, a transition of the bTB program from a State classification system to a science-based zoning approach addressing disease risk is proposed in the USA (Anon, 2009c). In consequence, traditional control/eradication strategies will likely be reinforced with more targeted control efforts requiring additional tools (e.g. extended pre-movement testing and cost-efficient diagnostic tests with high accuracy in individual animals).

The objective of this review is to provide an overview of current and emerging bTB testing tools/strategies and a vision for incorporation of emerging technologies into the current control/eradication programs.

Current Testing Strategies

Brief description of current bTB status and diagnostic strategies in various countries

In the EU, the overall prevalence of bTB is currently slightly increasing. In 2007, 0.53% of cattle herds were bTB positive compared to 0.48% in 2006 (Anon, 2009c). Both officially bTB-free (OTF) and non-OTF countries reported an increase in the proportion of bTB positive cattle herds. Ireland and the United Kingdom are currently facing the highest herd prevalences in Europe (4.37 and 3.27%, respectively, Anon, 2009c). In Great Britain, the incidence of bTB is increasing (Anon, 2009e). Eradication of bTB is expected to take at least 20 years (Anon, 2009b). In face of a reservoir of infection in badgers, vaccination will likely become a major component of Great Britain's bTB eradication strategy. Recently, a field trial evaluating the practicalities of delivering an injectable *M. bovis* BCG vaccine for badgers has been initiated in England, with licensing expected in mid-2010 and that of an oral vaccine probably in 2014 (Anon, 2009b). Vaccine strategies for cattle in the United Kingdom are also under consideration (Anon, 2007a). Complete eradication is not only difficult to achieve in high prevalence countries but also in countries with low proportions of bTB-infected herds (Anon, 2009c). Even in some OTF countries bTB prevalences are currently slightly rising. Trade and wildlife reservoirs both in non-OTF and OTF countries are major factors for re-infections of and spillover to livestock.

New Zealand undertook a great effort over the last 15 years to eradicate bTB, and the herd point prevalence was reduced from 2.4% in 1993 to 0.35% in 2004 (Ryan et al., 2006). Complete eradication, however, has failed so far, most likely because of the presence of wildlife reservoirs. Attempts to halt disease transmission from wildlife to livestock by oral vaccination of possums with lipid-formulated *M. bovis* BCG are currently under evaluation (Ramsey et al., 2009). A recent field trial demonstrated that oral vaccination with BCG significantly protected possums against natural exposure to *M. bovis* (Tompkins et al., 2009). Vaccination is being considered as a possible adjunct to the current practice of reducing the population of possums (annual costs of possum control accounting for US$ 30 million; Anon, 2005) to hasten the eradication of the disease from wildlife reservoirs.

In the USA, the bTB eradication campaign has been highly successful. The prevalence of bTB has decreased from 5% in 1917 to <0.001% in 2009. Eradication, however, remains elusive and new strategies are proposed to specifically address the continued challenges of imported cases of bTB from Mexico and the presence of a wildlife reservoir (i.e. white-tailed deer) in Michigan and potentially Minnesota. These strategies include more stringent testing of cattle entering from Mexico, enhanced efforts to mitigate risks from wildlife, accelerating diagnostic test development to support increased surveillance, transition from a state-based to a regional or zoning classification system for designation of bTB status, and increasing the options for managing bTB-affected herds. These changes will most likely result in several opportunities for application of new testing strategies, particularly with pre-movement testing – both at the USA/Mexico border and for interstate shipment. Additionally, increased numbers of bTB-affected herds will be managed via a test and slaughter as opposed to a whole herd depopulation strategy.

In Canada, eradication efforts are ongoing based upon slaughter surveillance and test/removal strategies, similar to the USA. As with other countries, spillover from bTB-infected wildlife (e.g. elk in Riding Mountain National Park and bison in Wood Buffalo National Park) hinders eradication efforts (Nishi et al., 2006).

In Mexico, procedures equivalent to the USA bTB program were implemented in 2001 to enhance the capacity for export of beef cattle to the USA; however, focal areas of high prevalence still exist especially in dairy cattle. Additionally, bTB program activity varies greatly in Mexican states.

The current status of control efforts in Central and South America, Indonesia, Africa, and Eastern European countries is not clearly defined, yet bTB is a prominent concern for the livestock industries of many of these countries.

Primary screening for bTB in live cattle is performed using one of the variants of the TST: the caudal fold test (CFT), the (mid) cervical intradermal test (CIT), or the comparative cervical test (CCT). In addition, the interferon gamma (IFN-γ) assay is applied either as a confirmatory test of reactors to the CFT or CIT (serial testing), or in alongside TSTs to increase diagnostic sensitivity (parallel testing). Diagnostic strategies including requirements for removal of individual animals testing positive, whole herd depopulation, and pre-movement testing differ largely between countries and regions and are summarized in Table 1. In OTF countries, control programs are usually limited to passive surveillance by post-mortem examination of all slaughtered cattle. The following section gives an overview of current ante- and post-mortem testing tools and indicates modifications which could contribute to optimize disease control and eradication.

Tuberculin skin test (TST)

The TST represents the OIE prescribed test for international trade and constitutes a delayed type hypersensitivity test (Anon, 2008a). It measures dermal swelling primarily because of a cell-mediated immune response (CMI) three days after intradermal injection of purified protein derivative (PPD) in the skin of the caudal fold (CFT) or neck (CIT), respectively. The skin of the neck is regarded to be more sensitive to a tuberculin-related hypersensitivity reaction than the skin of the caudal fold. To compensate for this difference, higher doses of PPD may be used in the caudal fold. Because animals are frequently exposed to or infected with various non-tuberculous mycobacteria, cross-reactive responses to PPD-B may occur as many antigens contained within PPD-B are shared between non-tuberculous and tuberculous mycobacteria. The CCT is used to differentiate between animals infected with *M. bovis* and those sensitized to PPD-B as a result of exposure to other mycobacteria. Responses to bovine and avian tuberculins are compared according to OIE guidelines or those developed by national eradication programs (e.g. use of a scattergram in the USA).

Historically, the TST has been the primary antemortem test available to support bTB eradication campaigns. Advantages of the TST and reasons for its wide use are low costs, high availability, long history of use and, for a long time, the lack of alternative methods to detect bTB. Still, this test has many known limitations including difficulties in administration and interpretation of results, need for a second-step visit, low degree of standardization, and imperfect test accuracy (De la Rua-Domenech et al., 2006).

Performance of TST techniques may vary because of differences in tuberculin doses, PPD preparations, site of application, and interpretation schemes. In fact, disparate performances have been found in many international studies (Monaghan et al., 1994): 68–96.8% sensitivity and 96–98.8% specificity for CFT, 80–91% sensitivity and 75.5–96.8% specificity for CIT, 55.1–93.5% sensitivity and 88.8–100% specificity for CCT. Recent data from some countries or regions indicate a markedly reduced sensitivity of TST in certain situations. A study in Northern Ireland showed that as few as 59% of animals with confirmed *M. bovis* infection were detected by CCT in some herds (Welsh, M., and McNair, J., unpublished results). Similarly, herd ($n = 42$) sensitivity of CCT in fighting bulls in Camargue, France, was 58% (10.6% sensitivity for individuals, $n \geq 9000$) with more than 80% of bTB cases detected upon subsequent slaughter inspection (Keck, N., unpublished results). Twenty-eight of the 42 herds contained at least one culture-positive animal and 142 animals had tuberculous lesions upon slaughter inspection. Immunosuppression caused by stress (handling of fighting bulls) or anergy because of an advanced stage of bTB possibly account for the low sensitivity of TST in some circumstances. These data may represent the lower end of the performance spectrum of TST and not average values. Still, a low sensitivity constitutes a serious problem for the sustainable success of control and eradication programs.

Worldwide, the *M. bovis* strain AN5 is used for bovine tuberculin production (details and also concerns are summarized in the supporting information). Tuberculin potency is estimated by biological methods (*in vivo* testing of tuberculin batches in sensitized guinea pigs or cattle against an international reference standard; Anon, 2008b). Standardization of tuberculins is critical for test accuracy. Variations in PPD production methods may lead to different antigenic profiles and therefore to qualitative differences between PPDs (Seibert and DuFour, 1940; Landi and McClure, 1969; Haagsma et al., 1982; Tameni et al., 1998). Moreover, the in vivo potency tests are inherently variable and of questionable precision, demonstrated by studies with repeated batch testing (Good et al., 2008). Importantly, a recent comparison of

commercially available tuberculins has shown that if applied in a dose of 1 mg/ml, the majority of these tuberculins would not meet the required minimum dose of 2000 International Units (Bakker et al., 2009). In this study, potencies largely varied among the bovine tuberculins and, to a lesser degree, among the avian tuberculins. Marked variations were also found with regard to the relative potencies of tuberculin pairs (PPD-B and PPD-A) from the different manufacturers. As these tuberculins are used in current control and eradication programs, the marked variability of potencies may have direct implications for the diagnosis of bTB cases.

Improvements in the production and quality control of tuberculins would have the potential to enhance sensitivity and specificity of TST. In fact, improved and extended control of PPD activity after production by standardized methods resulting in higher reproducibility than *in vivo* guinea pig potency testing could markedly improve TST. Furthermore, more active and more specific tuberculins would be desirable. To date, limited progress has been achieved in this field, mainly because of the ill-defined nature of the antigens present in tuberculins as well as the complexity of PPD production.

Attempts to improve the TST by the use of defined antigens such as ESAT-6 (Pollock et al., 2003) have resulted in increased specificity compared to the PPD-based test (100 compared to 90%) but decreased sensitivity (82 compared to 86%). Only a high dose of the recombinant protein (400 μg) was able to induce these responses, which would make the test unaffordable. Co-administration of a synthetic lipopeptide resulted in induction of antigen-specific CMI using lower concentrations of ESAT-6. However, an adjuvant application harbours the risk of sensitization and constitutes, therefore, not a practical solution (Whelan et al., 2003). The combined use of ESAT-6, CFP-10, Rv3615c, and MPB83 in a cocktail of recombinant proteins with low protein doses (10 μg each) showed comparable sensitivity levels as the PPD-based TST in naturally infected cattle (Whelan et al., 2009). Encouragingly, a peptide cocktail containing 21 overlapping peptides of the same antigens (10 μg each) also enhanced sensitivity compared to recombinant proteins (Whelan et al., 2009). Additionally, an improved antigen cocktail (ESAT-6 and CFP-10 based) is currently being tested for use in humans (P. Anderson, personal communication) and application of these antigens to the TST for cattle is also currently under investigation (R. Waters, P. Anderson and K. Lyashchenko; research in progress). However, these promising results will need to be validated in large-scale field trials before such antigens can be used as part of control strategies.

Alternative tools for the read out of TST may improve its ease of use. Infrared thermography (IRT) is currently being evaluated in the USA to determine skin test reactivity without the need for direct measurement of skin thickness (Johnson and Dunbar, 2008). Preliminary data from 15 cattle sensitized with PPD-B, PPD-A, or nothing (control) indicate that 86% of cattle were correctly classified with infrared thermography compared to 80% with skin thickness measurements using callipers. In addition, infrared thermography may be performed 24 h after PPD injection when compared to 72 h with traditional reading; and, importantly, this technique may represent a more objective method. However, further studies are required to determine the specificity of the 24-h vs 72-h readings of infrared thermography as early arthus-type reactions may be falsely interpreted as positive at 24 h.

Interferon gamma assay

The IFN-γ assay (Rothel et al., 1990) is OIE listed as an alternative test for international trade (Anon, 2008a) and approved as a complementary bTB test by the United States Animal Health Association as described in USDA, APHIS, Bovine Tuberculosis Eradication, Uniform Methods and Rules (Anon, 2004a), and by the European Union (Anon, 2002). This in vitro assay is a laboratory-based test detecting specific cell-mediated immune responses by circulating lymphocytes. Briefly, the assay consists of two stages. First, heparinized whole blood is incubated with antigens (i.e., PPDs, specific antigens) for ~18–24 h. Antigenic stimulation induces production and release of IFN-γ by predominantly T lymphocytes. Second, IFN-γ present in the plasma supernatants is quantified in a sandwich ELISA. With PPDs, a differential optical density (OD) value is determined by subtracting the OD value achieved with PPD-A stimulation from that of PPD-B, thereby, analogous to an *in vitro* comparative skin test. In general, most laboratories also include a no stimulation negative control (e.g. PBS or media) and a mitogen or superantigen positive control [e.g. pokeweed mitogen (PWM) or staphylococcal enterotoxin B (SEB)].

The IFN-γ assay (Bovigam®, Prionics, Switzerland) is being incorporated into bTB eradication programs in many countries (reviewed by De la Rua-Domenech et al., 2006; Vordermeier et al., 2008), either in a serial testing regime as confirmatory test after CFT to enhance specificity or in a parallel testing regime to enhance sensitivity of TSTs (Table 1). Advantages of the IFN-γ assay are its increased sensitivity, the possibility of more rapid repeat testing, no need for a second visit to the farm and more objective test procedures and interpretation in comparison to the TST. Limitations comprise a reduced specificity, high logistical demands (culture start is required within 24 h after blood sampling), an increased likelihood of non-specific response in young animals (owing to natural

killer (NK) cell activity) and its high costs (reviewed by De la Rua-Domenech et al., 2006; Vordermeier et al., 2006), as well as the difficulties in the standardization of tuberculins already discussed in relation to the TST.

The combined use of the in vivo and in vitro CMI assays raises the question whether the IFN-γ responses are influenced by injection of PPDs for TST. Briefly, no significant effect can be observed after CCT (Doherty et al., 1995; Gormley et al., 2004; Coad et al., 2010). In contrast, injection of PPD-B for CFT boosts IFN-γ responses in cattle experimentally infected with *M. bovis* (Palmer et al., 2006). The boost was shorter than previously observed with sensitized cattle (Whipple et al., 2001) and lasted only from 3 to 7 days after the CFT. Importantly, there was no effect on the differential response to tuberculin and therefore no interference with the final assay interpretation. In naturally infected cattle, however, CFT-related boosting selectively increased the in vitro *M. bovis* PPD (PPD-B) response 3 days after CFT, resulting in an increased PPD-B response relative to the response to PPD-A (Coad et al., 2010). Additional studies are warranted to evaluate the influence of CFT on the IFN-γ responses (i) with tuberculous cattle exhibiting weak IFN-γ responses, (ii) with uninfected cattle, and (iii) to evaluate the effect in view of a possible depression of IFN-γ production.

Sensitivity and specificity of the IFN-γ assay have been estimated in a great number of international studies (reviewed by De la Rua-Domenech et al., 2006). Estimates of test sensitivity range from 73.0 to 100%, with a median value of 87.6%, and specificity from 85.0 to 99.6%, with a median of 96.6%. Variations in assay protocols may have resulted in disparate results in test accuracy between studies. More standardized procedures would be needed to strengthen reliability of screening tools within TB programs. In recent studies, parameters of the IFN-γ assay have been analyzed in view of defining a range of possible conditions (summarized in the supporting information). Importantly, assay conditions which have been identified to be of significant influence on the IFN-γ test performance and which have already been defined to optimize the assay (e.g. sample and culture conditions; validity controls and their interpretations) should be integrated in the commercial assay. Thus, users could refer to consolidated, standardized procedures at the implementation of the assay in their laboratory quality system. This would also increase the likelihood that the assay will be used in a more standardized way and it would facilitate national approvals of the IFN-γ (as it would not be efficient to repeat the validation of all relevant assay parameters in each country).

Many studies have demonstrated that the sensitivity of the IFN-γ assay is superior to that of the skin test (data from most relevant published studies and additional unpublished data are summarized in the supporting information). Concerns, however, exist about Bovigam specificity (Lauzi et al., 2000; Pollock et al., 2000; Palmer et al., 2006). The replacement of PPDs by defined antigens offers the greatest potential for the improvement of specificity (Pollock et al., 2000; Buddle et al., 2001). ESAT-6 and CFP-10 are outstanding diagnostic target proteins in the whole blood IFN-γ assay (Pollock et al., 2000; Vordermeier et al., 2001; Aagaard et al., 2003; Buddle et al., 2003; Waters et al., 2004; Vordermeier et al., 2005; Aagaard et al., 2006; Cockle et al., 2006). Results from a recent field trial in Great Britain indicate that a peptide cocktail composed of peptides from ESAT-6 and CFP-10 (E/C) achieved only a slightly better specificity (97.0%; Vordermeier, H., unpublished results) than the conventional PPD-based IFN-γ assay (96.6%; Anon, 2008f); IFN-γ sensitivities in this field trial were 91.0% for PPDs and 81.0% for E/C. The gain in specificity by the use of E/C may be especially striking in individual animals and in regions with a low bTB prevalence and in OTF countries. A study performed in Switzerland (OTF) testing a selected herd ($n = 69$) with a high percentage of non-specific reactors (i.e. false positives) in the PPD-based assay (specificity of 66.7%) resulted in an E/C-specificity of 97.1% (Vordermeier, H., and Schiller, I.; unpublished results), again, demonstrating the potential for improved specificity of the test when specific antigens are used in place of PPDs.

Defined mycobacterial antigens have not only the potential to increase specificity but also sensitivity: in a PPD-based IFN-γ assay the diagnosis of bTB may be masked by a high response to avian tuberculin superseding that to PPD-B, as may occur early after infection (Schiller et al., 2009c) or in animals co-infected with non-tuberculous mycobacteria such as *M. avium* ssp. paratuberculosis (Aranaz et al., 2006). As indicated earlier, however, overall results of current studies with defined antigens using ESAT-6 and CFP-10 demonstrated a reduced sensitivity compared to tuberculins, reduced by approximately 10% (Pollock et al., 2000; Buddle et al., 2003; Vordermeier et al., 2006; Vordermeier, H., unpublished results). Therefore, a wider range of antigens in addition to ESAT-6 and CFP-10 is needed for improving the sensitivity of specific antigen-based IFN-γ assays. In recent studies, promising antigen combinations for the IFN-γ assay resulting in improved sensitivity and specificity have been described (Cockle et al., 2006; Sidders et al., 2008; Schiller et al., 2009b). Importantly, Rv3615c and OmpATb were found to detect cattle with confirmed bTB but not responding to E/C. Recent data indicate a sensitivity of 89.0% and specificity of 97.0% for E/C combined with Rv3615c (Sidders et al., 2008; Vordermeier et al.,

2009). These data have been achieved after 24-h storage of blood samples. A shortening of pre-culture time, however, has the potential to further increase sensitivity of a peptide-based IFN-γ assay. As shown with E/C in experimentally infected cattle, 24-h storage of blood samples resulted in slightly decreased OD values and reduced predictive outcome using E/C compared to 8 h storage (Whelan et al., 2004). This warrants further examination in field reactors and with additional defined antigens.

High logistical demands limit the use of the IFN-γ assay. Currently, blood samples need to be transported to a laboratory and to be processed within 24 h. An ''in-tube'' or ''in-plate'' stimulation device allowing rapid stimulation of lymphocytes after blood collection would possibly have a positive impact on diagnostic sensitivity using defined antigens for stimulation. Such a device would surely be beneficial to overcome logistical difficulties and to reduce costs now required for ''express transportation''.

Current costs of the IFN-γ assay constitute a major disadvantage in relation to TST. Regarding full cost calculations of TST (e.g. $7-10 in the USA (M. Dutcher, personal communication, 2007) and £7.5 in GB (R. De la Rua-Domenech, personal communication, 2007), the difference between in vitro and in vivo CMI assays might be smaller than anticipated, particularly considering rising costs associated with on-farm visits. Yet constraints in terms of costs should be mitigated to make the IFN-γ assay more widely available. Increased use of the assay might allow high scale production at a lower price. Importantly, additional cost reduction could be achieved by applying automation at production and application (i.e. in diagnostic laboratories). Modification of the ELISA as a rapid ELISA would allow further reduction of laboratory costs.

The current interpretation of the Bovigam assay relies exclusively on the OD values of specific (PPD-based) stimulation and non-stimulation (PBS), without considering standards correcting possible plate-to-plate variations. The kit positive control exhibits a strong positive control and may produce a wide range of OD readings (Jungersen et al., 2002; Robbe-Austerman et al., 2006). Therefore, a different control would be needed to calibrate plate-to-plate variations. Alternative read-outs (such as percentage positivity) based on a standardized positive control should be considered. An alternative calculation of the cut-off may contribute to improve test accuracy.

The IFN-γ assay is generally applied to animals of more than 6 months of age. The reason for this age restriction is a high likelihood of non-specific responses to mycobacterial antigens in young animals triggered mainly by natural killer (NK) cells (Olsen et al., 2005). This phenomenon may possibly be overcome by the depletion of NK cells, thus allowing application of the IFN-γ assay with samples from animals <6 months of age (H.M. Vordermeier and A. Storset, research in progress).

In summary, improving specificity by the replacement of PPDs by defined antigens for stimulation, their application as an in-tube/in-plate stimulation device, in combination with a modified interpretation/cut-off and cost reduction may represent useful developments for the IFN-γ assay. Thus, the assay may be adapted to provide a highly specific and sensitive screening test for use as a stand-alone test or in conjunction with other screening tests. In addition, a multispecies IFN-γ assay for non-bovine species, such as camelids, cervids, dogs, and cats, would be a welcome tool for bTB screening and control in those species and for overall bTB control.

Post-mortem diagnosis of bTB

Abattoir surveillance with lesion detection during commercial slaughter is used as cost-efficient method for passive surveillance of bTB both in OTF and in non-OTF countries, in the latter to supplement live cattle testing. The finding of a tuberculous animal at slaughter initiates an investigation through TST of the herd of origin and any other potentially exposed animals (Whipple et al., 1996; Olea-Popelka et al., 2008). The success of such investigations is highly variable. In the USA, because of the lack of uniform animal identification regulations combined with inconsistent record-keeping, only 50–70% of such investigations result in identification of the herd of origin and in finding all exposed animals (Kaneene et al., 2006). In Australia, which has successfully eradicated bTB, meticulous animal identification was credited as a major factor in successful eradication (Cousins and Roberts, 2001; Radunz, 2006). In the British Isles, national cattle tracing systems allow accurate back-tracing following identification by meat inspectors of any suspect granuloma in commercially slaughtered cattle. The herds of origin of such animals are placed under precautionary movement restrictions pending confirmation of bTB in slaughter lesions by histopathology, culture and/or PCR.

Generally speaking, lesion detection exhibits a major lack in sensitivity. The sensitivity of abattoir surveillance may be as low as 28.5%, as indicated by a recent study in the USA (Anon, 2009a). Possibilities to improve the sensitivity of post-mortem detection of bTB may be limited. Continuous education and training of slaughter inspectors are certainly of major importance. In addition, the combined use of liquid and solid culture media has been reported to improve culture sensitivity (Hines et al., 2006). The addition of assays such as PCR detection of *M. bovis* DNA from formalin-fixed specimens has further enhanced some surveillance programs (Miller et al.,

2002). PCR assays to detect MTB complex bacteria are currently less sensitive than culture techniques. Therefore, important further steps would be to improve PCR sensitivity and to standardize PCR methods.

Genotyping of bacterial isolates or PCR products is increasingly becoming a standard tool for epidemiological disease control and eradication. Distinguishing *M. bovis* strains on a molecular basis provides important insights into the sources of infection and identification of practices or environments which may aid the spread and maintenance of tuberculosis. Importantly, transmission routes between livestock and wildlife may be identified by strain typing. In addition, transmission routes of bTB within livestock via animal movements become evident, a prerequisite for targeted disease control aiming at testing all potentially exposed animals. More details for post-mortem diagnosis of bTB (lesion detection, culture, PCR, and strain typing) are included in the supporting information.

Emerging Testing Strategies

Antibody detection assays

Antibody-based bTB assays offer the possibility for convenient, flexible, and generally cost effective platforms for bTB surveillance. The development path for an accurate antibody-based bTB test has been particularly arduous and disappointing, most likely because of the inherent nature of the disease. *Mycobacterium bovis* infection of cattle elicits an early and robust CMI response and a peculiarly weak antibody response (Pollock et al., 2001; Welsh et al., 2005). In early studies, the use of crude mycobacterial preparations that provided generally satisfactory test sensitivity resulted in poor assay specificity because of broad cross-reactivity with non-TB mycobacteria, such as *M. avium* (O'Loan et al., 1994; Gaborick et al., 1996). Many attempts have been made to identify immunodominant proteins with improved specificity. Antigen discovery efforts have unveiled numerous seroreactive targets (Fifis et al., 1992; Cataldi et al., 1994; Lyashchenko et al., 1998; Amadori et al., 2002; Koo et al., 2005). So far, MPB70 and MPB83 proteins of *M. bovis* appear to be the major serodominant antigens for detection of tuberculous cattle (Wood et al., 1992; Lightbody et al., 1998; McNair et al., 2001; Liu et al., 2007; Wiker, 2009).

Recent advances in both antigen discovery and immunoassay technology have facilitated progress in developing novel antibody-based tests for bTB. Importantly, studies have demonstrated the benefits of multi-antigen approaches, relying primarily on MPB83 plus additional proteins (Greenwald et al., 2003; Waters et al., 2006; Whelan et al., 2008). Cocktails of carefully selected antigens or multi-epitope fusion proteins have been used to demonstrate improved test sensitivity (Waters et al., 2006; Lyashchenko et al., 2008; Whelan et al., 2008). Regardless of the antigen cocktail or single-antigen approach, improved detection technologies offer opportunities for serological assays not previously realized with standard technologies (Waters et al., 2007; Whelan et al., 2008; Green et al., 2009). Apart from the selection of *M. bovis* -specific antigens for antibody detection, the immunoassay format and detection technology are crucial for developing accurate serodiagnostics. Promising recent developments include MAPIA (Waters et al., 2006), a fluorescence polarization assay (FPA) (Jolley et al., 2007), a rapid immunochromatographic test (Lyashchenko et al., 2008), a 96-well plate multiplex system (Whelan et al., 2008), a dual path platform assay (Greenwald et al., 2009; K. Lyashchenko, personal communication), a chemiluminescent platform (Green et al., 2009), and an improved ELISA (John Lawrence, IDEXX, Portland, Maine, personal communication, 2009), which are listed in a chronological order of development. Some may be used as a quick animal-side test for field use (Waters et al., 2006; Lyashchenko et al., 2008), whereas others are laboratory-based for automated large-scale testing (Jolley et al., 2007; Whelan et al., 2008; Green et al., 2009). MAPIA (Chembio Diagnostic Systems, Inc., Medford, NY, USA) is an efficient tool for large-scale antigen screening as well as for characterization of serological responses in cattle and other host species infected with *M. bovis* (Lyashchenko et al., 2004; Waters et al., 2006; Lyashchenko et al., 2008). The multiplex immunoassay (Enfer Scientific, Naas, Ireland) simultaneously detects and analyzes antibody responses to multiple antigens within a single well of a 96-well plate array format (Whelan et al., 2008). With the multiplex chemiluminescence (Enfer Scientific), single antigen chemiluminescence (Seralyte-Mbv, Pritest, Redmond, Wa), rapid immunochromatographic and dual path platform (Chembio Diagnostic Systems) tests, antibody responses are detected as early as 2 weeks – 2 months after infection with samples from experimentally infected cattle (Waters et al., 2006; Whelan et al., 2008; Green et al., 2009; K. Lyashchenko, unpublished observations). Additionally, field studies with each of these assays are encouraging (Meyer and Orloski, 2007; Whelan et al., 2008; K. Lyashchenko, unpublished observations); however, exact estimates of test accuracy under varying herd prevalence rates is still pending. The FPA (Diachemix, Grayslake, IL, USA) detects antibody responses to a small polypeptide of MPB70 using a convenient and automated platform. The FPA is highly specific (>99%), however, lacks sensitivity (26%) for use as an individual animal test (Meyer and Orloski, 2007; Ngandolo et al., 2009). The low sensitivity of the test may be because of the nature of the bovine immune response

to MPB70 (i.e. generally occurs later during the course of disease), the limited antigenicity of the peptide used for the test and lower sensitivity of FPA compared to other technologies. Possibilities to include several antigens in the FPA platform would be very limited.

In summary, preliminary studies have indicated the potential for antibody-based tests primarily with positive samples from experimental infection trials, limited numbers of samples from naturally-infected cattle, and in certain instances, with un-blinded sample sets. Large-scale field trials are required to clearly define serological test accuracy, especially in direct comparison to CMI-based tests. Testing should include samples from appropriate populations of animals, as test accuracy may be impacted by environmental and host factors such as disease prevalence, nutrition, handling, stress, environmental mycobacterial exposure, and parasite burden. Effects of PPD administration for TSTs (e.g. CFT versus CCT, time after TST, tuberculin source and dose) on serological test results should also be considered. As with TST and IFN-γ -based assays, serological test performance will likely vary considerably between field test sites; thus, multiple studies at varying locales may be required. More details for antibody-detection assay are included in the supporting information.

Research Tests

Potential for new readouts of infection

New research tools are rapidly emerging for the characterization of bovine immune responses. Numerous primer sets are available for characterizing bovine cytokine/chemokine/transcription factor/etc. responses to bTB via real-time PCR techniques (Thacker et al., 2007) and bovine immune microarrays (Coussens and Nobis, 2002; Tao et al., 2004; McGuire and Glass, 2005; Almeida et al., 2007; Machugh et al., 2009). In addition, strategies for sequencing transcriptomes are being developed (Thacker, T., and Vordermeier, H.; unpublished results). The development of bovine cytokine and chemokine multiplex systems detecting several parameters in a single sample (Coad et al., 2010) raise the possibility to enhance sensitivity and/or specificity of antemortem cellular immunity diagnostic systems based on parameters like IL-10. The increased availability of monoclonal antibodies recognizing bovine cytokines also provides the opportunity to intervene in assays such as the IFN-γ assay to overcome modulating effects of cytokines such as IL-10 to increase sensitivity as has been demonstrated by Denis et al. (2007) for bTB and by Buza et al. (2004) in the case of Johne's disease. Such reagents and read-out systems may also be useful to counter the observed potential loss of sensitivity of TH1-cell-mediated diagnostic systems (like TST and IFN-γ assays) reported recently in the case of experimental Fasciola hepatica/*M. bovis* co-infections (Flynn et al., 2009). Thus, further cellular immunity-based tests may offer opportunities to improve test sensitivity in certain indications.

Options for Future Control Strategies

Incorporation of modified existing and emerging tests into traditional eradication campaign algorithms

Diagnostic tests may be used in different ways within control and eradication programs, depending on different epidemiological settings (for details please refer to the supporting information). Improved diagnostic tests will be a crucial factor for future eradication of bTB. There is a special need for individual animal tests, as high test accuracy represents a pre-condition for cost-efficient, targeted disease control. The positioning of modified existing and/or emerging tests as screening tests within bTB control/eradication programs will mainly depend on their performances and costs.

Changes required for the IFN-γ assay to overcome its current disadvantages and to allow its use as individual animal test comprise improvements of specificity, overall test accuracy and ease of use, in addition to cost reductions. New antigen combinations have been found which complement the current lead diagnostic antigens ESAT-6 and CFP-10 and result in superior sensitivity, specificity, and predictive values compared to PPD-based tests (Sidders et al., 2008; Schiller et al., 2009b; Vordermeier et al., 2009). Interestingly, recent data, using relatively small sample numbers that need to be confirmed in larger trials, indicate that even ESAT-6 and CFP-10 alone (without further antigens) could result in superior performance of the IFN-γ assay compared to that of CCT. Options how to utilize a new IFN-γ assay in bTB programs will largely depend on its costs. Currently, a major part of total IFN-γ test costs is attributed to logistics and laboratory costs. An automatable version of the IFN-γ assay, e.g. comprising in-tube/in-plate whole blood stimulation applicable in the field or in the laboratory, could significantly reduce these costs. Thus, an improved version of the IFN-γ assay may represent a powerful primary screening tool for bTB.

Antibody-based assays offer additional opportunities for bTB programs. Major advantages are ease of testing, ability to achieve quick results, and low costs. Possibilities for application of emerging antibody-based assays within programs will mainly depend on their performances. In countries with limited resources, where large-scale culling is not cost effective, serological tests are appealing as a cost-efficient means for removal of cattle with increased risk of bacterial shedding because of advanced disease (Pollock et al., 2005; Ngandolo et al., 2009). In countries

with established bTB eradication programs, serological tests could complement current CMI-based testing strategies and offer additional opportunities for herd, movement, sale barn, and abattoir testing. One important application of serological tests is the potential to detect animals anergic on CMI-based assays (Coad et al., 2008). As a pre-movement test, large numbers of animals could be rapidly tested at holding sites prior to movement. For slaughter surveillance, a serological assay would need to be highly specific (e.g. 99.9%) as false positive results would result in costly trace-back/epidemiological investigations. However, possibilities to use antibody-based assays to enhance slaughter surveillance may be limited for financial reasons. A rough cost/benefit calculation showed that costs per antibody-based test (including laboratory costs) must be very low (lower than $ 0.8) to achieve added value (increased overall sensitivity) at competitive costs compared with traditional slaughter surveillance (inspectors visual exams and follow-up of granulomas). This clearly limits the use of an antibody-based test as complimentary test for passive surveillance at slaughter. This conclusion is especially surprising, as we calculated a rather ''optimistic case'' in our cost/benefit analysis by assuming 90.0% sensitivity and 99.9% specificity for a potential serological assay (all other numbers for the cost/benefit analysis were derived from USA's slaughter surveillance costs for histopathology, PCR or culture and slaughter surveillance numbers from 2008; for details please refer to supplemental Table S1).

In countries lacking industry or government indemnity support for condemned animals, a test that only detects animals that are most likely shedding the organism may be used to diminish risks of transmission to other animals or humans. In this case, a high-specificity/low-sensitivity test at low cost may be applied even when disease prevalence is high. Herds may then be identified for follow-up testing with TST and IFN-γ assay. This procedure could also be applied as a complementary pre-movement test, especially when animals are moved from ''at risk'' regions. As with slaughter surveillance (i.e. inspectors visual exams), a lower sensitivity test would be used to identify herds for follow-up testing. This alternative strategy may be considered for countries with limited financial resources to significantly reduce (not necessarily to eradicate) bTB and its economic and zoonotic damage.

In summary, new screening tests are likely to be soon available and to offer further opportunities for control/eradication of bTB.

Conclusions

Considering current trends associated with bTB control/eradication programs (e.g. global increase in live animal trade, alternative strategies for whole herd depopulation, general financial limitations to fund programs) will be important to focus resources increasingly to targeted control strategies. Thereby, pre-movement testing on a national and international basis is becoming more important. Effective pre-movement testing, however, requires diagnostic tests with a high reliability as individual animal tests. Currently used screening tests (TST and IFN-γ assay) have originally been designed to meet demands as herd tests and their performances depict a broad range of sensitivity and specificity. A prerequisite to improve the accuracy of screening tests in individual animals would be to stipulate performances to the upper end of the performance spectrum. New or modified screening tests will be needed for cost-efficient, targeted disease control. Importantly, the replacement of tuberculins by defined antigens has the potential to significantly improve the performance of diagnostic tests. New testing technologies for disease screening are likely to be soon available. Authorities and other stakeholders that benefit from bTB programs should support the development and field validations needed to have improved diagnostic tools. Lacking a gold standard for the overall diagnosis of bTB (culture is regarded as gold standard for confirmatory diagnosis of bTB), the validation of new immunological assays requires further statistical tools (e.g. Bayesian analysis, calculation of Odds ratios).

Great progress has been achieved in the fields of culture technologies (e.g. liquid culture systems) and typing of mycobacterial strains. Molecular typing of mycobacteria isolated from domestic livestock and from wildlife allows establishing epidemiological links, necessary for the development of successful control strategies. Thereby, not only potential transmission routes between livestock and wildlife are identified but also the spread of bTB by trade. Epidemiological identification of infection sources and routes are a necessary part of targeted and effective disease control.

International trade inevitably represents a risk to introduce bTB. According to the current EU legislation, Member States or regions may achieve an OTF status if the percentage of infected bovine herds has not exceeded 0.1% per year and if at least 99.9% of herds have been OTF; both conditions are required for six consecutive years (Anon, 2004b). Individual herds are declared OTF if all cattle over six weeks of age reacted negatively to two TSTs (the first six months after the elimination of any infection and the second six months later). OTF countries or regions may be dispensed from tuberculin testing, meaning that surveillance is limited to post-mortem examination of all slaughtered cattle. For trade, cattle must originate from OTF countries or regions, or they must originate from an OTF herd and have been

subjected to a TST with negative result within 30 days prior to shipment. Animals under six weeks of age are exempt from TST. There are four major possibilities how bTB may be spread by trade: First, re-infections or residual undisclosed infection of herds declared as OTF in non-OTF regions are not detected because of long periods between tests or false negative test results, especially at low disease prevalence (e.g. low sensitivity at early infection stage) or in cases of advanced infection with anergy. Second, infections of calves under six weeks of age are not detected as they exempt from testing. Third, new infections of herds in OTF regions (e.g. originating from wildlife, from extended animal contact on shared pastures, or from trade) may be undetected for a long period, as testing is optional in these regions. These issues warrant the question whether future approaches to manage the risk of introducing bTB should consider a lowering of the pre-export testing age, more sensitive pre-export testing of traded cattle (through parallel TST and blood testing and/or more severe interpretation of the TST), compulsory post-import testing, channeling imported cattle into approved quarantine units, and supplemental bTB surveillance in geographic areas that have an increased risk for exposure to wildlife TB. For economic reasons and competitiveness of agriculture, it is crucial to declare herds OTF as soon as possible to facilitate trade. More sensitive methods than TST, however, might be needed to prevent movement of animals infected with bTB, especially in the presence of wildlife reservoirs. Fourth, South American camelids form a special risk to transmit TB to cattle, as antemortem diagnosis of TB is problematic in camelids. Directive 92/65/EEC (Anon, 1992) states that the TST is the official method for certification of TB freedom in South American camelids intended for international trade in the EU. However, TST has not been fully validated in camelids, and recent studies indicate an extremely low sensitivity of this test in llamas. Dean et al. (2009) found that only 2 of 14 llamas with confirmed TB were positive by TST, as opposed to all of the 14 animals reacted positive by antibody-detection. Camelids are increasingly kept on pastures in close proximity to livestock cattle, illustrating that infected camelids represent a great risk to transmit TB to cattle. Therefore, diagnostic tests with reasonable sensitivity/specificity in camelids are highly needed for disease control and for international trade, and should be integrated into current legislation.

In the USA, requirements to achieve an OTF status are more stringent than in the EU. A state or zone must comply with the provisions of the Uniform Methods and Rules (Anon, 2004a) to qualify for OTF status. According to these rules, a state or zone must have zero percent apparent prevalence of infected cattle and bison herds and no findings of TB in cattle or bison in the state or zone for the previous 2–5 years, depending on the past recent history of TB in the state or zone. Points of testing for eradication/control campaigns include importation, within region movement, herd certification, and slaughter surveillance. For importation, OTF countries/states/zones may ban or restrict importation of cattle from non-OTF countries/states/zones. The USA imports cattle only from low prevalence states in Mexico; however, bTB-infected cattle still enter the USA from Mexico. While the vast majority of these cattle are targeted for short-term feedlots, occasionally they are housed near adult cattle and transmit the disease to USA stock. Although not possible for political reasons, banning importation of cattle from Mexico would diminish the number of cases of bTB within the USA. Likewise, states may consider denying access of cattle from bTB-affected regions/states within the USA. OTF regions may also impose stringent testing protocols to limit the risk of bTB-infected cattle from entering, especially when animals are from non-OTF or ''at-risk'' regions. Politically, defining regions that are ''at risk'' for bTB may provide leverage for policy makers and regulators to target populations for increased testing. Prior to entry into OTF regions, ''at risk'' cattle may then be subjected to additional testing and stricter interpretation(s). Additionally, regions may adopt bTB testing policies for within region movement and herd certification; thereby, providing additional assurance of bTB status that may benefit trade possibilities. Blood-based tests are particularly convenient for increased frequency and wide-scale testing, especially with improved tests offering high specificity.

In summary, new diagnostics with technological advances offer great promise and will be essential to fight bTB. Focus should be laid on the cost-effectiveness of diagnostic tests within bTB programs. A cost-effective test does not generally mean the cheapest test. Sustainable use of improved bTB diagnostics should go along with mid-term and long-term overall cost reductions spent for the eradication of bTB. Cost-effectiveness analysis including emerging tests, as soon as these are available, may help to identify testing strategies that provide the greatest impact with the lowest cost per unit of output.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Comparative performance of diagnostic techniques for bTB in different epidemiological settings.

Table S1. Numbers and assumptions used for cost/benefit evaluation of antibody-based tests integrated in slaughter surveillance

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