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Evaluation of the cut-off point of the interferon-y assay (IFN-y) for eradication of bovine tuberculosis

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Key words
Bovine tuberculosis, *Mycobacterium bovis/caprae*, IFN-y assay, *Mycobacterium avium* subsp. *paratuberculosis*, nontuberculous Mycobacteria

Aim of the study
This study aimed to: (i) verify, based on the epidemiological conditions in Switzerland, if a more stringent cut-off point of 0.05 for the interferon-y assay (IFN-y) compared to 0.1 is suitable and ethically acceptable; (ii) evaluate the performance of three commercial IFN-y test kits on blood-samples from cattle confirmed by specific culture for Mycobacteria of the *Mycobacterium tuberculosis* Complex (MTBC), nontuberculous mycobacteria (NTM) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

Material and methods
From a total of 118 healthy cows older than 6 months two blood samples were collected *ante mortem* for the IFN-γ. The animal testing was approved by the Animal Welfare Committee of the Canton of Zurich under licence number ZH185/18. Unforeseen, due to an insufficient number of animals which were stabled one day before slaughtering, the planned sample number of 200 could not be achieved. The commercial IFN-γ kits Bovigam TB (Thermo Fisher Scientific, Schlieren-Zurich, Switzerland), Bovigam 2G (Thermo Fisher Scientific) and ID Screen Ruminant IFN-γ ELISA test (IDVet, Grabels, France) were tested for each blood sample according to the manufacturers' instructions. The blood samples were stimulated with PPDs [Avian PPD, Bovine PPD (CZ Veterinaria for IDVet and Lelystad for Thermo Fisher Scientific)], Pokeweed Mitogen (PWM) as positive and PBS as negative stimulation control (NIL) according to the manufacturers' protocols. The stimulation of the blood samples occurred within 6h and 22-24h after collection respectively. A 24h delay in processing samples has been described to affect assay performance, decreasing the optical density (OD) of the values obtained in both tuberculin skin-test reactor and non-reactor animals [1]. The mentioned delay was deliberately included into the project in order to simulate a sample delivery overnight. Interpretation of the three kits was done using the following formulas as recommended by Thermo Fisher Scientific:

Positive result: cut-off 0.1: $OD_{PPDB} - OD_{NIL} \geq 0.1$ **AND** $OD_{PPDB} - OD_{PPDA} \geq 0.1$
Positive result: cut-off 0.05: $OD_{PPDB} - OD_{NIL} \geq 0.05$ **AND** $OD_{PPDB} - OD_{PPDA} \geq 0.05$

The results of the IDVet kit were additionally evaluated using the S/P ratio, as recommended by the manufacturer.

Positive result: $S/P\% = ((OD_{PPDB} - OD_{PPDA}) / (OD_{PK} - OD_{NK}))*100 \geq 35\%$

The performance of the three different kits at different stimulation times and cut-offs was compared with a Wilson binomial proportion confidence interval. In order to assess the viability of the cells, stimulation with a Mitogen control (PWM) was included for each test. Only if the values were valid according to the manufacturers' protocols, interpretation was done.

To confirm the bTB-free status of the tested 118 animals and to detect potential cross-reaction with NTM species a pool of pulmonary lymph nodes (left bronchial and caudal mediastinal) and a pool of intestinal tissues (ileum mucosa and jejunal/caecal lymph nodes) from each cattle were collected after slaughtering. The pulmonary lymph nodes were cultured at 37°C for eight weeks as described elsewhere [2]. The pool of intestinal tissues was cultured at 37°C for 16 weeks on Herrold's Egg Yolk agar with mycobactin J and ANV (BD, Basel, Switzerland), on BBL Stonebrink agar slants (BD) and on liquid MGIT supplemented with PANTA (BD) and mycobactin J (IDvet) as culture media.
All tubes showing growth of presumptive mycobacterial colonies were investigated for acid-fast bacilli after Ziehl-Neelsen (ZN) staining. The colonies were identified using MALDI-TOF MS and presumptive positive MAP colonies were confirmed by the ID Gene Paratuberculosis Duplex PCR (IDVet). For two isolates sequencing of housekeeping genes (*hsp65*/16S) was performed [2].

Results and significance

All the tissue samples were negative for MTBC whereas NTM were found in 17 of the 118 cultured pulmonary lymph node pools with *M. avium* subsp. *hominissuis* (MAH) as the predominant species (n = 14). In the remaining three pools *M. kansasii, M. lentiflavum* and a member of the *M. chimaera/intracellulare* group were isolated. In six out of the 118 intestine tissue pools MAP was cultured and one animal was positive for *M. engbaekii.*

The cattle positive for *M. kansasii* showed a clear positive result with all three kits, stimulation times and cut-offs. Our results confirm that *M. kansasii* leads to false-positive results in the interferon-y assay, as already shown in previous studies [3,4], whereas MAH and MAP seem to play a minor role in cross reaction. Cattle with positive MAP results were negative with all kits and test conditions with the exception of two false-positive results with the lower cut-off of 0.05 (all three kits). The majority of the MAH positive animals showed negative results with the IFN-y assay; only two animals were positive using the stricter cut-off of 0.05 (Bovigam 2G and ID Screen Ruminant), whereas one animal was also positive using the cut-off of 0.1 and the Bovigam 2G kit. The avian PPD values of the six MAP positive animals were compared with the corresponding values of the culture negative animals (Figure 1). Although the number of MAP positive cows is low, no significant variation could be observed.

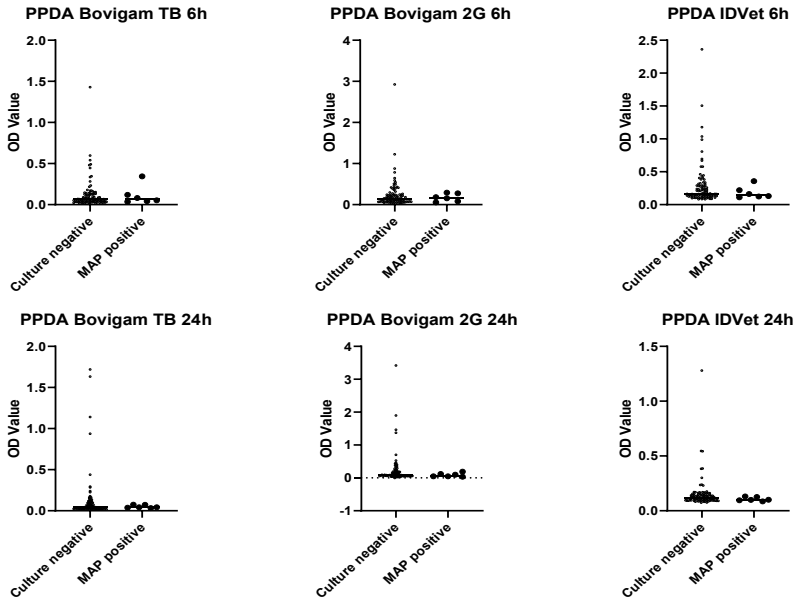

Figure 1: Distribution of the OD-values from the avian PPD (MAP infected animals versus culture negative animals). No significant difference between the six MAP infected animals and the culture negative animals were observed.

Taken the culture as gold standard and assuming the likelihood of a MTBC infection in Swiss cattle as very unlikely, every single positive test result can be assumed as a false positive result. The false positive ratios (FPR) for the three kits, stimulation times and cut-off were evaluated. The ID Screen Ruminant kit from IDVet with the S/P% evaluation showed the best performance with a FPR of 2.5% ($CI_{95\%}$ 0.8-7.2), independently from the stimulation times. The other two kits showed alarming high amounts of false-positive results, especially when applying the more stringent cut-off of 0.05 and the stimulation time of 6h after collection (Figure 2). For

instance, with the Bovigam 2G kit, a FPR of 32.1% ($CI_{95\%}$ 24.2-41.3) was observed with a stimulation time of 6h after collection and cut-off at 0.05, meaning that one third of the tested animals was classified as positive even though MTBC could not be cultured in any sample.

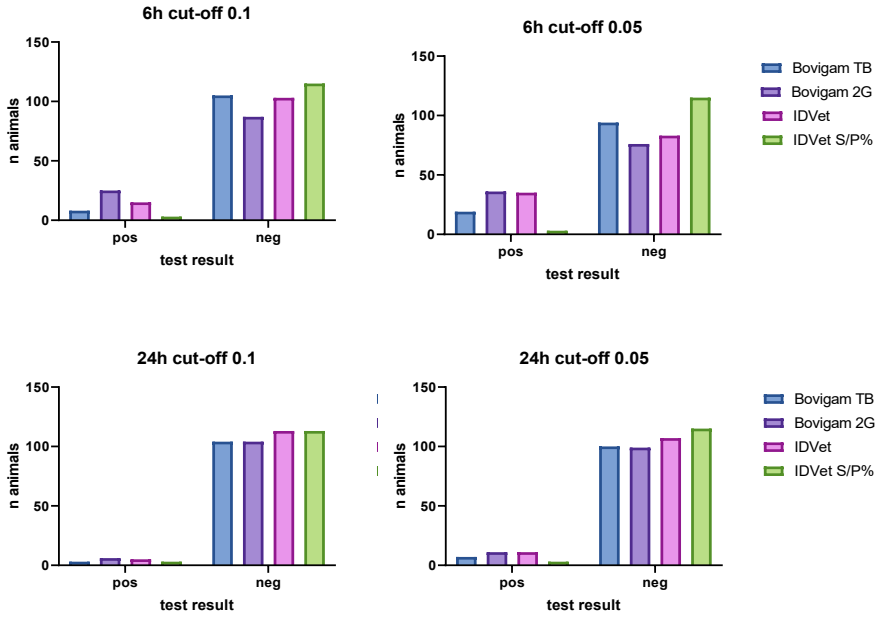

Figure 2: Test results of the three kits, two stimulation times and different interpretations. Based on the fact that no MTBC infections were detected in the tested animals, the number of false positive results in the different IFN-γ kit is noteworthy.

Comparison of the valid PWM values revealed, that the Bovigam TB kit had the most invalid results compared to the other two kits. With the stimulation time of 24h the Bovigam TB kit had a total of ten invalid results, whereas with the stimulation time of 6h there were only four invalid results. The Bovigam 2G kit only had two invalid results at the stimulation time of 6h, in contrast the IDVet kit showed no invalid results. Under realistic conditions, an invalid result would require an additional farm visit for blood sample collection, resulting in increased time and cost efforts.

Nevertheless, in order to evaluate the cut-off of the different kits, sensitivity data have to be considered. This can only be achieved by using samples originating from known infected herds or experimentally infected animals.

Publications, posters and presentations
The publication is under preparation.

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References

1 European Union Reference Laboratory for Bovine Tuberculosis. Technical report for the harmonization of the IFN-γ test protocol. (Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense de Madrid).

2 Ghielmetti, G. *et al.* Non-tuberculous *Mycobacteria* isolated from lymph nodes and faecal samples of healthy slaughtered cattle and the abattoir environment. *Transboundary and Emerging Diseases*, doi:10.1111/tbed.12793 (2017).

3 Scherrer, S., Landolt, P., Friedel, U. & Stephan, R. Distribution and expression of *esat-6* and *cfp-10* in non-tuberculous mycobacteria isolated from lymph nodes of slaughtered cattle in Switzerland. *Journal of Veterinary Diagnostic Investigation* **31**, 217-221, doi:10.1177/1040638718824074 (2019).

4 Waters, W. R. *et al.* Immune responses to defined antigens of *Mycobacterium bovis* in cattle experimentally infected with *Mycobacterium kansasii*. *Clinical and Vaccine Immunology* **13**, 611-619, doi:10.1128/CVI.00054-06 (2006).