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Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*)

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ABSTRACT

The application of diagnostic tests for bovine tuberculosis in wildlife poses formidable technical difficulties and the use of the gamma interferon assay offers a simplified approach to testing wild animal species. We compared the performance of the gamma interferon assay in African buffalo (*Syncerus caffer*) under the recommended guidelines for interpretation of test results and found a high sensitivity (92.1%) at the cost of a greatly reduced specificity (68.3%). The optimised cut-off value for positive test results under local conditions was identified at an optical density of 0.385 at wavelength 450 nm as the preferred compromise between sensitivity and specificity. Additional optimisation approaches to improve test performance were examined and showed that the application of 'a priori exclusions' of test results on the basis of reactivity to fortuitum PPD (sensitin produced from *Mycobacterium fortuitum*) and to a lesser degree, avian PPD, increased specificity without losing sensitivity. The implications of these findings on a modified testing protocol adjusted to include measurement of immune responsiveness to fortuitum PPD and other interpretation schemes are discussed.

1. Introduction

The intradermal tuberculin test (IDT), or skin test, is still the most widely used method to diagnose bovine tuberculosis (BTB) in cattle in countries worldwide. Limitations of the IDT in cattle have been mainly described in developed countries and include aspects relating to test performance (Wood et al., 1992; Neill et al., 1992; Monaghan et al., 1994), source of tuberculin PPD (Cagiola et al., 2004) as well as to logistical drawbacks in terms of repeated handling of animals and the minimum testing interval (Radunz and Lepper, 1985).

Developing countries face a number of constraints in implementing and maintaining a bovine tuberculosis control scheme. In remote areas difficult accessibility, long travelling distances and large scattered herds are aggravating logistical constraints in addition to the frequent lack of veterinary capacity and handling facilities for cattle. In the communal farming systems of sub-Saharan Africa, BTB testing is typically performed at communal diptank sta-

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tions where cattle owners in the district muster their herds in weekly or two-weekly intervals to receive general veterinary extension services. Failure of owners to present the injected cattle for test interpretation three days later is among the most common causes of the limited efficacy of BTB control in those areas. These factors constitute a high financial burden for governments and render BTB testing in developing countries less efficient and affordable.

The development of the gamma interferon (IFNγ) assay as an ancillary test for BTB diagnosis has improved the sensitivity of BTB testing (Wood et al., 1991). Cattle with early *M. bovis* infections are more readily detected by the IFNγ assay than the IDT (Neill et al., 1994) and parallel interpretation of both tests exceeded their individual diagnostic sensitivities (Whipple et al., 1995). The achieved specificity of approximately 96% was initially considered sufficient for BTB control purposes in cattle and could not be increased further without compromising the test's sensitivity (Wood et al., 1991; Buddle et al., 2001). As the incidence of *M. bovis* declined, a need for improved specificity arose and alternative antigens, i.e. early secreted antigenic target 6 kDa protein (ESAT-6) and culture filtrate protein (CFP)-10 were introduced in some laboratories to replace the tuberculin purified protein derivatives (PPDs), often at a loss in sensitivity (Buddle et al., 2009). As an objective, laboratory based test the IFNγ assay is not designed to consider differential interpretation for infected and uninfected herds, respectively.

Once bovine tuberculosis has established itself in an indigenous wildlife population it is difficult to control and probably impossible to eradicate. Despite its status as maintenance host for *M. bovis* the African buffalo is of high commercial and ecological value and diagnostic tools used towards BTB control are required to offer maximum sensitivity and specificity (Michel et al., 2006). We have previously observed false positive test results in free-ranging buffaloes when using the standard protocol for the gamma interferon assay. We have further established that false positive test results may be caused by sensitisation of the animals with environmental mycobacteria (Michel, 2008a). Subsequently the commercial assay was modified into a triple comparative test set-up. In addition to the standard test format based on stimulation of whole blood with bovine and avian tuberculin PPD, IFNγ produced in response to sensitin derived from *M. fortuitum* (fortuitum PPD), was explored. The results suggested that fortuitum PPD could be of potential value in detecting non-specific sensitisation in cattle and buffalo, hence possibly allowing improved test specificity in uninfected herds and populations. This modified IFNγ test protocol has been in use in the Kruger National Park (KNP) and other projects since 2000, but has to date not been formally validated. It was therefore the aim of this study to use data sets generated from the field application of the IFNγ assay in buffalo to determine measures to predict the BTB status and subsequently to improve test validity for African buffaloes by determining the most appropriate cut-off value(s) for the IFNγ test under local conditions. Our validation analyses furthermore include a comparison of the standard IFNγ test protocol with the modified protocol including fortuitum reactivity, and protocols optimized based on our findings in this study.

2. Materials and methods

2.1. Animals

In total, IFNγ test data from 1875 known uninfected buffaloes from 20 farms and parks were collated between 2001 and 2005, from which 344 samples from 14 herds were selected for analysis based on availability of skin test records and repeated test occasions to substantiate the negative BTB history of the herds. All farms were registered for breeding buffaloes which are free from specified controlled diseases, including bovine tuberculosis. All testing formed part of the routine statutory testing protocol for buffaloes. The test protocol for these breeding projects is enforced by the responsible state veterinarian and prescribes that all breeding stock are sourced from certified tuberculosis free herds and their infection status is monitored by means of a herd test once every three years. Offspring are tested for BTB according to a five-phase protocol applied over a two year period using a combination of the IFNγ assay and a comparative IDT before they can be classified as BTB negative.

Data from 149 infected buffaloes were selected from herds examined during bovine tuberculosis surveys in the endemically infected KNP (19) and Hluhluwe-iMfolozi Park (HiP) (71) between 1998 and 2007 (Grobler et al., 2002; Hofmeyr et al., 2003; Michel et al., 2006). Additional samples were sourced between 2000 and 2004 from three different research trials involving experimentally or naturally infected buffaloes (59) (De Klerk et al., 2006, 2010; Michel et al., 2007).

2.2. Necropsy and bacteriological confirmation

All culled buffaloes from infected herds were subjected to a detailed *post mortem* examination and in the majority of cases tissue samples were collected for bacterial culture. Isolation and identification of mycobacteria was performed as described previously (Bengis et al., 1996; Michel et al., 2008b).

2.3. Production of sensitin from M. fortuitum

Mycobacterium fortuitum cultures (ATCC strain 6841) were grown in 7H9 Middelbrook medium supplemented with OADC (oleic acid, albumin, dextrose, and catalase) (Biolab Diagnostics, Wadeville, South Africa) at a final concentration of 0.1%. The cultures were incubated at 37 °C for three to four weeks with loosened caps and occasional shaking of the flasks. Before harvesting, the cultures were autoclaved at 121 °C for 20 min and filtered through a Whatman 40 filter paper. The culture filtrates were precipitated overnight with trichloracetic acid (TCA) at a final concentration of 4%. On the following day the protein precipitate was concentrated by centrifugation (4000 rpm, Beckman-Coulter Allegra X22R) and washed in succession twice with 1% TCA and once with phosphate buffered

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Table 1
Interpretation criteria for reactor classification in a study on gamma interferon assays to diagnose *M. bovis* infections in African buffaloes. OD_{bovine}, OD_{av}, OD_{fort}: optical densities measured for plasma stimulated with bovine, avian, fortuitum PPDs, respectively. OD_{nil}: optical density measured for unstimulated plasma. The test result was considered valid if OD_{nil} or OD_{fort} was lower than or equal to 0.35.

Bovine reactor	Avian reactor	Multiple reactor	Equal reactor
$OD_{bovine} - OD_{av} > 0.2$ and $OD_{fort} - OD_{nil} \leq 0.15$	$OD_{av} > (OD_{bovine} + 0.1 \times OD_{bovine})$	$OD_{bovine} - OD_{av} > 0.2$ and $OD_{fort} - OD_{nil} > 0.15$	$(OD_{bovine} + 0.1 \times OD_{bovine}) > OD_{av} > (OD_{bovine} - 0.1 \times OD_{bovine})$

saline (PBS), pH 7.2. The concentrated, wet pellet was weighed and dissolved in PBS containing 0.01% Tween 20, pH 7.2, to give a final concentration of 20 mg/ml (wet weight/volume).

2.4. Assay for bovine gamma interferon

The standard testing protocol for the Bovigam IFNγ assay was performed as per manufacturer's instructions and for the interpretation of test results the criteria reported by Whipple et al. (2001) were adopted. In brief, animals were classified as BTB positive if $OD_{bovine} - OD$-control was greater than 0.049 and if OD_{bovine} was greater than OD_{avian}.

The modified testing protocol comprised the following changes to the above protocol. When setting up blood cultures an additional 1.5 ml aliquot of whole blood was stimulated with 500 μg of fortuitum PPD and incubated as recommended for the standard blood cultures. All plasma samples were assayed in parallel according to the manufacturer's instructions. The data was analysed taking into consideration the optimized cut-off value for OD_{bovine}, as well as the reactivity to fortuitum PPD, avian PPD and the nil control (unstimulated sample), using the criteria listed in Table 1 for classifying reactor animals.

2.5. Data analysis

The sensitivity of the IFNγ assay was determined from data collected from known infected buffaloes, whereby their infection status was confirmed by culture in the majority of cases. Where this was not possible for logistical reasons, diagnosis was made by means of a detailed post mortem examination by experienced veterinary professionals. The sensitivity was calculated as the proportion of test positive infected animals from the total number of infected animals examined (Toma et al., 1999).

The specificity of the IFNγ assay was determined using test data from buffalo herds with a history free of bovine tuberculosis and with sustained negative IFNγ and IDT test records. The specificity was defined as the proportion of test negative animals from the total number of negative animals examined.

Confidence intervals for specificities and sensitivities were calculated following Thrusfield (2005): we used the standard formula for proportions unless the sensitivity or specificity in question exceeded 95%, in which case the method of Wilson (1927) was used. The Systat software was used for all statistical analyses (©Systat Software 2008).

2.5.1. Predictive value for establishing buffalo BTB status of IFNγ response to stimulation with bovine, avian and fortuitum PPD, and circulating (unstimulated) IFNγ

We used logistic regression analysis with BTB status as the dependent variable and each of the optical density values (OD_{bovine}, OD_{avian}, $OD_{fortuitum}$, OD_{nil}) as independent variables to evaluate which variables contributed to predicting individual BTB status. The fit of single-factor and multiple factor models was assessed using Akaike's Information Criterion (AIC; lower AIC score indicates better fit of model to data). This initial analysis is aimed only at establishing which of the OD values have significant utility in TB diagnosis. We are not using these models to predict actual cut-off values for positive versus negative test results, because host responses to different antigens must be evaluated by comparing them against the animal's response to bovine tuberculin. Model-determined cut-off values for the various antigens would be misleading, as they would be treating all OD values as independent pieces of information, not as comparative against OD_{bovine}.

2.5.2. Optimizing test criteria to maximize test validity

We structured our test validity optimization procedure in two sequential steps. First, using OD_{bovine} alone as the primary indicator for BTB status, we asked: Given the data from our study population of buffalo, what OD_{bovine} cut-off value maximizes the proportion of animals that are correctly diagnosed as BTB-positive or BTB-negative? To answer this question, we compiled frequency distributions of BTB-negative and BTB-positive animals found in each 0.01 interval of OD_{bovine} readings. We then converted these frequency distributions to percentages (rather than numbers of animals in each interval of OD_{bovine} readings), allowing us to pick an optimal OD_{bovine} cut-off value based on the shapes of the two distributions alone, not on the prevalence of BTB in our study population. For a given OD_{bovine} cut-off value, test sensitivity is the cumulative percentage of BTB-positive animals with OD_{bovine} readings larger than the cut-off value. Test specificity is given by the cumulative percentage of BTB-negative animals with OD_{bovine} readings smaller than the cut-off value. The optimal cut-off value is the value that maximizes total correct diagnoses, i.e. that maximizes overall test validity (the arithmetic mean of sensitivity and specificity).

Second, we investigated whether including information on γ responses to stimulation with avian or fortuitum PPD (OD_{avian}, $OD_{fortuitum}$), or circulating IFNγ (OD_{nil}) could further improve test validity. We assigned test-negative status to buffalo with OD_{bovine} readings that failed to exceed their OD_{avian}, $OD_{fortuitum}$ or OD_{nil} by at least 10%, regardless of the absolute OD_{bovine} value. The rationale behind these "a

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priori exclusions" is that OD_{avian}, $OD_{fortuitum}$ or OD_{nil} readings of comparable magnitude as an individual's OD_{bovine} reading may indicate cross-reactivity due to sensitisation by *M. avium*, *M. fortuitum*, or in the case of high circulating IFNγ – any other pathogen stimulating a pronounced IFNγ response. This rationale follows established BTB diagnostic procedures (e.g. Bovigam assay), which include interpreting as test-negative animals with similarly high OD_{bovine} and OD_{avian} readings (Wood et al., 1992; Whipple et al., 2001). We considered three scenarios in evaluating the utility of information on OD_{avian}, $OD_{fortuitum}$ and OD_{nil} in form of a priori exclusions, evaluating: (i) the contribution of this supplementary information to the overall validity of the test; (ii) the potential role of such information in alleviating the loss of sensitivity incurred by choosing a high cut-off value for OD_{bovine}; and conversely, (iii) the potential role of such information in reducing the loss of specificity associated with setting a low cut-off value for OD_{bovine}. The latter two options reflect the fact that priorities for choosing an OD_{bovine} cut-off value may vary according to test application. Very high sensitivity may be desirable in situations where identifying every BTB-positive animal is critical, even at the cost of removing some false-positives (e.g. disease control in large infected populations). Emphasis may be placed on maximizing test specificity in situations where unnecessary animal removals due to false positive test results are not acceptable (e.g. routine testing in small disease-free populations).

3. Results

3.1. Animals

3.1.1. African buffalo

Data from 149 infected buffaloes from known infected herds were examined, which included 80 animals with culture confirmed *M. bovis* infection. In the remaining 69 animals (from HiP) bovine tuberculosis was diagnosed macroscopically at necropsy and 67 of those had been tested with the comparative intradermal tuberculin test and found positive. No buffalo from the uninfected group were culled.

3.2. Assay for gamma interferon

The data set of 149 infected and 344 uninfected buffaloes comprised the optical densities measured in response to whole blood stimulation with bovine, avian, fortuitum PPD and a nil control and subsequent detection of IFNγ. For the standard test interpretation the criteria reported by Whipple et al. (2001) were used. Standard test interpretation correctly identified 138/149 infected animals and 235/344 uninfected animals, resulting in a sensitivity and specificity of 92.6% (±4.2%) and 68.3% (±4.9%), respectively, giving an overall (combined average) test validity of 80.2%. Of the 109 false positive reactors, 32 showed a pronounced reactivity to bovine tuberculin of $OD_{bovine} > 0.40$ (Figs. 1c and 2).

3.2.1. Predictive value of the different optical density values in determining TB status

Using the single-factor model with OD_{bovine} as predictor for BTB status as a baseline, we assessed whether composite single factors taking the control value (OD_{nil}) into account, improved our ability to predict BTB status. Including OD_{nil} in composite single factors ($OD_{bovine} - OD_{nil}$) or $\log(OD_{bovine}/OD_{nil})$ reflects the standard practice of using the *difference* or *proportionate change* in IFNγ titre following stimulation with bovine tuberculin as predictor for BTB status. Contrary to our expectation, neither of these composite variables outperformed OD_{bovine} as a predictor for BTB status (Table 2).

In line with the established practice of including reactivity to avian tuberculin in the diagnosis of bovine TB, we found that including OD_{avian}, and to a lesser degree OD_{nil}, improved our ability to distinguish TB-positive from TB-negative animals: Two-factor models (OD_{bovine} plus each of the others in turn) showed that adding OD_{avian} improved model fit substantially, while including OD_{nil} resulted in slightly better model fit, and $OD_{fortuitum}$ did not improve model fit as measured by AIC. Consistent with this result, the three-variable model including OD_{nil} marginally outperformed the two-factor model with OD_{bovine} and OD_{avian} alone, while including $OD_{fortuitum}$ in three- and four-factor models did not improve model fit (Table 2).

3.2.2. Optimizing test criteria to maximize test validity

We used a two-step approach to optimize the validity of the IFNγ test for buffalo by first establishing the optimal OD_{bovine} cut-off value and second, assessing how additional information on OD_{avian}, $OD_{fortuitum}$ and OD_{nil} might help maximize test validity.

Step 1: What OD_{bovine} cut-off value maximizes the proportion of animals that are correctly diagnosed as BTB-positive or BTB-negative? Optical density frequency distributions of BTB-negative and BTB-positive overlapped, but BTB-negative animals typically showed much lower OD_{bovine} readings than BTB-positive animals (Fig. 2). The shapes of the two distributions were quite distinct. The distribution curve derived from uninfected buffalo had an aggregated shape, with most animals (>80%) displaying low (≤ 0.25) OD_{bovine} readings, and the remaining ~20% distributed outside this typical range in the distribution's long tail, reaching OD_{bovine} values of up to 3.25. By contrast, the infected distribution was much more even, covering a range of OD_{bovine} readings between 0.1 and 3.4 with no distinct peak indicating a "typical" OD_{bovine} interval for infected buffaloes. Based on the distributions of OD_{bovine} readings in uninfected and infected buffaloes, the optimal OD_{bovine} cut-off which correctly identifies BTB status for the largest proportion of buffalo, is 0.385. When OD_{bovine} readings < 0.385 are interpreted as test negative, and OD_{bovine} readings ≥ 0.385 are interpreted as test positive, a test specificity of 91.9% and sensitivity of 86.5% are achieved, giving an overall validity of 89.2% (Fig. 3). Setting the cut-off lower leads to an increase in sensitivity and a larger decrease in specificity resulting in a higher total error rate; while selection of a higher cut-off value yields a higher specificity at the cost of sensitivity (Fig. 3).

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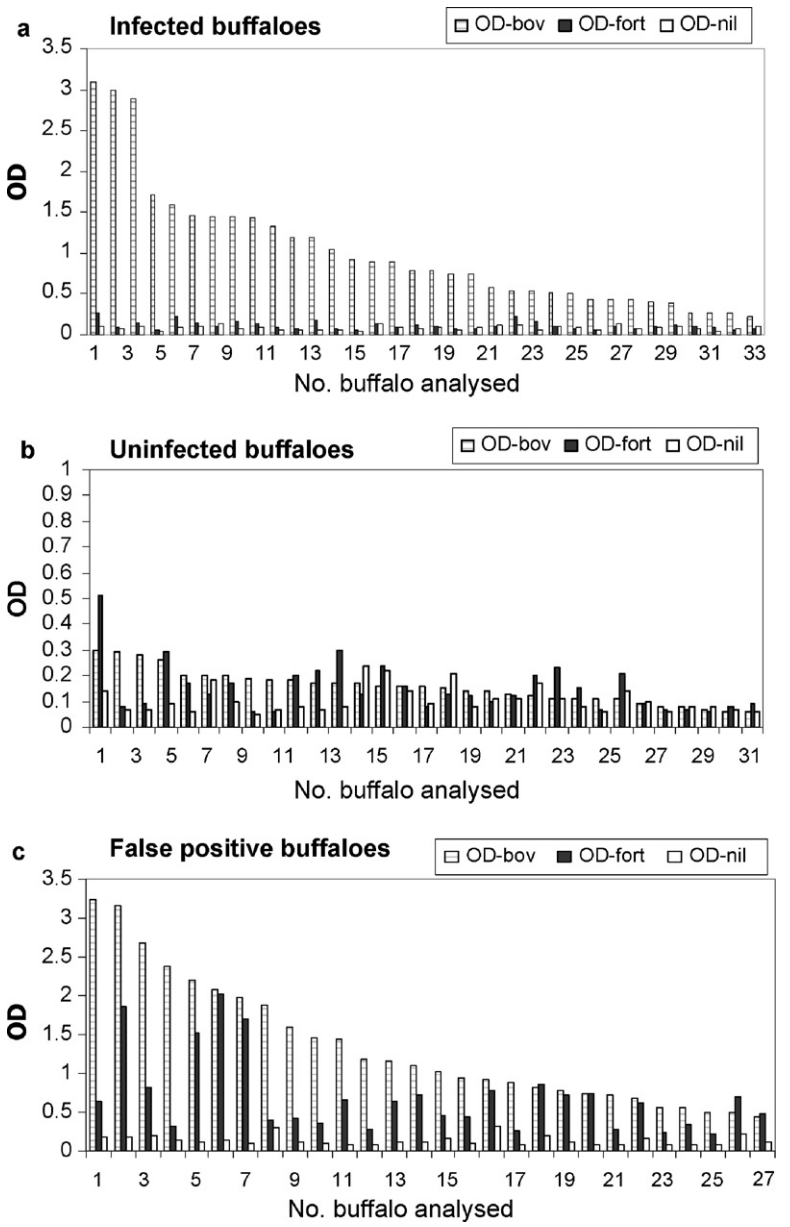

Fig. 1. IFNγ reactivity profiles among groups of (a) infected (b) uninfected and (c) uninfected, false positive reactor buffaloes following stimulation with bovine, avian, fortuitum PPD as well as unstimulated control samples. Note different scalings of (b) compared to (a) and (c).

Step 2: Can information on IFNγ responses to stimulation with avian or fortuitum PPD (OD_{avian}, $OD_{fortuitum}$), or circulating IFNγ (independent from infection with cross-reacting *Mycobacterium* spp.) (OD_{nil}) further improve test validity? Taking into account information on OD_{avian}, $OD_{fortuitum}$ and OD_{nil} in form of a priori exclusions did not increase the overall validity of the test significantly. Test validity was nominally higher at 89.6% in the best-performing protocol allowing exclusions based on $OD_{fortuitum}$, compared to the no-exclusions protocol with overall validity of 89.2% (Fig. 4a, Table 3); however confidence intervals for both sensitivity and specificity between the protocols overlapped broadly, indicating a lack of difference between the two (Table 3). Moreover, if high test sensitivity (e.g. >95%) is the primary priority, adding a priori exclusions based on OD_{avian}, $OD_{fortuitum}$ and OD_{nil} to the protocol is unhelpful, because these tend to increase test specificity but decrease sensitivity (Fig. 4b, Table 3). By contrast, if high specificity is paramount, a priori exclusions can improve overall test validity, because they allow a lower OD_{bovine} cut-off to be picked for a given target specificity (Fig. 4c). This reduces the loss of sensitivity associated with prioritizing high specificity. When the primary test selection criterion is a specificity exceeding 95%, the protocol allowing for designation as BTB-negative, based on fortuitum cross-reactivity, thus performs best, improving test

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Table 2
Comparison of logistic regression models predicting buffalo BTB status in a study on gamma interferon assays to diagnose *M. bovis* infections in African buffaloes. IFNγ response to stimulation with bovine, avian and fortuitum tuberculin, and circulating (control) IFNγ titers – (OD_{bovine}, OD_{avian}, $OD_{fortuitum}$, OD_{nil}, respectively) were compared.

Model: TB status	AIC	Parameter	Estimate	*SE*	*Z*	*p*
OD_{bovine}	172.1	OD_{bovine}	8.091	1.1	7.6	<0.0001
$(OD_{bovine} - OD_{nil})$	231.4	$(OD_{bovine} - OD_{nil})$	5.113	0.7	7.466	<0.0001
$\log(OD_{bovine}/OD_{nil})$	253.8	$\log(OD_{bovine}/OD_{nil})$	1.785	0.2	9.292	<0.0001
$OD_{bovine} + OD_{avian}$	160.8	OD_{bovine}	10.2	1.4	7.319	<0.0001
		OD_{avian}	−3.253	1.2	−2.708	0.007
$OD_{bovine} + OD_{fortuitum}$	174.0	OD_{bovine}	8.151	1.1	7.464	<0.0001
		$OD_{fortuitum}$	−0.363	1.4	−0.257	0.797
$OD_{bovine} + OD_{nil}$	170.8	OD_{bovine}	8.292	1.1	7.48	<0.0001
		OD_{nil}	2.381	1.2	2.007	0.045
$OD_{bovine} + OD_{avian} + OD_{fortuitum}$	162.4	OD_{bovine}	10.18	1.4	7.197	<0.0001
		OD_{avian}	−3.594	1.4	−2.565	0.01
		$OD_{fortuitum}$	0.989	1.4	0.692	0.489
$OD_{bovine} + OD_{avian} + OD_{nil}$	158.6	OD_{bovine}	10.59	1.5	7.145	<0.0001
		OD_{avian}	−3.598	1.4	−2.635	0.008
		OD_{nil}	2.668	1.2	2.254	0.024
$OD_{bovine} + OD_{avian} + OD_{fortuitum} + OD_{nil}$	160.6	OD_{bovine}	10.6	1.5	7.144	<0.0001
		OD_{avian}	−3.576	1.4	−2.462	0.014
		OD_{nil}	2.692	1.3	2.071	0.038
		$OD_{fortuitum}$	−0.071	1.6	−0.044	0.965

sensitivity by almost 10% compared to the no-exclusions protocol, while maintaining test specificity around 95% (Table 3).

The practical value for selecting a priori exclusion based on fortuitum reactivity has also been tested across a random set of 1531 routine diagnostic IFNγ data from buffalo from eight herds with a negative BTB status. Twenty-six multiple reactors were identified (1.69%), compared to the 0.87% detected in the uninfected population in this study (Fig. 1c). The levels of reactivity to fortuitum PPD in this false positive reactor group were distinctly higher than in the infected and uninfected groups (Fig. 1a and b).

Table 3
OD_{bovine} cut-off values and a priori exclusions for different diagnostic priorities. Criteria for the choice of cut-off values are (i) fulfilling the stated priority and (ii) maximizing validity given the constraint of (i). The optimal test protocol for each priority scenario is given in bold italics.

Priority	A priori exclusions: TB-neg if $OD_{bovine}{}^{*} \leq 0.9$	Cut-off: TB-pos if $\geq OD_{bovine}$	Specificity [CI] (%)	Sensitivity [CI] (%)	Overall validity (%)
Optimize overall test validity	None	0.385	91.9 [89.0, 94.8]	86.5 [81.0, 92.0]	89.2
	OD_{avian}	0.375	93.5 [90.9, 96.1]	85.4 [79.7, 91.2]	89.5
	$OD_{fortuitum}$	***0.385***	***93.9*** [90.2, 97.5]	***85.4*** [79.7, 91.2]	***89.6***
	OD_{nil}	0.385	91.9 [88.9, 94.7]	85.8 [80.2, 91.4]	88.8
	OD_{avian} or OD_{nil}	0.375	93.5 [90.9, 96.2]	84.7 [78.8, 90.6]	89.1
	OD_{avian} or $OD_{fortuitum}$	0.375	95.1 [90.6, 97.5]	82.6 [76.4, 88.8]	88.9
	OD_{nil} or $OD_{fortuitum}$	0.385	93.9 [90.2, 97.5]	84.7 [78.8, 90.6]	89.3
	OD_{avian} or OD_{nil} or $OD_{fortuitum}$	0.375	95.1 [90.6, 97.5]	81.9 [75.7, 88.2]	88.5
To achieve sensitivity >95%	***None***	***0.235***	***79.1*** [74.8, 83.4]	***95.3*** [90.6, 97.7]	***87.2***
	OD_{avian}	n/a	n/a	Max < 95	n/a
	$OD_{fortuitum}$	n/a	n/a	Max < 95	n/a
	OD_{nil}	0.225	78.5 [74.1, 82.8]	95.3 [90.6, 97.7]	86.9
	OD_{avian} or OD_{nil}	n/a	n/a	Max < 95	n/a
	OD_{avian} or $OD_{fortuitum}$	n/a	n/a	Max < 95	n/a
	OD_{nil} or $OD_{fortuitum}$	n/a	n/a	Max < 95	n/a
	OD_{avian} or OD_{nil} or $OD_{fortuitum}$	n/a	n/a	Max < 95	n/a
To achieve specificity >95%	None	0.525	95.3 [92.6, 97.1]	73.6 [66.5, 80.7]	84.5
	OD_{avian}	0.425	95.3 [92.5, 97.1]	79.8 [73.3, 86.4]	87.6
	$OD_{fortuitum}$	***0.395***	***95.1*** [90.6, 97.5]	***83.3*** [77.2, 89.4]	***89.2***
	OD_{nil}	0.525	95.3 [92.6, 97.1]	73.0 [65.8, 80.1]	84.2
	OD_{avian} or OD_{nil}	0.425	95.3 [92.5, 97.1]	79.2 [72.5, 85.8]	87.2
	OD_{avian} or $OD_{fortuitum}$	0.375	95.1 [90.6, 97.5]	82.6 [76.4, 88.8]	88.9
	OD_{nil} or $OD_{fortuitum}$	0.395	95.1 [90.6, 97.5]	82.6 [76.4, 88.8]	88.9
	OD_{avian} or OD_{nil} or $OD_{fortuitum}$	0.375	95.1 [90.6, 97.5]	81.9 [75.7, 88.2]	88.5

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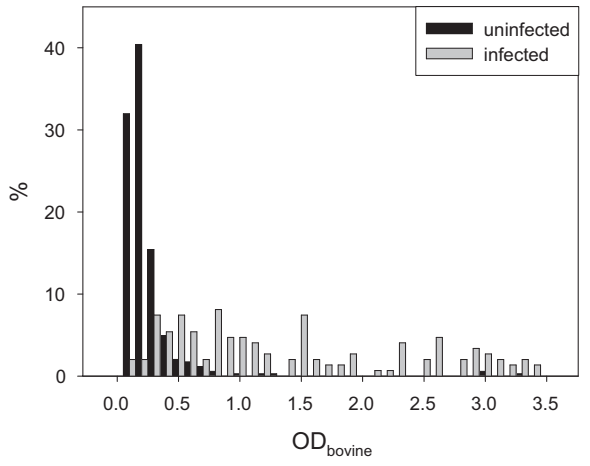

Fig. 2. IFNγ responses of TB-infected and uninfected African buffaloes to *in vitro* stimulation with bovine PPD. Frequency distributions of optical density readings (OD_{bovine}) are shown for both populations.

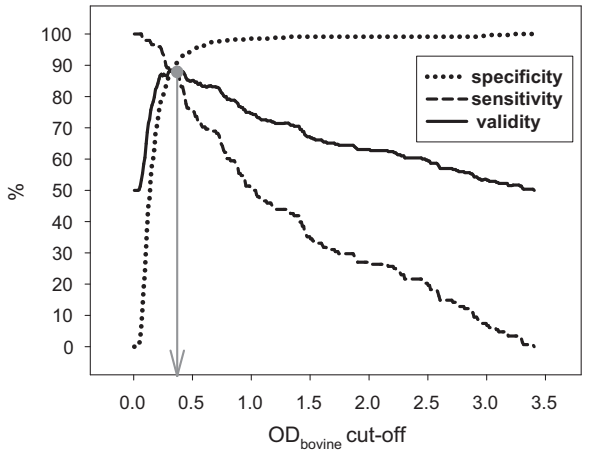

Fig. 3. Validity of a TB diagnostic test in African buffaloes, based on IFNγ responsiveness to *in vitro* stimulation with bovine PPD.

In combination, our results provide strong support for including information on reactivity to the environmental mycobacterium *M. fortuitum*, and tentative support for including avian reactivity in comparative skin testing protocols for buffalo.

4. Discussion

Bovine tuberculosis control in protected wildlife reservoir species such as the African buffalo in South Africa introduces a new challenge for government, conservation organisations and the wildlife industry. An overkill of buffaloes in order to reduce the herd and regional prevalence is only acceptable in known infected populations with a high prevalence, such as the Hluhluwe-iMfolozi Park (Michel et al., 2006). In all currently uninfected populations the culling of false positive buffaloes as a result of a lack of test specificity is an ethically and financially unacceptable sacrifice. The IFNγ assay has many practical advantages over the skin test, especially in wildlife, as shown in a preliminary evaluation in buffalo in the KNP (Michel, unpublished data). A complicating factor, however, is the demand for high specificity in valuable species and populations. To meet the diverging requirements for high specificity in certain management strategies and retaining the ability to provide good sensitivity in other populations, the ideal IFNγ test system would allow for a differential interpretation scheme according to the task at hand.

There is no perfect discrimination between infected and uninfected populations: frequency distributions for OD_{bovine} in uninfected and infected buffalo showed broad overlap. More specifically, diagnostic accuracy based on OD_{bovine} readings is limited by the distributions' shapes. The uninfected distribution has a long tail, with some animals returning very high OD_{bovine} readings, which will lead to false-positive test results given any reasonable OD_{bovine} cut-off value (Fig. 2). By contrast, infected animals showed an even distribution of OD_{bovine} readings covering a very broad range, with no obvious peak or typical OD_{bovine} value. This makes it very hard to achieve excellent test sensitivity without severely compromising specificity. A potential limitation of the data presented here may be that roughly half of the infected animals came from culls based on positive IDT results in a known infected population (HiP). The IDT test might, however, miss some animals with chronic infections such as in HiP, which may no longer show a pronounced reactivity to stimulation with bovine PPD due to anergy (Monaghan et al., 1994; Cross et al., 2009). Such anergic animals would have escaped detection and cull at HiP, and would therefore be missing from our sample of infected animals. Because anergic animals are likely to return low OD_{bovine} readings, this bias in our sample may have caused us to underestimate the proportion of false-negatives. It is, however, also true that calculating IFNγ sensitivity based exclusively on culture positive animals and animals showing macroscopic lesions could have caused an underestimation of the sensitivity as cases with early infections may have been detected by IFNγ and IDT but subsequently missed by necropsy and culture and would therefore have been disregarded for the study. As a result, we may argue that the overall test sensitivity across the wide spectrum of infection stages found in wildlife populations may have been estimated with satisfactory accuracy.

A second limitation of our data is that we had to source TB-infected and TB-uninfected animals from a number of different populations. This is not optimal, because test validity may vary between populations, based on differences in pathogen exposures as well as host immune responses, mediated by genetic and environmental factors. Our selection of study animals was driven by animal availability and the complexity of the gold standard for identifying TB-infected and uninfected animals: TB-positive populations, such as Hluhluwe-iMfolozi Park, typically offer access to culled positive-testing animals, while negative-testing animals are released and therefore are not available for necropsy. Rarely, TB surveys in KNP include uninfected buffaloes but in those instances all study animals are shot from the air and not suitable for whole blood collection and interferon gamma testing. Our uninfected animals were sourced exclusively from populations

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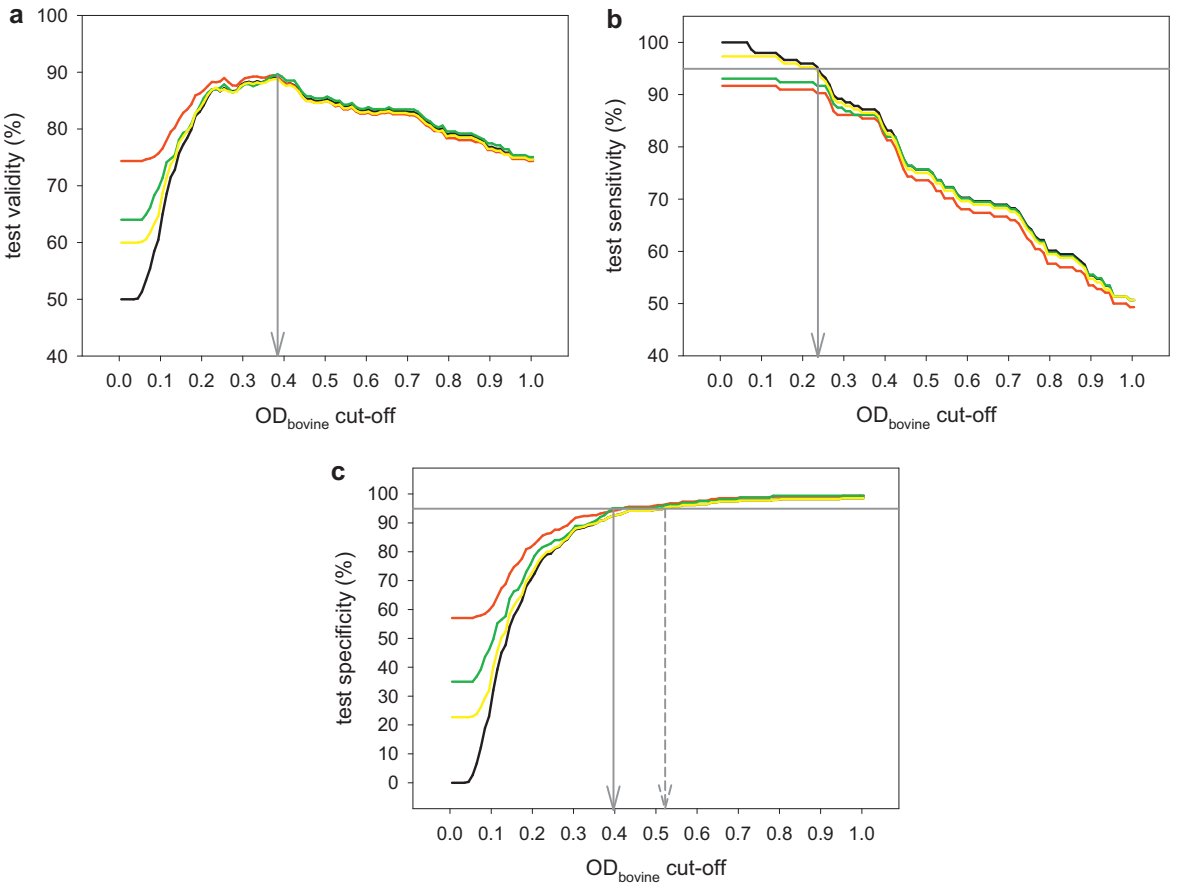

Fig. 4. Effect of including information on IFNγ responses to *in vitro* stimulation with avian and fortuitum PPD (OD_{avian}, $OD_{fortuitum}$), and IFNγ in unstimulated blood (OD_{nil}) on the validity of a TB diagnostic test in African buffaloes.

certified TB-negative due to negative founder animals and a sustained history of herd-wide negative TB test results. Our infected animals were sourced from distinct, infected populations. Within the infected and uninfected groups, we used animals from multiple farms and reserves to achieve the large sample sizes needed for detecting the contribution of different antigenic reactivities to TB diagnosis. This implies that exposures and environmental conditions surely differed between locations and we are hence confident that the analytic approaches presented here should be suitable and readily transferable to test data from any population, although the test protocols that performed best in this study may require some modification to optimize them for different buffalo populations.

The desired compromise in our situation should offer optimum specificity but at the same time the flexibility to opt for high sensitivity when required. When applying the standard interpretation criteria reported by other investigators it was found that the IFNγ assay could not meet these requirements. The test validity was improved in this study by firstly identifying the absorbance of bovine PPD as the dominant variable and by optimising the cut-off value for a positive test result. By applying this OD_{bovine} value (0.385) which is significantly higher than in the standard protocol, the specificity was increased from 68.3% to 91.9%. As expected this was accompanied by a decrease in sensitivity, from 92.1% to 86.5% (Table 3). Because the loss in sensitivity incurred by this optimization was much smaller than the improvement in specificity, overall test validity was substantially improved, from 80.2% in the established protocol to 89.2% using OD_{bovine} at the optimized cut-off value as the sole test interpretation criterion. To achieve a further increase in specificity without losing sensitivity it is important to understand the mechanisms which modulate the immune responses in cattle and buffalo.

Exposure of cattle to environmental mycobacteria has been previously implied as underlying cause of non-specific reactivity during skin testing as well as IFNγ testing (Kleeberg, 1960; Cagiola et al., 2004; Kormendy, 1995; Donoghue et al., 1997). We have recently reported the isolation of environmental mycobacteria from infected and uninfected buffalo as well as from surface water (Michel et al., 2007; Michel, 2008a) and some of the false positive reactivity in the buffaloes examined in the present study may have been caused by antigenic cross-reactivity with mycobacteria other than tuberculosis. Further optimisation of the IFNγ test validity was therefore pursued in this study by examining 'a priori' exclusions which allow for some bovine reactors to be classified as test negative, based on the level of reactivity to avian or fortuitum

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PPD. In this context, we found that indeed, allowing for BTB-negative classification of so-called multiple reactors (mounting an immune response to bovine and fortuitum PPDs), substantially improved validity of tests optimized for high specificity: With a cut-off value allowing for >95% specificity (OD_{bovine} = 0.525; specificity = 95.4%), the IFNγ test based on bovine reactivity alone achieved a sensitivity of only 73.7%, compared to 83.3% sensitivity in a test allowing for a priori exclusions based on fortuitum reactivity (OD_{bovine} cut-off 0.395; specificity 95.1%). Information on fortuitum reactivity thus substantially reduced the loss of sensitivity associated with constraining the OD_{bovine} cut-off to minimize false-positive test results. Tests including a priori exclusions based on fortuitum and avian reactivity also slightly outperformed the bovine-only protocol when optimized for overall validity.

Notably, fortuitum reactivity provided the greatest test improvement in this context, despite the lack of support for a role for fortuitum reactivity in defining BTB status in our logistic regression analysis. This apparent discrepancy is due to the fact that test protocols with a priori exclusions do not align structurally with generalized linear models such as logistic regressions. The information hierarchy in our logistic regression model simply starts with the strongest predictor for BTB status (i.e. OD_{bovine}) and then asks whether addition of further variables (in order of predictive information content) significantly improves prediction accuracy. Fortuitum reactivity did not add predictive power after bovine and avian reactivity had been taken into account. Contrasting with this statistical approach, the test protocol allowing for a priori exclusion from BTB-positive status of strong fortuitum reactors is biologically based. Here we ask, first, how the animal's bovine reaction compared to its fortuitum reaction, assigning TB-negative status to animals with suspected cross-reactivity. Only secondarily do these protocols evaluate OD_{bovine} readings to designate BTB status to the remaining animals that do not show evidence of cross-reactivity. In this case, our data suggested that fortuitum reactivity, and to a lesser degree avian reactivity, helped increase test validity.

A slight modification of this approach has already been applied very successfully in BTB surveys in buffalo in the low BTB incidence northern region of the KNP (Grobler et al., 2002; Hofmeyr et al., 2003; De Klerk, unpublished data) as well as in interpreting immune status of experimentally infected and vaccinated buffaloes as described recently (De Klerk et al., 2006; Michel et al., 2006; Michel et al., 2007; De Klerk et al., 2010). This serves to show the usefulness of the interpretation approach described in this study for an overall improvement in the IFNγ test performance in buffalo populations, whereby it is most advantageously applied to uninfected populations.

Out of 149 known *M. bovis* infected buffalo in this study, 41 buffaloes were classified as multiple reactors in the modified IFNγ assay, which would have escaped detection based on their classification as test negative. Their bovine and fortuitum reactivities were, most likely, the result of a mixed infection of these buffaloes with *M. bovis* and a non-tuberculous *Mycobacterium*. When managing infected populations where there is a greater need for sensitivity (accompanied by a tolerance for a decreased specificity) such as in HiP, the IFNγ assay offers the flexibility to use a test interpretation without a priori exclusion of multiple reactors (Table 3). The decision what error rate in either direction is acceptable, entirely depends on the specific epidemiological setting and management objective. In wildlife farming operations where buffaloes are individually identified and accessible for testing, it is a further possibility to diagnose and separate multiple reactors from non-responsive buffaloes and re-test them after eight weeks, in accordance with the kinetics of the cross-reactivity caused by exposure of buffaloes to non-tuberculosis mycobacteria (Michel, 2008a). According to our observations, uninfected buffaloes, previously non-specifically sensitized with the latter then no longer have detectable levels of IFNγ above the cut-off value, irrespective of the antigen used for stimulation. In contrast, multiple reactors constituting truly infected animals tested at a time of simultaneous non-specific sensitization, are likely to present unequivocally as bovine reactors upon re-testing 8 weeks later (Michel, unpublished data).

Nevertheless, the use of two different interpretation schemes for infected versus uninfected populations would add value to the use the IFNγ assay in supporting the control of BTB in buffalo in South Africa. The approach is not new and generally accepted for the IDT (Kleeberg, 1960) and it therefore remains our objective to determine appropriate cut-off values for the IFNγ assay in these contrasting situations.

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