"LIOdetect®Animal TB" rapid test as a serological ancillary test to tuberculin skin test can significantly improve overall sensitivity with high specificity for the routine screening of bovine tuberculosis in cattle herds.

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Keywords: Animal tuberculosis, Cattle, serology rapid test, Point-Of-Care, Lateral-Flow-Test

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Abstract

Lateral flow technology is an easy and cheap serological tool for the screening of animal Tuberculosis, exploitable as Point Of Care test. The LIOdetect®Animal TB is a rapid test for the diagnosis of bovine tuberculosis, containing recombinant MPB83 and MPB70 of Mycobacterium bovis and a highly purified cell wall antigen. The rapid assay was tested on sera from 114 healthy cattles and 74 animals from herd with confirmed *M. bovis* tuberculosis . Serum with antibodies to one or all of the antigens will show one or more pink-purple bands in the test zone of the test card within 15 minutes. All the TB-free animals showed negative results (100% of specificity); for infected animals, when reactions to either of the 3 bands were taken into account, the test displays a variable sensitivity reaching upto to 95.8 %. Considering the skin test negative infected animals, the LIOdetect®Animal TB rapid test could be an ancillary tool to detect false negative subjects emerged with the tuberculin skin test.

In order to compare the LIOdetect®Animal TB rapid test response with another serological assay, the new in-house LIONEX MPB70 - MPB83 ELISA kit was tested on the same serum samples from 54 healthy and 36 *M. bovis* infected animals. The best cut-off of the ELISA test was set for a sensitivity of 78% and a specificity of 89%. A substantial concordance between the two tests was found for all the results obtained in the 90 tested animals.

Highlights

- LIOdetect®Animal TB rapid test produces consistent and highly specific results in bovine tuberculosis.
- LIOdetect®Animal TB rapid test as an ancillary test to tuberculin skin test can significantly improve diagnosis of bovine tuberculosis.
- LIOdetect®Animal TB rapid test can be used as a Point-Of-Care-Test (POCT).
- LIOdetect®Animal TB rapid test is a cost effective, cheap test highly suitable for developing countries.

1. Introduction

The development of mycobacterial antigen-based tests is one of the main elements of tuberculosis research. Whilst most tests are aimed at measuring cell-mediated immunity, for example in skin tests or Interferon-gamma release assays (IGRA), serological tests are also attractive targets for development due to their easy of use and cost-advantages compared to IGRA tests. Serology, for example based on laterial flow technology, also offer the potential

for the development of a point of care test (POC) (Ranjan et al., 2018). This test format is relevant to human and animal tuberculosis. For example, a recent study in humans provided preliminary results of a new prototype POC rapid lateral flow TB test based on detection of antibodies to combination of the three immunodominant epitopes (peptides) of immunogenic *M. tuberculosis* cell-wall proteins with sensitivity and specificity >90% (Gonzalez et al., 2014). For use in veterinary species such as cattle and deer infected with *M. bovis*, lateral flow devices have been developed by several companies, such as Chembio. The sensitivity and specificity of the Chembio CervidTB STAT-PAK® assay, tested in fallow deer, roe deer and red deer from Great Britain were 85.7% and 94.8%, respectively (Gowtage-Sequeira et al., 2009). SeraLyte-*Mbv* system based on MPB83 antigen showed sensitivity 89% and specificity 98% (Green et al., 2009). Results of a study in Indian cattle population showed a positive response in up to 50% of serum samples from tuberculosis-prevalent farms to fusion CFP-10:MPB83 antigen (Veerasami et al., 2012). Immunochromatographic dual-path platform VetTB assay based on two antigens MPB83 and a CFP10/ESAT-6 fusion proein showed sensitivity of 65.1% and specificity 97.8% in naturally *M.bovis*-infected white-tailed deer (Lyashchenko et al., 2013). Sensitivity and specificity of ELISA with MPB83 and MPB70 reaches 63% and 98%, respectively (Waters et al., 2011). Recombinant MPT-64 can be a potencial antigen for serological diagnosis to detect pathogenicity and non-pathogenicity between infected cattle (Tashakkori et al., 2016). Multiantigen formats of serological tests using proteins such as ESAT-6, CFP-10, MPB83, MPB70 have improved the specificity and sensitivity (Whelan et al., 2008; Wadhwa et al., 2012). Antibodies to MPB83 were detectable in cattle after 3-4 weeks post experimental *M.bovis*-infection, while antibodies to other antigens (MPB70, ESAT-6, CFP-10, MPB59, MPB64, Acr1, 38 kDa) were detected 3-6 weeks later (Waters et al., 2006). Mycobacterial antigens can be used for serological testing of milk samples: sensitivity of 50.0% and specificity of 97.5% was shown on MPB70 and MPB83 ELISA (Buddle et al., 2013).

The sensitivity and specificity of TB ELISA may vary depending on geographical region where cattle are tested (Trost B et al., 2016) as well as on the epidemiological situations (Bezos et al., 2014). The antibody responses are associated with the antigen burden; improtantly, administration of PPD boosts antibody response in infected cattle (Waters et al., 2010). For example, the sensitivity of the serological tests *M. bovis* Ab Test (IDEXX, Westbrook, USA) and Enferplex™ TB assay (Enfer, Kildare, Ireland) increased from 24-33%, prior to intradermal test, to 67-85% 15 days post-intradermal test (Casal C et al., 2014). A study performed on TB-free red deer shows that repeated comparative skin testing do not cause non-specific progressive increments in antibody production (Che-Amat et al., 2016).

The aim of this study was to evaluate the LIOdetect®Animal TB rapid test (LIONEX GmbH, Germany; www.lionex.de) for the diagnosis of bovine tuberculosis. This rapid test contains in addition to the two protein antigens MPB83 and MPB70, a highly purified carbohydrate antigen located in the cell wall. This cell-wall antigen could considerably increases the sensitivity compared to the protein antigens alone.

2. Material and methods

2.1 Cattle serum samples from Italy.

Serum samples from 12 healthy animals (population 1), were tested in Italy as negative control for the test (Table 1).

Table 1. Italian study populations

Population	Number of animals tested	Region	Herd classification	SICCT	VL	M. bovis culture	Post SICCT sampling	Applied for
1	12	Non endemic	TB-free > 5 y	Negative	NA	NA	Yes	Specificity

SICCT single intradermal comparative cervical tuberculin test; VL animals with visible lesions.

2.2 Cattle serum samples from Russian Federation.

Serum samples from four cattle populations collected in Russia were used in this study (Table 2). Samples from 24 infected animals were collected from one farm (population 2), infection was confirmed in all cases by the presence of visible lesions typical of bovine tuberculosis and/or the culture of *M.bovis* from tissue samples, and they were skin-test positive reactor animals. A further group was composed of samples from 50 TB-free animals (long-term, > 5 years, TB-free herds, located in two different TB non-endemic areas of Republic of Tatarstan, Russian Federation), named as populations 3.

Table 2. Russian study populations

Population	Number of	Region	Herd classification	SICCT	VL	M. bovis culture	Post SICCT sampling	Applied for

	animals tested							
2	24	Endemic	Confirmed bTB	Positive	Yes	Yes	Yes	Sensitivity
3	50	Non endemic	TB-free > 5 y	Negative	NA	NA	Yes	Specificity

SICCT single intradermal comparative cervical tuberculin test; VL animals with visible lesions.

2.4 LIOdetect®Animal TB serological test

The LIOdetect®Animal TB is a membrane-based lateral flow test for the rapid detection of antibodies to *M.bovis* in serum from cattle or other animals (e.g. elephant, sheep, goat, badgers). The innovative and rapid screening test is based on lateral flow immunochromatography and is among the easiest diagnostic point-of-care (POC) tests yielding results within 15 minutes. Two specific recombinant antigens from *M.bovis* (MPB83 in test line 1 and MPB70 in test line 2) and one highly purified cell wall antigen (test line 3) are immobilized on the membrane in the «T» region (test zone). If the sample contains antibodies to one or all of these antigens, then the antibody-conjugate complex attaches itself to one or more of the test lines: one or more pink-purple bands then appears in the «T» zone of the test card. The remaining antibody-conjugate complex then passes through the card until it reaches the control zone «C». Again, a pink-purple band appears, indicating that the test has been performed properly.

All tests were performed according to the manufacturer's instructions, with the specification about the intrerpretation of results as follow: up to 4 pink-purple lines appear – one appears in control zone "C" and the others in test zone "T". If one or more test lines appear (figure 1), the result is POSITIVE, regardless on the position of the test line ("Test line 1", "Test line 2", "Test line 3"); examples are shown in figure 1, where C (control line) and test lines T1, T2 and T3 are shown by arrows.

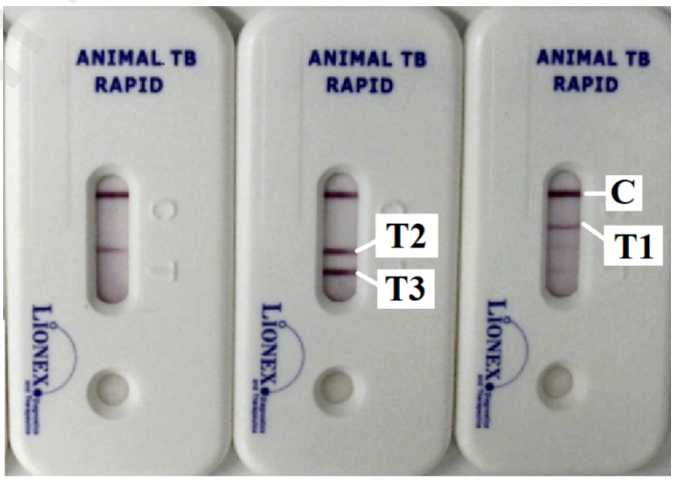

Figure 1. Examples of POSITIVE LIOdetect®Animal TB results

2.5 Lionex MPB70 - MPB83 ELISA kit in comparison to LIOdetect®Animal TB

In addition to the LIOdetect®Animal TB, sera from 54 healthy and 36 infected animals were also tested with Lionex in-house newly developed ELISA kits for test comparison. These ELISA kits contain the same highly amount of purified recombinant MPB70 and MPB83 as capture antigens, which were used in the manufacture of the LIOdetect®Animal TB, thus allowing a useful matching of the two formats. Lionex MPB70 - MPB83 ELISA kits were evaluated according to manufacturers instructions. Anti-MPB70 and anti-MPB83 antibodies production were determinated as optical density (OD) readings at 450 nm wavelength.

2.6 Statistical analysis.

All compared distributions were considered statistically significant if $p < 0.05$.

ELISA accuracy was evaluated by a ROC (Receiving Operating Characteristic) curve and the best cut off value to discriminate between healthy or infected animals, was calculated by the Youden's index. Correlation between tests were calculated by Cohen's Kappa coefficient.

Descriptive statistic, frequences, relative sensitivities and specificities (95% CI) values of the tests studied in this project were calculated using GraphPad Prizm (GraphPad, San Diego, CA, USA) and IBM SPSS Statistics version 27.

3. Results

3.1 Evaluation of test specificity in a TB-free population in Italy.

All the 12 sera tested with LIOdetect®Animal TB, derived from TB-free animals, showed negative results, reaching 100% of specificity (table 4).

Table 4. Results obtained with the LIOdetect®Animal TB Rapid test at Italian population.

	Population 1 (n=12)	
	% Specificity (95%CI)	N positive
Band 1, 2 or 3	100.0	0/12
Band 2 or 3	100.0	0/12

Band 1	100.0	0/12
Band 2	100.0	0/12
Band 3	100.0	0/12

3.2 Evaluation of the LIOdetect®Animal TB test in cattle in Russia.

The LIOdetect®Animal TB tests were supplied to the Central Research Laboratory of Kazan State Medical Academy and tested using sera from all four population groups. The results, summarised in table 5, show that, when reactions to either of the 3 bands were taken into account, the test displays a high degree of sensitivity of 95.8 % (population 4, table 5). The inclusion of band 1 in the analysis resulted in decreased specificy. Whether considering bands 2 and 3 for test interpreation had a deleterious effect on sensitivty reducing it from 95. 8 to 87.5 % (table 5). Consequently, disregarding band 1 in the test interpretation it improved specificity only marginal from 96 to 98 % (table 5).

Table 5. Results obtained with the LIOdetect®Animal TB Rapid test at Russian populations.

	Population 2 (n=24)		Population 2 and 3 (n=50)	
	% Sensitivity (95%CI)	N positive	% Specificity (95%CI)	N positive
Band 1, 2 or 3	95.8 (78.9, 99.9)	23/24	96.0	2/50 2/25
Band 2 or 3	87.5 (67.6, 97.3)	21/24	98.0	1/50
Band 1	75.0 (53.3, 90.2)	18/24	98.0	1/50
Band 2	83.3 (62.6, 95.3)	20/24	98.0	1/50
Band 3	20.8 (7.1, 42.2)	5/24	100.0	0/50

3.4 Evaluations of Lionex MPB70 and MPB83 ELISA kits

54 sera from healthy animals and 36 sera from *M. bovis* infected ones were evaluated both with Lionex in-house MPB70 - MPB83 ELISA test and with LIOdetect®Animal TB. Anti-MPB70 and anti-MPB83 antibodies production were determinated as optical density (OD) readings at 450 nm wavelength.

Mann-Whitney inferential test was used to compare MPB70-MPB83 ELISA OD results between healthy and *M. bovis* infected animals. As shown in table 7 and figure 2, a significant difference ($p<0.0001$, 95% IC) was found between the two groups.

Table 7. Sera responses to MPB50 and MPB83 detected by Lionex ELISA kits OD (450nm).

	OD readings for Healthy sera (N=54)	OD readings for Infected sera (N=36)
Mean ± SD	0.0044 ± 0.0105	0.2691 ± 0.3751
Median (IQ range)	0.0000 (0.0000 – 0.0022)	0.1377 (0.0146 – 0.3330)

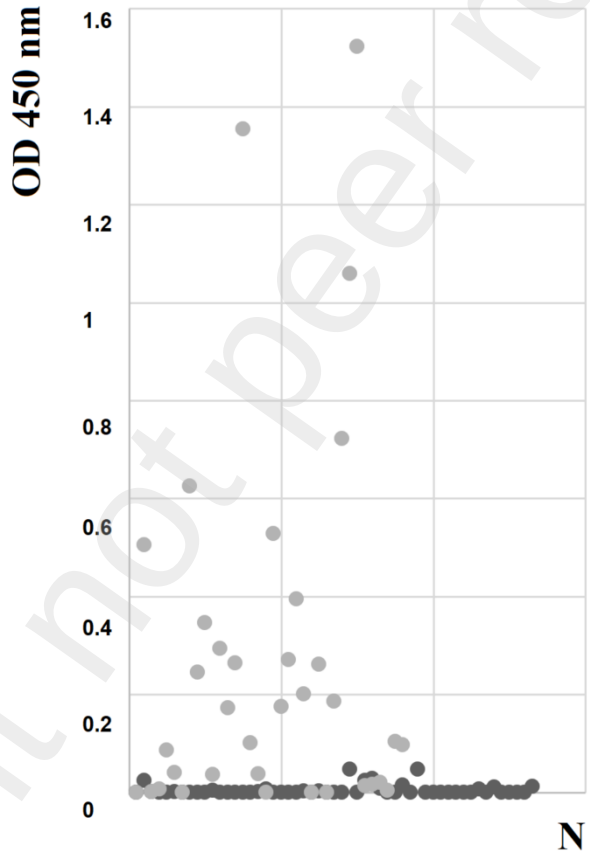

Figure 2. Distribution of OD (450 nm) results for MPB70 – MPB83 ELISA on sera from 54 healthy (dark grey dots) and 36 *M.bovis* infected (light grey dots) animals.

ROC analysis was carried out to assess the accuracy of the MPB70 – MPB83 ELISA test and to set, by Youden's index (= Sensitivity± [1- Specificity]), the best test cut-off. Thus, as shown in figure 3, AUC was find to be 0.88 (highly accurate) and sera were considered reactive for a OD cut-off value of 0.013 (Sensitivity 78%; Specificity 89%).

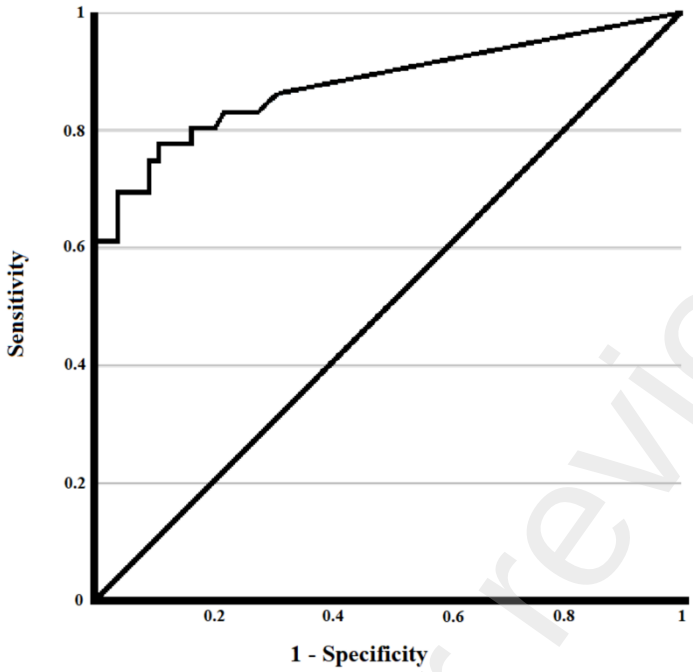

Figure 3. ROC curve computed for the MPB70 – MPB83 ELISA test.

Once the best threshold value for the MPB70-MPB83 ELISA was set, the correlation with the microbiological diagnosis was assessed by the Cohen's Kappa coefficient: a substantial concordance was found for all the results obtained in the 90 tested animals (k = 0.67). The same substantial concordance (k=0.72) resulted between the LIOdetect®Animal TB rapid test and the microbiological diagnosis.

4. Discussion

In this study, we could demonstrate that the specificity of the LIOdetect® Animal TB Rapid test kit could be improved by disregarding reactions to band 1. In an independent study using Non-tuberculous mycobacteria (NTM) infected cattle, no significant cross reaction was observed (results to be published elsewhere). Compared to other similar tests, both sensitivity and specificity of this test are comparable, with the sensitivity on the higher end of the reported result spectrum. Considering the skin test negative animals, it is important to underline that these animals would have all been missed and thus these data highlight that serological ancillary will improve overall test sensitivity when used in combination (parallel) with the tuberculin skin test. Moreover, one also needs to state that all these skin test negative animals were detected positive by routine IGRA testing confirming that these were truly infected animals

(unpublished results). In Russian populations specificity and sensitivity of the LIOdetect® Animal TB Rapid test is relatively high (92.0-100.0% and 95.8%, respectively). It also detects bTB infected cattle, which were missed by skin test.

5. Conclusion

LIOdetect® Animal TB Rapid test is an instrument free, easy to handle blood test presenting results within 15 minutes. The results obtained with LIOdetect® Animal TB Rapid test show that this test can be considered as a reliable tool to diagnose tuberculosis in bovines.
In conclusion, the LIOdetect® Animal TB rapid test can be regarded as a serological ancillary test which can significantly improve overall test sensitivity with high specificity when used in combination (parallel) with the tuberculin skin test for the screening of cattle herds for bovine tuberculosis.

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Author contributions

Eduard A. Shuralev: Conceptualization, Methodology, Data collection, Analysis. Chiara Della Bella: Conceptualization, Methodology, Data collection, Analysis, Writing: Malik N. Mukminov: Data collection. Nail I. Khammadov: Data collection. Kamil S. Khaertynov: Data analysis. Susanne Kaempfer: Data collection. Mario Milco D'Elios: Conceptualisation, Writing – review & editing. Mahavir Singh: Writing – review & editing.

Declaration of Competing Interest

Susanne Kaempfer (S.K) and Mahavir Singh (M.S) are scientists working at Lionex Diagnostics and Therapeutics GmbH. M.S and S.K had no influence on results and interpretations concerning LIOdetect® Animal TB rapid test. The other authors declare that they have no conflict of interest.

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