Evaluation of the use of the BOVIGAM TB Kit in water buffalo

Summary

The results presented in this paper show that:

- The Applied Biosystems™ BOVIGAM™ TB Kit detects bovine tuberculosis in water buffalo
- The test showed a higher sensitivity on positive animals than the single intradermal tuberculin (SIT) skin test
- The kit demonstrates good laboratory reproducibility and almost perfect repeatability on water buffalo samples

Introduction

The BOVIGAM test has been used routinely for over 20 years to diagnose bovine tuberculosis (TB) in cattle, where it is widely used as an ancillary test to the SIT test or as a stand-alone test. The test is registered by the OIE (World Organisation for Animal Health) for use in cattle, goat, buffalo (*Syncerus caffer*), and sheep, both as a primary test and as an ancillary test to the SIT test.

Water buffalo (*Bubalus bubalis*) can also be infected with *Mycobacterium bovis*. The SIT test is routinely used for detection of the presence of the bacterium in this species. This test, however, is difficult to interpret in buffaloes due to the thickness, composition, and color of the animals' skin. In addition, a much higher number of doubtful and false-positive results have been reported in buffalo compared to cattle, because of environmental mycobacteria that transiently stimulate the immunity of the host in a nonspecific way.

For these reasons, the BOVIGAM test was investigated for the diagnosis of TB in water buffalo as an ancillary test to the SIT test. The experiments were performed by the Italian Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM), which coordinated and performed experiments, and the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSTO), which was responsible for the statistical analysis of the data, to evaluate performance of the test with respect to diagnostic sensitivity and specificity, repeatability, and reproducibility.

BOVIGAM TB Kit

The BOVIGAM kit is a blood-based assay of cell-mediated immunity. Animals infected with *Mycobacterium bovis* can be identified by measuring the cytokine interferon gamma (IFN-γ).

This method detects the amount of the cytokine produced by T lymphocytes of infected animals, in response to stimulation with tuberculin antigens derived from *M. bovis*. The result is compared with the nonspecific reaction induced by tuberculin purified protein derivative (PPD) extracted from *Mycobacterium avium* subsp. *paratuberculosis*. The cytokine produced is then quantified in a sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies.

Results

The BOVIGAM TB Kit shows excellent diagnostic accuracy

A total of 878 serum samples, previously determined negative by the SIT test (465 samples) or positive by postmortem tests (412 samples), were tested with the BOVIGAM TB Kit. The 878 samples were stimulated with tuberculin PPD from *M. bovis* (PPDB) and from *M. avium* (PPDA), and IFN-γ production was measured with the BOVIGAM TB Kit. The results for 80 animals could not be included in the interpretation, because the negative control didn't fulfill the kit's criteria (i.e., the optical density (OD) exceeded 0.13). The remaining 797 animals, 385 negatives and 412 positives, were classified with the BOVIGAM TB Kit.

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The test result is interpreted on the basis of a comparison between the production of IFN-γ following stimulation of the blood sample with PPDB and PPDA. See the materials and methods section for the interpretation criteria of the kit's instructions for use.

The BOVIGAM TB Kit correctly identified the status of 763 out of 797 animals (Table 1). Table 2 shows the estimates of the parameters used to calculate the kit's accuracy. The BOVIGAM TB Kit showed specificity of 97.9%, sensitivity of 93.7%, and overall accuracy of 95.7%. The kappa value, calculated as an index of agreement between the expected and obtained results, was 0.9147 (IC95% 0.8867–0.9427), which is very high agreement.

Table 1. Results of the BOVIGAM TB Kit test.

True sample status	Result with BOVIGAM test		
	Negative	Positive	Total
Negative	377	8	385
Positive	26	386	412
Total	403	394	797

Unclassified samples = 80

Table 2. Summary of the diagnostic accuracy indexes.

Parameter	Criterion	Value
Sensitivity	Suitable samples	412
	False negatives	26
	True positives	386
	Sensitivity	93.7%
	IC95% lower limit	90.9%
	IC95% upper limit	95.9%
Specificity	Suitable samples	385
	True negatives	377
	False positives	8
	Specificity	97.9%
	IC95% lower limit	96.0%
	IC95% upper limit	99.1%
Total	Accuracy	95.7%
	Kappa	0.91
	Total eligible	798
	Corrected	764
	Invalid data points	34
	Not classified	80

On the animals that tested positive, evaluations were also carried out with the single intradermal comparative cervical tuberculin (SICCT) test. The results are shown in Table 3. The SICCT test showed a lower sensitivity than the BOVIGAM test.

BOVIGAM kit results are highly reproducible between laboratories

The BOVIGAM TB Kit was tested for reproducibility of results. Two laboratories performed the test on 32 samples, 16 positive and 16 negative, and their qualitative (negative, positive) and quantitative (OD) results were compared. Table 4 shows that the qualitative results obtained by the two laboratories had very high agreement. The kappa value was 0.81 (IC95% 0.61–1.00), and the proportion of agreement observed was 90%.

We also determined the variance in OD values between laboratory 1 and 2 in order to get an even more robust evaluation of the reproducibility. Table 5 shows the values of the chi-square test and the *P* values for the analysis of quantitative variance performed with the Savage score technique. *P* values were >0.05 for both PPDA and PPDB. The tuberculin PPD measurements made by the two laboratories are therefore not statistically different from each other, so it can be concluded that the measurements are reproducible.

Table 5. Reproducibility of the BOVIGAM test, quantitative results.

Measured parameter	Chi-square	Degrees of freedom	*P* value
PPDA	0.8422	1	0.3588
PPDB	1.4834	1	0.2232

Table 3. Comparison of SICCT and BOVIGAM tests on positive samples.

Test	Suitable samples	False negatives	True positives	Sensitivity	IC95% lower limit	IC95% upper limit
SICCT	412	42	371	89.9	86.5%	92.6%
BOVIGAM kit	412	26	386	93.7	90.9%	95.9%

Table 4. Reproducibility of the BOVIGAM test, qualitative results.

	Laboratory 2		
Laboratory 1	Negative	Positive	Total
Negative	16	3	19
Positive	0	13	13
Total	16	16	32

BOVIGAM kit results are repeatable

Repeatability is estimated by measuring the variation between results of replicates of the same sample. For each sample, the minimum number of replicates is three. For the estimation of repeatability, 10 serum samples from 10 animals, 3 positive and 7 negative, with three replicates of each, were tested by the same operator under the same conditions. For the qualitative assessment, the Fleiss kappa index was calculated. The Fleiss kappa value was 1.00 (IC95% 0.64–1.00), indicating an almost perfect match. The reading of the results of the BOVIGAM test is therefore repeatable.

Repeatability was also estimated for OD readings obtained with the BOVIGAM kit from the 10 samples tested with three replicates. Table 6 shows the values of the chi-square test and the *P* values for the analysis of nonparametric variance performed with the Savage score technique. *P* values were >0.05 for both PPDA and PPDB. The three replicates are not statistically different from each other, so it can be concluded that the measurements are repeatable.

Table 6. Repeatability of the BOVIGAM test.

Measured parameter	Chi-square	Degrees of freedom	*P* value
PPDA	0.7699	2	0.6805
PPDB	0.3532	2	0.8381

Conclusions

The BOVIGAM TB kit is a robust and convenient test for detection of tuberculosis in cattle, goat, buffalo (*S. caffer*), and sheep. Here we provide data that show that the test can also be applied in water buffalo (*B. bubalis*). The BOVIGAM test showed higher sensitivity than the SICCT test that is routinely used for detection of *M. bovis* infection in this species. As the BOVIGAM kit is an OIE-registered test for species closely related to the water buffalo, the results are as expected. These results provide a foundation to enable more accurate testing for *M. bovis* infection in water buffalo.

Materials and methods

Diagnostic accuracy

For the estimation of diagnostic specificity, 465 buffaloes from 4 officially tuberculosis-free herds, all negative by the SIT test, were selected. The farms were in different Italian regions: 52 animals came from a Lombardian herd, 64 animals from a Piemonte herd, 80 animals from a Lazio herd, and the remaining 269 animals from a Campania herd.

For the estimation of diagnostic sensitivity, 412 animals were selected from 59 farms in Campania, where an outbreak of tuberculosis occurred. All the animals were positive in the postmortem tests: 386 with anatomo-pathological lesions related to tuberculosis and 60 with isolation of *M. bovis*. The average age of the animals was 7.6 (SD = 4.2) years, varying from 3 months to 26 years.

For the estimation of diagnostic accuracy, the following parameters were calculated: sensitivity, specificity, proportion of false positives, proportion of false negatives, accuracy, and all related confidence intervals.

The BOVIGAM TB test results were interpreted according to the kit instructions for use. In short, the test is interpreted on the basis of a comparison of the production of IFN-γ following stimulation of a sample with tuberculin PPDB and PPDA (Figure 1).

Positive	OD of PPDB – OD of nil antigen (PBS) ≥ 0.1 and OD of PPDB – OD of PPDA ≥ 0.1
Negative	OD of PPDB – OD of nil antigen (PBS) < 0.1 or OD of PPDB – OD of PPDA < 0.1

Figure 1. Interpretation of BOVIGAM TB Kit results.

Reproducibility and repeatability

Two categories of results were considered: qualitative (negative, positive) and quantitative (OD). For the estimation of reproducibility, 32 serum samples from 32 buffalo heads were selected, 16 of which were positive and 16 of which were negative. The tests were performed by two different laboratories (IZSME-Salerno, IZSUM-Perugia). For results expressed on a nominal scale (negative, positive), the Fleiss kappa statistical index can be used to quantify the degree of agreement between the results of a test. The Fleiss kappa varies from 0 (no agreement) to 1 (perfect agreement) [1,2]. For the purposes of qualitative evaluation, reproducibility has been defined as the degree of agreement between different laboratories on the same sample. It was calculated on 32 samples from two different laboratories with the Fleiss kappa [1]. For the interpretation of the kappa value, the table of Landis and Koch [2] was used.

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For the quantitative evaluation of reproducibility, the variances between the two laboratories in the measurements obtained for PBS, PPDA, and PPDB were compared. Since all parameters are considered to have an exponential distribution, the comparison between laboratories was made using the analysis of nonparametric variance of Savage [3], and the values of the scores between laboratories were compared using the chi-square test.

Repeatability is estimated by measuring the variation between the results of replicates of the same sample; for each sample the minimum number of replicates is three. For the estimation of repeatability, 10 serum samples from 10 animals, of which 3 were positive and 7 negative, and three replicates were carried out by the same operator under the same test conditions. For qualitative assessment, repeatability was calculated on 10 samples in 3 replicates by the same operator with the Fleiss kappa.

For the quantitative evaluation of the repeatability, the variances of the 3 series of measurements carried out for PBS, PPDA, and PPDB were compared. Since all parameters are considered to have an exponential distribution, the comparison between replicates was made using the analysis of nonparametric variance of Savage [3], and the scores of the three replicates were compared using the chi-square test.

Description of method

Samples of heparinized blood were collected from all buffaloes and transported to the laboratory immediately after collection and never more than 8 hours later. The quantitative detection of IFN-γ with the BOVIGAM TB Kit consists of two steps and was performed according to the kit instructions. In the first step, 1 mL of heparinized blood is dispensed into a 24-well culture plate and treated with 66 µL of PPD antigens to stimulate lymphocytes to produce IFN-γ. As a control for the animal's immunological condition, each sample is treated with 100 µL of phosphate buffered saline (PBS). The cell culture plates are incubated for 16 or 24 hours at 37°C in a humidified atmosphere, preferably with addition of 5% CO_2. After 18–24 hours, the plates are centrifuged for 5–10 minutes at 500 rpm. In the second step, the IFN-γ present in the sensitized plasma samples is quantified using a sandwich ELISA according to the instructions. The OD is determined at 450 nm. The different control values (negative controls and positive controls) used to validate the ELISA assay were defined according to the kit instructions.

The SICCT test was performed using Italian tuberculin PPD (PPDA It, 50 µg/mL; and PPDB It, 100 µg/mL). These are approved and used for official SICCT tests in Italy.

Ordering information

Product	Quantity	Cat. No.
BOVIGAM TB Kit	150 samples	63320
	450 samples	63326
BOVIGAM 2G TB Kit	300 samples	63330
BOVIGAM Tuberculin PPD Stimulating Antigen, bovine (30,000 I.U./mL)	5 mL	7600060
BOVIGAM Tuberculin PPD Stimulating Antigen, avian (25,000 I.U./mL)	5 mL	7600065
BOVIGAM Pokeweed Mitogen	3.2 mg	5108777

References

1. Fleiss JL (1981) *Statistical Methods for Rates and Proportions*. New York: John Wiley & Sons.
2. Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. *Biometrics* 33:159–174.
3. Hajek J (1969) *A Course in Nonparametric Statistics*. San Francisco: Holden-Day.

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