

COMPARISON OF THE ESP CULTURE SYSTEM II WITH PARA-JEM BROTH WITH THE MODIFIED LÖWENSTEIN-JENSEN MEDIA CULTURE FOR DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN SHEDDERS

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TREK ESP culture system, Löwenstein-Jensen, Map, shedders

Introduction

Culture of *Mycobacterium avium* subsp. *paratuberculosis* (Map) on conventional solid agars is time-consuming due to the slow growth of Map. Therefore systems with a reduced incubation time, such as the TREK ESP para-JEM Culture System II, may allow earlier detection of Map shedders in certification-, surveillance and control programs for Map. Comparison of both systems revealed a good agreement if, after incubation for 42 days, all samples in the TREK ESP were tested by Ziehl-Neelsen (ZN) staining, and ZN-positive samples were confirmed by IS900-PCR (1).

The aim of this study was to compare the culture of Map fecal samples from Dutch cattle which were known to be shedders in the following two culture methods: on a conventional solid agar, modified Löwenstein-Jensen media (LJ), and in the TREK ESP para-JEM Culture System II.

Materials & methods

Fecal samples were collected from 60 cows expected to be intermediate to high Map shedders based on previous positive ELISA or culture results. Of each sample, 2 g was cultured on modified Löwenstein-Jensen media (LJ) or used in the TREK ESP system.

Briefly, for the culture on LJ the samples were decontaminated and treated with antibiotics. From each sample 4 tubes of modified Löwenstein-Jensen media (with mycobactin) were inoculated, incubated for up to 16 weeks and inspected at 8, 12 and 16 weeks. Suspected growth was confirmed by IS900 PCR. At the time of detection of Map the number of colonies were counted and were used for scoring the load of Map in the sample like the following: A = 1-10 colonies; B = 10-100, C = more than 100 colonies Map in the tubes after 8 (08), 12 (12) and 16 (16) weeks of incubation.

The samples were each inoculated in a flask Para-Jem (TREK diagnostic systems) after using a modified sedimentation-centrifugation method for the decontamination as described by Stable et al. (2) and incubated in the TREK ESP system for up to 6 weeks. Every 20 minutes the ESP TREK system measures changes in the gas pressure which might be an indication of the presence of Map in the sample and gives a signal. To determine the time to detection (TTD) of Map the signalling and the graphs of the gas pressure of the system were used. High shedding was determined as a TTD of 7-21, intermediate 22-28 and light shedding 29-42 days. After 6 weeks of incubation all samples were further investigated via Ziehl-Neelsen staining and the IS900-PCR.

Table 1: Comparison of the classification in light, intermediate and high shedding by both methods (n=45): ESP TREK system (ESP) and classical culture method using Löwenstein-Jensen media (LJ)

| ESP | LJ classification | | | total |
|---------------------------|-------------------|------------------|-----------|-------|
| | Light (A) | Intermediate (B) | Heavy (C) | |
| Light (29-42 days) | 0 | 5 | 2 | 7 |
| Intermediate (22-28 days) | 0 | 1 | 11 | 12 |
| Heavy (7-21 days) | 3 | 7 | 16 | 26 |
| Total | 3 | 13 | 29 | 45 |

Results

All samples but one were positive with both culture methods. In one sample Map was only detected by the TREK ESP system. Using the classification of the LJ culture method 31 cows were classified as heavy (C08-C12), 21 medium (B08-12) and 7 light (A08-12) shedders of Map.

The heavy shedders were also detected the ESP TREK system itself using the graphics of the gas pressure measurement. The mean time to detection TTD was 20,5 days by high shedders following the LJ classification system.

The TREK ESP system gives a TTD in 45 sample by signal and/of graph. The results of the classification both culture methods are shown in table 1 and figure 1.

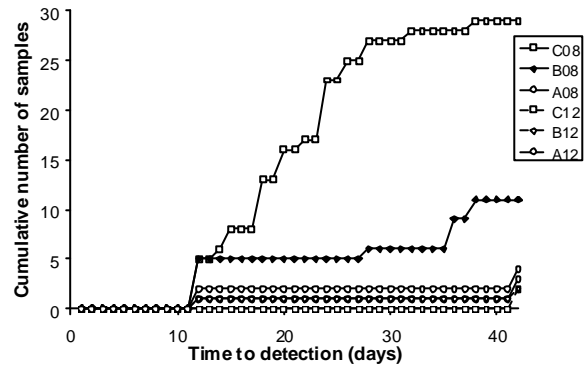


Figure 1: Time to detection in the TREK ESP system in comparison of the LJ classification

Discussion & conclusions

For the detection of Map shedders both systems give comparable results. All sample which were classified as heavy shedders following the LJ classification were also detected by the ESP system itself. Some light shedders were detected by the ESP system after a short period of incubation which could be interpreted as high shedders using semi-quantitative time of detection by the ESP system.

Both systems can be used for the detection of shedders. However, there is a poor agreement between both tests between the classifications of shedders as light, intermediate or heavy shedder. This could be due to the not equally distributed Map in fecal samples.

Acknowledgements

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References

- Rothkamp, A, van Maanen, C, Weber, M (2009). Fecal culture for *Mycobacterium avium* subsp. *paratuberculosis*: ESP culture system II with para-JEM broth versus modified Löwenstein-Jensen media culture, 10th International Colloquium on Paratuberculosis, Minneapolis, USA.
- Stabel JR. (1997). An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. *J Vet Diagn. Invest.*, 9 (4), 375-80.

VALIDATION OF A NEW COMMERCIALLY AVAILABLE BTV ELISA FOR THE DETECTION OF ANTIBODIES AGAINST BLUETONGUE VIRUS

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Blue Tongue Virus, early detection, serology, validation

Introduction

Blue Tongue is a non-contagious, vector-transmitted viral disease of domestic and wild ruminants. Disease symptoms are characterized by inflammation of the mucous membranes, congestion, swelling and haemorrhages. The aim of this study was to compare a new serological assay with the assay which is used at the laboratory since 2006. The assays are used for the detection of antibodies against *Bluetongue virus*.

Materials & methods

In this study the PrioCHECK® BTV DR test was validated and compared with the ID Screen® Bluetongue Competition ELISA.

Because of the pronounced high analytical sensitivity of the PrioCHECK® BTV DR test kit the validation started with testing 1081 negative serum samples. The validation has been performed by determining detection limit, relative sensitivity, specificity, selectivity, repeatability and reproducibility.

For determining the relative sensitivity a test panel of sera from vaccinated animals was used (70 cattle, 77 sheep and 24 goats). The detection limit was determined in two ways: by testing serum samples taken at different time point after experimental infection and by testing serially diluted serum samples.

Results

The PrioCHECK® BTV DR ELISA is highly specific (99.4%) and highly sensitive (96.5%) for the detection of BTV-specific antibodies in serum from cattle, sheep and goat.

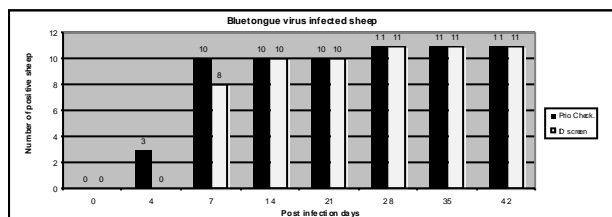


Figure 1: Results of sera taken at different time points after experimental infection

The PrioCHECK® BTV DR ELISA detects anti-BTV antibodies at post infection day 4 in sheep. This means an earlier detection comparing to the ID Screen® Bluetongue Competition ELISA (day 7 p.i.).

The coefficient of variation under repeatability and reproducibility conditions was found to be 6% and 14%, respectively

Discussion & conclusions

The PrioCHECK® BTV DR ELISA is a simple, highly specific and highly sensitive assay for the detection of BTV-specific antibodies in serum. The sensitivity of this test appears to be superior to that of other serological diagnostic procedures.

VALIDATION OF A WESTERN BLOT FOR THE DETECTION OF ANTI-*TRICHINELLA* SPP. ANTIBODIES IN DOMESTIC PIGS

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Trichinella spp., domestic pigs, serology, Western Blot

Introduction

Trichinellosis is a zoonotic disease in humans caused by *Trichinella* spp.. Measures to protect consumer health include testing pigs at slaughter for the presence of muscle stage larvae of *Trichinella* spp.. Also, the EU regulation as well as guidelines of the World Organisation for Animal Health (OIE) foresee the possibility of serological surveillance to demonstrate the absence of *Trichinella* spp. in a defined domestic pig population. Most ELISA tests presently available do not yield 100% specificity, and therefore there is a need for a complementary test to confirm the specificity of any initial ELISA-seropositivity. The goal of the present study was to evaluate the sensitivity and specificity of a Western Blot assay.

Materials & methods

The Western Blot, using somatic *Trichinella spiralis* muscle stage (L1) antigen, was tested as a confirmatory method to validate seropositive ELISA findings in the framework of serological surveillance for *Trichinella* infections in domestic pigs. Bayesian modeling techniques were used to account for the absence of a true gold standard test, as well as to correct for conditional dependence between serological tests. A total of 295 *Trichinella*-larvae negative samples and 93 *Trichinella*-larvae positive samples were included in the study. The *Trichinella*-larvae negative samples included 74 potentially cross-reacting samples from pigs with known other nematode infections..

Results

The diagnostic sensitivity and specificity of the Western Blot were 95.8-96.0% and 99.5-99.6%, respectively (1). It was also demonstrated that the diagnostic sensitivity of the routine artificial digestion test was below 100%, even when the larval density of the samples exceeded the limit of detection.

Discussion & conclusions

A sensitivity analysis showed that the model outcomes were hardly influenced by changes in the prior distributions, providing a high confidence in the outcomes of the models. This validation study demonstrated that the Western Blot is a suitable method to confirm samples that reacted positively in an initial ELISA.

References

1. Frey, CF, Schuppers, ME, Nöckler, K, Marinculić, A, Pozio, E, Kihm, U, Gottstein, B (2009). Validation of a Western Blot for the detection of anti-*Trichinella* spp. antibodies in domestic pigs. *Parasitol Res*, 104, 1269-1277

TEST VALIDATION – A VLA PERSPECTIVE

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Validation, fit-for-purpose, ELISA, *Trichinella***Introduction**

The changing requirements of quality systems, as applied in science, have influenced the necessity for tests used in both clinical and veterinary contexts to undergo some form of validation prior to use. The primary purpose of validating a diagnostic test is to provide assurance to both user and customer that the test is considered 'fit for purpose'. According to the OIE Manual (2009) 'a validated assay consistently provides test results that identify animals as positive or negative for an analyte or process... and by inference accurately predicts the infection and/or exposure status of animals with a degree of statistical certainty'.

Developing optimised and standardised approaches to both undertaking and assessing validation is challenging. The validation approach currently in place at the Veterinary Laboratories Agency, UK (VLA) may be demonstrated using a recently validated commercial ELISA for presumptive detection of *Trichinella spiralis* antibodies in porcine serum. For practical reasons the VLA wished to adopt a commercial test to replace the current in-house test. After validation of two commercial ELISAs, the assay considered fit for purpose was subsequently adopted as part of the VLA test portfolio.

Materials & methods**Study Design**

An initial feasibility study was undertaken using two commercial ELISAs compared against an 'in-house' ELISA. This test comparison was based on the testing of approximately 100 negative field samples, 25 *Trichinella* positive serum samples of varying titres (kindly provided by the *Trichinella* CRL and BfR) and samples from several possible cross reacting conditions including *Ascaris*, *Strongyloides*, *Trichuris*, *Hyostromyloides*. Positive samples used in this study were confirmed independently using digestion methods. There is no national or international standard serum for *Trichinella* species.

Repeatability studies were undertaken to demonstrate that the tests could be repeated on several occasions by a single operator with the same result outcome. Additionally, several different operators undertook testing using the test ELISAs against a panel of positive and negative serum samples.

Manufacturer test validation was provided; this information provided supporting information from independent studies.

Data analysis – 2X2 box analysis and statistical analyses of the resultant dataset.

Results

Analysis of diagnostic sensitivity and specificity demonstrated that the chosen test was 96.3% sensitive and 100% specific.

Discussion & conclusions

The chosen *Trichinella* test was considered cost effective, easy to use and implement within an existing testing laboratory whilst providing good sensitivity and specificity. This validated test will undergo regular review to confirm continued suitability.

References

1) World Organisation for animal Health (OIE) (2009). Principles of validation of diagnostic assays for infectious diseases. In OIE Manual of Standards for Diagnostic Tests and Vaccines, Edition. OIE, Paris, France, 34-45.

Table 1: 2X2 Box Analysis comparing the *Trichinella* gold standard test with the test ELISA of choice

| 2 x 2 Box Analysis | | Gold Standard Test – Digest / NRL ELISA | | |
|--------------------|----------|---|---------------|-------|
| | | Positive | Negative | Total |
| Test ELISA | Positive | 26 | 0 | 27 |
| | Negative | 1 | 93 | 94 |
| | Total | 27 | 93 | 121 |
| Analysis Term | | Value | Calculation | |
| Sensitivity | | 96.3 | (26÷27) x 100 | |
| Specificity | | 100 | (93÷93) x 100 | |

CALCULATION OF MEASUREMENT UNCERTAINTY IN QUANTITATIVE ANALYSIS OF GENETICALLY MODIFIED ORGANISMS BY 'GUIDANCE DOCUMENT ON MEASUREMENT UNCERTAINTY FOR GMO TESTING LABORATORIES'

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GMO, measurement uncertainty, within-laboratory reproducibility

Introduction

The measurement uncertainty (MU) is a parameter associated with the results of a measurement, and it characterizes the dispersion of the values that could reasonably be attributed to measurand (1). The MU is an important factor to be considered in GMO quantitative analysis. The importance of MU was recognized by European Regulation EC 882/2004 (2), where it is mentioned in relation to validation of methods used for official control. The same regulation defines that the national laboratory performing analysis of samples taken during official control must consequently be accredited.

Accreditation according to International Organization for Standardization (ISO) 17025 is introduced to many GMO testing laboratories around the world. Testing laboratories, therefore, have to apply procedures for evaluating the MU (3).

When the nature of test method may preclude rigorous, metrologically and statistically valid calculation of MU the laboratory has to identify all components of uncertainty and to make a reasonable estimation, and has to ensure that the form of reporting the results does not give a wrong impression about the uncertainty. MU can be reasonably estimated with the knowledge of the performance of the method and on the measurement scope using previous experience and validation data of the laboratory (4). Uncertainty calculation can be done in different ways: 1. bottom-up or deconstructive approach – which calculate uncertainty propagation, 2. top-down or holistic approach – which use validation data. The bottom-up approach provides 4 steps: measurement specification, uncertainty sources identification, uncertainty quantification, combined uncertainty. The top-down approach can be applied only with interlaboratory validation data, especially, considering the following parameters: Bias, repeatability (S_r), reproducibility (S_R). It is required to compare our validation data with a certified value. The Bottom-up method is surely precise but complex and expensive, the top-down method give more elevated values of uncertainty and a suitable selection of data is necessary to be used for the calculation.

Within Network of Italian GMO Laboratories (NILO) uncertainty is calculated in various ways. Differences are derived from the laboratory history, e.g. chemical laboratories have a bottom-up or metrologic approach. This approach is very complex to apply for a PCR method where many factors can affect uncertainty. Recently a novel method was proposed for calculating MU in environmental laboratories (5) based on within-laboratory reproducibility from Community Reference Laboratory. It's important that MU estimation include all phases of the analytical method. For this reason within-laboratory reproducibility is determined by independent sample measurements repeated in the same laboratory but varying factors like operator, time, equipment, calibration.

Samples should be representative of all matrixes and concentration that can be analyzed in the laboratory routine and include samples with a GMO level near legal limit (0,9%).

Our laboratory decided to apply CRL method (5) for MU calculation.

This method is based on: within-laboratory reproducibility Relative Standard Deviation (RSD_R), verification of Bias absence, Bias associated error calcul (U_{Bias}).

Therefore uncertainty measurement associated is calculated with combination of RSD_R and U_{Bias} .

Materials and Methods

In this work field samples and reference material were analysed by CRL analysis protocols for following transformation event: soy roundup-ready 40-3-2; maize MON810, Bt11, NK603, GA21, Bt176. For every event quantification was performed by real time PCR. The tests were carried out by testing each sample fivefold. The calculations for the measurement uncertainty followed according to the Guidance Document on Measurement

Uncertainty for GMO Testing Laboratories. To estimate the bias of the methods 1% certified material was used.

Results

Table 1 Calculated MU

| Matrix | Event | MU |
|--------|--------|--------|
| Soy | RRS | 19,96% |
| | MON810 | 26,04% |
| | Bt11 | 22,90% |
| Maize | NK603 | 8,30% |
| | GA21 | 12,08% |
| | Bt176 | 12,58% |

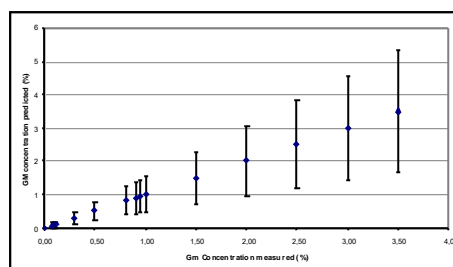


Fig.1 Uncertainty distribution for maize MON810 along a set of quantification value for maize MON810; the uncertainty increases with increasing value.

Discussion and Conclusions

All validated GMO detection methods need a in-house validation before the use in european community laboratories to establish the characteristic performances of the method under conditions of laboratory in-house. Our results (Table 1) show MU values that fully fulfil the values required by the ENGL document (maximum value 30%). MU for RRS, Mon810, Bt11 are higher than other values. For RRS and Mon810 this is due to the use also of data from field samples, while for maize Bt11 the PCR method itself is less efficient. Maizes NK603, Ga21 and Bt176 have lowest MUs, but in these cases data validation derived only from reference materials for lack of positive field samples.

This method is very useful for GMO laboratories and easy to apply, but it is not applicable for methods where reference materials with certified uncertainty are not available.

Bibliography

- (1) Guide to the expression of uncertainty in measurement (1995) International organization for Standardization, Geneva, Switzerland.
- (2) Regulation (EC) 882/04 (2004) Off J. Eur. Commun. L19, 1-52
- (3) ISO/IEC 17025 (2005) International Organization for Standardisation, Geneva, Switzerland
- (4) Zel, J., Gruden, K., Cankar, K., Stebih, D., Blejec, A. (2007). Calculation of Measurement Uncertainty in Quantitative Analysis of Genetically Modified Organisms Using Intermediate Precision-A Practical Approach. Journal of AOAC International, Vol.90, 582-586
- (5) Guidance Document on measurement Uncertainty for GMO Testing Laboratories (2008).

PROFICIENCY TESTING (RING TRIAL) TO CONTROL THE PERFORMANCES AND QUALITY OF DIAGNOSTIC TEST METHODS

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Proficiency tests, serology, ILAC-G13:2007

Introduction

Proficiency testing is a valuable tool to control test performances of laboratories. In addition, it gives information on the quality of the test systems used. Each quality laboratory needs to implement control procedures to monitor the validity of their tests. According to NEN-EN-ISO 17025, laboratories should participate in inter-laboratory comparison or proficiency tests, and laboratories are even obligated to participate in case the proficiency tests are accredited according to ILAC-G13:2007.

At this moment, different accredited proficiency tests are available such as proficiency tests for the qualitative and (semi)quantitative analyses of antibodies directed against Infectious Bursal Disease Virus, Infectious Bronchitis Virus, *Mycoplasma gallisepticum* / *Mycoplasma synovia*, New castle Disease Virus, *Salmonella enterica* (poultry), Avian Influenza virus, REO-virus, Avian Metapneumovirus / Turkey Rhinotracheitis virus (poultry), Porcine Reproductive Respiratory Syndrome Virus, *Salmonella enterica* (swine), Small Ruminant Lentivirus (SRLV: goat and sheep), and *Mycobacterium avium* subspecies *paratuberculosis* antibodies in serum and milk (cattle).

These proficiency tests can be performed using different test systems such as ELISA, VNT, HI-test, RPA-test and AGPT. Results from these different test systems can be used for comparison. In general, the purpose of proficiency testing is to determine the performances of individual laboratories for specific tests.

Materials & methods

In our set up, participants of proficiency tests (e.g. 40-125 participants from countries in Africa, Asia, Europe, Oceania and Latin America) will receive between 8 and 16 lyophilised serum samples (checked for homogeneity and stability). In most cases, the serum panels consist of mono-specific sera (well-defined field or vaccine-related sera), sera raised in SPF animals (isolators), field sera (known history) and sera from SPF animals. Serum panels should also mimic field situations (negative, low, medium and high antibody levels), including potential cross-reacting sera.

For analyses by participants, serum panels are transported by express post, and the dead-line for screening and reporting is in general 6 weeks. Sera need to be analysed in duplicate in two different test runs.

Results

Participants will receive a report after each proficiency test. The reports include the determination of precision and trueness, e.g. the within lab-reproducibility.

The deviation of trueness of the test results of a laboratory is defined as the difference in their total mean titre or S/P value (of all sera) compared to the total mean titre (or S/P ratio) of all laboratories (after exclusion of outliers) together using the same system

The within laboratory reproducibility (sR_{within}) or precision of the test results of a laboratory is defined as the square root of the mean variances of the duplicates of the test results (expressed as e.g. log₂ titres) of the duplicates.

The between laboratory reproducibility (sR_{between}) is defined as the square root of the mean variances in reported titres (or S/P ratio) for all sera by all laboratories using the same test system. Test results are subjected to two statistical tests for detecting outliers, namely the Cochran tests and the Grubbs test. The Grubbs test is used for detecting extreme mean values. The Cochran test is used to detect extreme within lab variation within each pair of serum samples. Detected outliers are excluded from the calculations of the mean results of a group of laboratories

using a particular test. Z-scores for all samples and labs with significant outliers will be marked.

Scatterplot average z-values

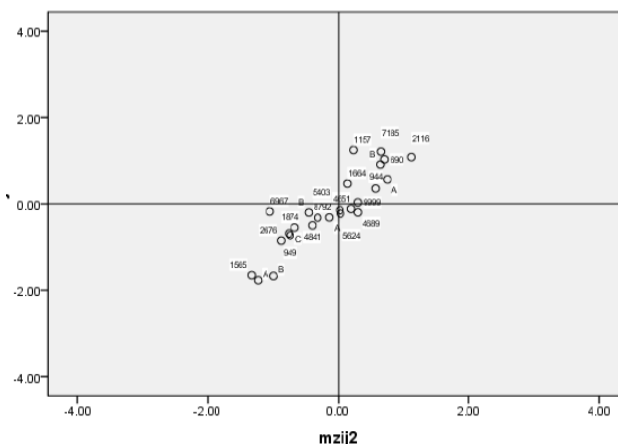


Figure 1: XY diagram: the difference expressed in Z-scores for the average titer of the first test run (z1) and second test run (z2) compared to the average result (titer) of all laboratories using the same test system.

Table 1: Results with outliers marking sR_{within} and sR_{between}

| Labcode | Mean value | Deviation from trueness | Within Lab reproducibility sd |
|---------|------------|-------------------------|-------------------------------|
| 4938 | 3.1 | -1.8 | 0.0 |
| 5202 | 4.3 | -0.6 | 0.4 |
| 5231 | 4.5 | -0.5 | 0.0 |
| 5403 | 4.6 | -0.3 | 0.5 |
| 5458 | 4.6 | -0.4 | 0.3 |
| 5624 | 4.9 | -0.1 | 0.4 |
| 6016 | 3.4 | -1.6 | 0.4 |
| 6325 | 7.9 | 2.9 | 0.0 |

Discussion & conclusions

The results of the proficiency tests, titers, OD- or S/P-values, can be compared with other labs. Information on e.g. "random errors" will be provided.

The advantages of this set up of proficiency tests are that every lab can join proficiency tests (global participation), easy way of participation by downloading the digital application from the website, digital data transfer, comparison of many kind of test systems, technical support and labs can examine their own SOP. Codes of laboratories are confidential and will not be given to third parties.

In summary, proficiency testing can be used to examine whether your laboratory is on "quality level" and it gives you and your customers the confidence that your test methods are accurate.

References

1. De Wit, JJ, van de Sande HW, Counotte GH, Wellenberg GJ. 2007. Analyses of the results of different test systems in the 2005 global proficiency testing schemes for infectious bursal disease virus and Newcastle disease virus antibody detection in chicken serum Avian Pathol. 36(2):177-83.

COMPARISON OF SEROLOGICAL ASSAYS FOR THE DETECTION OF ANTIBODIES AGAINST
COXIELLA BURNETII IN SERUM OF RUMINANTS

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Coxiella burnetii, serology, validation

Introduction

Coxiella burnetii is an intracellular gram-negative bacterium causing Q-fever in humans and animals. The aim of this study was to compare available serological assays for the detection of antibodies against *Coxiella burnetii* in ruminants to assess their relative performance.

Materials & methods

In this study seven specialized/veterinary laboratories performed the serological tests available for routine diagnostics, which are semi in-house IFTs, semi in-house CFTs and commercially available ELISAs. These assays were used to test a panel of 26 sera composed of 9 sera from goats, 6 from sheep and 11 from cattle. In all assays the sera were tested in dilutions to estimate the titer of the serum. This allows comparison of assays with different background.

Results

Results of all commercially available ELISAs and in-house IFT are comparable and have a minimum variation compared to the CFT. The performed in-house CFTs show lower agreement. The ELISAs show a better sensitivity and specificity compared to the CFTs having a larger Area Under the ROC curve with less variation. The CFTs have a higher specificity compared to their sensitivity with ROC curves tending to the left.

Discussion & Conclusions

The panel of sera used was small and tend to have strong positive and strong negative sera. Low positive sera around the cut off of the ELISAs were lacking. Therefore, strong conclusions can not be drawn. Commercially available ELISA show a better performance compared to CFTs on this panel. For a better comparison of the sensitivity and specificity of the ELISAs further research, with sera with titers around the cut off, is needed.

SELECTION AND VALIDATION OF A REAL-TIME PCR ASSAY FOR DIRECT DETECTION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN FAECES

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Paratuberculosis, real-time PCR, DNA extraction, faeces, validation

Introduction

Mycobacterium avium ssp. *paratuberculosis* (Map) is the etiologic agent of Johne's disease in cattle. The disease causes diarrhoea, reduced milk production, poor reproductivity, emaciation, and eventually death. Culture is considered to be the definitive test for diagnosis of Johne's in cattle. This method has moderate sensitivity (30 to 50%) and is considered 100% specific; however, it can take up to 16 weeks due to the slow growth of Map. Currently, serum and milk ELISAs are used to screen herds for Johne's disease, but positive tests must be confirmed. Development of molecular methods as an adjunct to or replacement of culture has been a challenge over the last few decades, especially with respect to sensitivity and robustness. Direct detection in faeces by PCR has until recently been reported inferior to culture (5), especially for detection of low to moderate faecal shedders. Performance of PCR methods was often hampered by low sample input and sample inhibition. In recent years, however, new DNA extraction methods and real-time PCR technology have yielded more promising results (1-4). Therefore, we decided to evaluate a selection of promising PCR assays, either available as commercial kits, or taken from the literature. If this evaluation would yield one or more promising candidate tests, a full validation was foreseen.

Materials & methods

A ring trial was organised with 45 faecal samples from known shedders as confirmed by solid and liquid culture, and 20 faecal samples from herds with a long history of repeatedly negative coprocultures for Map. Three commercial PCR assays and two in-house PCR assays were included in the comparison. Faecal samples were aliquoted and stored at -80 °C, samples were randomised and evaluated blinded, also for the technicians in our own institute that evaluated two commercial PCR assays. The assay of choice (AB MagMAX™ Total Nucleic Acid Isolation Kit + AgPath-ID™ MAP Reagent Kit) was extensively validated. First a panel of faecal samples from ELISA positive cattle (n=199) submitted for confirmation by culture was also tested by PCR. For specificity evaluation a panel of 50 individual faecal samples originating from 10 different Map certified herds were tested by PCR. Also a panel of environmental samples (slurry, n=232) from dairy herds with one or more ELISA positive cows was tested both by culture and PCR. Culture had already been performed on fresh samples, whereas PCR had to be carried out on samples that were either stored at -20 °C (n=92) or -80 °C (n=140). Finally a panel of faecal samples originating from light shedders or culture negative cattle (n=377), all originating from Map infected herds in a previous cohort study and stored for more than three years at -20 °C, was evaluated by PCR.

Results

The ring trial revealed remarkable differences between the five PCR assays with relative sensitivities as compared to culture ranging from 9 – 100%, and relative specificities ranging from 85 – 100%. Two commercial PCR assays yielded very good results (sensitivities of 96 and 100%, specificity 100%), but the assay with 100% concordance with culture results was far more practical and less laborious than the other assay with respect to the DNA extraction procedure. Therefore, the AB MagMAX™ Total Nucleic Acid Isolation Kit + AgPath-ID™ MAP Reagent Kit was chosen for further validation.

A panel of individual faecal samples from ELISA positive cattle showed almost equivalent results between culture and PCR with a kappa-value of 0.80 (CI 0.65 – 0.93), 83% positive by culture, and 85% positive by PCR. There was a clear correlation between semi-quantitative culture results (light, moderate, or heavy shedder), and the Ct-values of the PCR.

A panel of 50 samples originating from 10 different Map certified herds scored all negative by PCR (specificity = 100%).

A panel of environmental samples (slurry, n=232) from dairy herds with one or more ELISA positive cattle was tested by culture and PCR. The agreement was very good (kappa = 0.83, CI 0.70 – 0.95). ELISA positive cattle in these herds were also tested by individual faecal culture. For the environmental samples of herds where one or more cattle were confirmed by culture (n=216), kappa was 0.72 with a relative sensitivity of PCR as compared with culture of 97%, and a relative sensitivity of culture as compared with PCR of 96%. Test performance of PCR was equivalent for samples stored at either -20 °C or -80 °C. Since faecal samples from light shedders are most challenging for any agent detection test, a panel of faecal samples originating from light shedders or culture negative cattle (n=377), all originating from Map infected herds in a previous cohort study, was evaluated by PCR. Kappa was rather low (0.37, CI 0.27 - 0.47), but PCR scored more samples positive (n=207) than culture (n=167), although the samples had been stored for several years at -20 °C.

Discussion & conclusions

With advances in PCR and extraction technology, direct detection of Map in faeces has become feasible nowadays (1-4). We were able to select a practical semi-automated commercial PCR assay with high-throughput capacity for direct detection of Map in faeces. The assay can be used semi-quantitatively like culture, indicating high, moderate or low shedding. The assay has been implemented in our laboratory now for 1.5 years, and has almost completely replaced culture. This is not surprising, since costs for the farmer did not rise while the turn-around-time diminished from up to 16 weeks to a few days. An internal control enables to check for inhibition, but the percentage of samples that can not be reported due to inhibition is very low.

As a consequence of the biology of this organism (clumping, hydrophobic, intracellular organisms), the distribution in a faecal sample is inhomogeneous. In moderate and high shedders, the impact on test performance is rather low, but in light shedders results of any test (culture and PCR) are unpredictable, and a single negative test result is per definition not reliable.

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References

1. Alinovi CA, Ward MP, Lin TL, Moore GE, Wu CC. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*. *Vet Microbiol.* 2009 Apr 14;136(1-2):177-9.
2. Bögli-Stuber K, Kohler C, Seitert G, Glanemann B, Antognoli MC, Salman MD, Wittenbrink MM, Wittwer M, Wassenaar T, Jemmi T, Bissig-Choisat B. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by real-time PCR and culture: a comparison of the two assays. *J Appl Microbiol.* 2005;99(3):587-97.
3. Clark DL Jr, Koziczkowski JJ, Radcliff RP, Carlson RA, Ellingson JL. Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. *J Dairy Sci.* 2008 Jul;91(7):2620-7.
4. Scott HM, Fosgate GT, Libal MC, Sneed LW, Erol E, Angulo AB, Jordan ER. Field testing of an enhanced direct-fecal polymerase chain reaction procedure, bacterial culture of feces, and a serum enzyme-linked immunosorbent assay for detecting *Mycobacterium avium* subsp. *paratuberculosis* infection in adult dairy cattle. *Am J Vet Res.* 2007 Mar;68(3):236-45.
5. Wells SJ, Collins MT, Faaberg KS, Wees C, Tavoranpanich S, Petriani KR, Collins JE, Cernicchiaro N, Whitlock RH. Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Clin Vaccine Immunol.* 2006 Oct;13(10):1125-30.

TWO MONITORING PROJECTS IN THE NETHERLANDS: THE RELEVANCE OF EQUINE INFLUENZA VIRUS INFECTIONS IN OUTBREAKS OF INFECTIOUS UPPER RESPIRATORY TRACT DISEASE

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monitoring, AURD, equine, influenza, real-time PCR

Introduction

Antigenic drift of equine influenza viruses has regularly compromised vaccine efficacy. Vaccines including out-of-date strains can be ineffective in controlling virus excretion, and therefore equine influenza is still transmitted by subclinically infected vaccinated horses. For these reasons international surveillance and characterization of virus strains has become critical. Although influenza outbreaks in immunologically naive horses can be quite typical, clinical symptoms in partially immune horses are less pronounced and can also be caused by f.e. EHV1, EHV4, ERV1, ERV2, or EAV (4,5).

To investigate the role of equine influenza in equine acute Infectious Upper Respiratory tract Disease (IURD), a monitoring project was carried out in the Benelux region (collaboration between Animal Health Service in the Netherlands and Fort Dodge) and again in 2007/2008 in the Netherlands (collaboration between Animal Health Service and Merial).

Materials & methods

In the first monitoring project 110 diagnostic kits were distributed among 55 veterinary practices in the Benelux region, and in the second monitoring project 23 diagnostic kits were supplied on request of the practitioner. Practitioners were asked to take nasal swabs and paired blood samples from three acutely diseased horses per outbreak of acute respiratory disease. Also, many details regarding clinical symptoms, vaccination status, clinical diagnosis, days between sampling and onset of disease etc. were registered.

Nasal swab extracts were pooled per outbreak and tested with PCR for influenza A viruses. In the first project a conventional PCR was used (1), while in the second project a real-time PCR was used (2,3). Both PCRs target the matrix gene of influenza A virus. Individual nasal swab extracts of positive pools were retested and virus isolation was attempted from PCR positive individual swab extracts. Influenza virus strain characterization was carried out at the AHT, UK. Serological investigations were carried out at the Equine Irish Centre, Ireland, and at Boese labor, Germany.

Results

In the first monitoring project samples from 50 outbreaks were received. PCR screening was positive for influenza in 26 pooled samples (52%). Of 22 influenza PCR positive submissions with paired sera available, 12 were confirmed by serology, while for 7 submissions HI titres were high in both samples without significant increase, suggesting recent seroconversion before the first sample was collected. The interval between onset of disease and sampling was significantly lower for PCR positive submissions that could be confirmed by serology. Influenza virus isolation was successful for 8 out of 19 swab extracts. All strains submitted to the Animal Health Trust, UK belonged to the recent clusters in the European lineage.

In the second monitoring project samples from 23 outbreaks were received (46 individual samples). Seven out of 23 pooled samples (30%), and 14/46 (30%) individual samples were influenza PCR positive. When results of PCR and serology were combined an influenza infection was confirmed in 10 out of 23 outbreaks (43%), and in 17 out of 46 horses (37%). Ten samples from individual horses with the lowest Ct-values in the real-time PCR were used for virus isolation. Despite extensive attempts (6 passages on embryonated SPF chicken eggs) no influenza viruses were isolated. However, PCR results were confirmed by an independent real-time PCR carried out by the Irish Equine Research Centre (2).

Discussion

Despite obligatory vaccination of competition horses in the Netherlands, influenza appears to be endemic in the Netherlands both in vaccinated and unvaccinated horses. Until now the prevalence and relevance is underestimated by practitioners. In partially immune horses, clinical signs induced by influenza virus infections appear to be rather aspecific and are undistinguishable from IURD induced by other respiratory pathogens. Sampling in the early stage is important and continued monitoring is necessary. In both monitoring projects a significant number of horses had already seroconverted, and showed mucopurulent discharge indicating secondary bacterial infections. This suggests that samples are often collected rather late in the diagnostic window of virus excretion, which can be only a couple of days in partially immune horses. Delayed sampling also compromises successful virus isolation by diminishing virus excretion and neutralization of virus by mucosal antibodies. Virus isolation, however, is important for characterization of viruses, providing insight in genetic evolution and antigenic drift.

Conclusions

PCR screening of pooled nasal swab extracts is a useful tool for early influenza diagnosis in equine IURD.

In the Netherlands, equine influenza is a major factor in outbreaks of acute respiratory disease.

Diagnosis of influenza depends on strategic sampling of febrile horses in the very early stage of disease.

Virus isolation is often difficult, but continued surveillance and strain characterization remains necessary.

Acknowledgements

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References

1. Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, Osterhaus AD. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J Clin Microbiol.* 2000 Nov;38(11):4096-101.
2. Quinlivan M, Cullinane A, Nelly M, Van Maanen K, Heldens J, Arkins S. Comparison of sensitivities of virus isolation, antigen detection, and nucleic acid amplification for detection of equine influenza virus. *J Clin Microbiol.* 2004 Feb;42(2):759-63.
3. Van Maanen C, van Essen GJ, Minke J, Daly JM, Yates PJ. Diagnostic methods applied to analysis of an outbreak of equine influenza in a riding school in which vaccine failure occurred. *Vet Microbiol.* 2003 Jun 10;93(4):291-306.
4. Van Maanen C, Cullinane A. Equine influenza virus infections: an update. *Vet Q.* 2002 Jun;24(2):79-94.
5. Munford EL, Traub-Dargatz JL, Salman MD, Collins JK, Getzy DM, Carman J. Monitoring and detection of acute viral respiratory tract disease in horses. *J Am Vet Med Assoc.* 1998 Aug 1;213(3):385-90.

SELECTION OF A REAL-TIME PCR AND VALIDATION AS COMPARED WITH CULTURE FOR DETECTION OF VIRULENT *RHODOCOCCLUS EQUI* IN FOALS WITH PNEUMONIA

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Rhodococcus equi, foals, pneumonia, PCR, culture

Introduction

Rhodococcus equi is an important cause of subacute or chronic abscessating bronchopneumonia of foals up to 3-5 months of age. The possession of a large virulence plasmid in isolates recovered from pneumonic foals is crucial for virulence. *R. equi* are inhaled in dust and are ingested into macrophages in the lung. The basis of pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages. Infectivity is largely or exclusively limited to cells of the monocyte-macrophage lineage. Epidemiological studies have revealed that avirulent *R. equi* are widespread in the faeces of horses and their environment on every farm, that the faeces of horses and the environment of the horse farms having endemic *R. equi* infections demonstrated heavy contamination with virulent *R. equi*, that only virulent *R. equi* are isolated from lesions of naturally infected foals, and that infected foals which constantly shed large quantities of virulent *R. equi* in their faeces are the major source of virulent *R. equi* in farms with a potential for endemic infection. Regular examination of foals and their environment by virulence markers might be the most practical approach to control *R. equi* infection on endemic farms. Commonly used diagnostic techniques like radiology, ultrasonography and clinical chemistry do not unambiguously identify the causal agent, while culture has several drawbacks (1,5). Therefore we selected a real-time PCR assay targeting the *vapA* gene, and validated this assay for relevant biological matrices such as nasal swabs, transtracheal aspirates, purulent material, and faecal samples.

Materials & methods

Decimal dilutions of two different virulent *R. equi* strains (ATCC 33701 and Dutch field strain SZ2003-2470, concentration in cfu/ml predetermined by plate counting) were prepared in peptone-NaCl 0.9% on one hand and a faecal suspension on the other hand. From the first dilution series DNA was extracted with the Dneasy Blood&Tissue kit (Quiagen) and the MagMax Total Nucleic Acid Kit (AM1840, Applied Biosystems). For the faecal dilution series DNA was extracted with the QIAamp DNA Stool Mini Kit (Quiagen), the Møller-van der Heijden method, and the MagMax Total Nucleic Acid Kit (Applied Biosystems). For the latter extraction method elution volumes of 25 and 75 µl were compared. De 75 µl eluates were also tested undiluted and diluted 1:10 in BSA 2,5 mg/ml. Three different real-time PCR methods (2,3,4) were subsequently evaluated for detection limit and efficiency.

Two PCR methods were subsequently used to evaluate diagnostic performance on foals suspected of *R. Equi* infections (n=25). Participating veterinary practitioners received a diagnostic kit containing sampling material for two nasal swabs (bacteriology and PCR), a faecal sample, and – if possible a transtracheal aspirate. One veterinary practice received 100 plastic containers for faecal samples for longitudinal sampling of a cohort of 17 foals on an endemically infected farm.

Results

One PCR method (2) yielded in our hands inferior results with very high detection limits in both dilution series, regardless the extraction method, and was therefore excluded from further investigations. The other two PCR methods (3,4) yielded detection limits of 5-50 cfu/ml and 1000-5000 cfu/g for peptone-NaCl and faecal suspensions, respectively. The MagMax Total Nucleic Acid Kit (Applied Biosystems) was the most optimal extraction method for all sample matrices. The 75 µl eluates of most sample types could be tested undiluted, faecal eluates had to be tested diluted 1:10 in BSA 2,5 mg/ml.

Samples were received from 24 foals suspected of *R. equi* infection. Foals originated from 6 different premises. From all foals nasal swabs were collected, whereas faecal samples and transtracheal aspirates were collected from 22 and 7 foals,

respectively. Results for the PCR assay according to Rodriguez-Lazaro et al. are shown in tables 1a-c.

Table 1a. *R. equi* testresults for nasal swabs

| Culture | PCR | | Total |
|----------|----------|----------|-------|
| | Positive | Negative | |
| Positive | 6 | 2 | 8 |
| Negative | 9 | 7 | 16 |
| Total | 15 | 9 | 24 |

Table 1b. *R. equi* testresults for faecal samples

| Culture | PCR | | Total |
|----------|----------|----------|-------|
| | Positive | Negative | |
| Positive | 6 | 0 | 6 |
| Negative | 9 | 7 | 16 |
| Total | 17 | 7 | 22 |

Table 1c. *R. equi* testresults for transtracheal aspirates

| Culture | PCR | | Total |
|----------|----------|----------|-------|
| | Positive | Negative | |
| Positive | 3 | 0 | 3 |
| Negative | 1 | 3 | 4 |
| Total | 4 | 3 | 7 |

For transtracheal aspirates both PCR assays scored equivalent results, whereas for faecal samples and nasal swabs the PCR assay according to Rodriguez-Lazaro et al. (4) outperformed the other PCR assay (3). The former PCR combined with the most optimal DNA extraction method appeared to be useful to measure the infection pressure/bacterial load in sequential faecal samples from a group of foals originating from a farm with recurrent *R. equi* cases.

Discussion & conclusions

For the sensitive detection of virulent *R. Equi* in all relevant sample matrices, a real-time PCR assay in combination with a robust DNA extraction method was selected and validated. This assay showed consistently a better diagnostic performance than culture, and can also be used for quantification of faecal or nasal shedding. However, culture combined with an antibiogram can still be deemed necessary in cases of treatment failure. While a positive PCR results in a transtracheal sample can be considered unequivocal proof of infection, environmental contamination can not be ruled out in case of PCR positive nasal swabs and faecal samples. However, a positive PCR result always demonstrates the presence of virulent *R. Equi* on a farm level.

References

- Anzai T, Wada R, Nakanishi A, Kamada M, Takai S, Shindo Y, Tsubaki S. Comparison of tracheal aspiration with other tests for diagnosis of *Rhodococcus equi* pneumonia in foals. *Vet Microbiol.* 1997;56(3-4):335-45.
- Harrington JR, Golding MC, Martens RJ, Halbert ND, Cohen ND. Evaluation of a real-time quantitative polymerase chain reaction assay for detection and quantitation of virulent *Rhodococcus equi*. *Am J Vet Res.* 2005 May;66(5):755-61. Erratum in: *Am J Vet Res.* 2005;66(7):1139.
- Pusterla N, Wilson WD, Mapes S, Leutenegger CM. Diagnostic evaluation of real-time PCR in the detection of *Rhodococcus equi* in faeces and nasopharyngeal swabs from foals with pneumonia. *Vet Rec.* 2007;161(8):272-5.
- Rodríguez-Lázaro D, Lewis DA, Ocampo-Sosa AA, Fogarty U, Makrai L, Navas J, Scortti M, Hernández M, Vázquez-Boland JA. Internally controlled real-time PCR method for quantitative species-specific detection and *vapA* genotyping of *Rhodococcus equi*. *Appl Environ Microbiol.* 2006;72(6):4256-63.
- Venner M, Heyers P, Strutzberg-Minder K, Lorenz N, Verspohl J, Klug E. [Detection of *rhodococcus equi* by microbiological culture and by polymerase chain reaction in samples of tracheobronchial secretions of foals] *Berl Munch Tierarztl Wochenschr.* 2007;120(3-4):126-33.

SENSIVET™ PARATUB SYSTEM: AN INNOVATIVE TOOL FOR THE EFFICIENT PROPHYLAXIS OF THE BOVINE PARATUBERCULOSIS

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Bovine Paratuberculosis, para-Jem®, PCR, Sensitivity

Introduction

Despite various control strategies, the *Mycobacterium avium paratuberculosis* (MAP) infected herds fail to recover a free status. The lack of sensitivity of the standard or usual techniques can not effectively detect the infected animals before they become strong shedders. A new tool named SensiVet™ Paratub System, which contains an enrichment process that allows the multiplication of MAP in fecal samples of cattle, is shown to improve RT-PCR sensitivity. The aim of the study is to evaluate this increase of sensitivity enabling efficient control of MAP in herds.

Results

Table 1: Distribution of the results of the three different methods on the 174 animals

| | Total number of tested animals | Number of positive animals with RT-PCR diagnostic | Number of positive animals with para-JEM® + RT-PCR diagnostic | Number of positive animals with serology diagnostic |
|----------|--------------------------------|---|---|---|
| Herd n°1 | 55 | 2 | 21 | 4 |
| Herd n°2 | 49 | 2 | 15 | 3 |
| Herd n°3 | 70 | 7 | 40 | 10 |

Table 2: Sensitivity (Se) and specificity (Sp) of the serology compared to the para-JEM® + RT-PCR

| | | para-JEM® + RT-PCR | | Total |
|----------|-----|--------------------|-----|-------|
| | | POS | neg | |
| serology | POS | 17 | 0 | 17 |
| | neg | 59 | 98 | 157 |
| Total | | 76 | 98 | 174 |

Material & Methods

The study is conducted on 174 animals coming from three farms (two dairy and one beef cattle) which regularly present clinical cases of Johne's disease for many years. Three tests have been performed on each animal:

- Serology (ELISA, Institut Pourquier - France),
- Direct RT-PCR on feces (TaqVet™-M. Paratuberculosis, LSI - France),
- Enrichment of feces with a MAP specific process for 42 days, combined to RT-PCR (SensiVet™ Paratub System, LSI-France).

The table 2 shows :

Se (serology vs para-JEM®+RT-PCR) = 22.4%

Sp (serology vs para-JEM®+RT-PCR) = 100%

Table 3: Sensitivity (Se) and specificity (Sp) of the direct RT-PCR compared to the para-JEM® + RT-PCR

| | | para-JEM® + RT-PCR | | Total |
|---------------|-----|--------------------|-----|-------|
| | | POS | neg | |
| direct RT-PCR | POS | 11 | 0 | 11 |
| | neg | 65 | 98 | 163 |
| Total | | 76 | 98 | 174 |

The table 3 shows :

Se (direct RT-PCR vs para-JEM®+RT-PCR) = 14.47%

Sp (direct RT-PCR vs para-JEM®+RT-PCR) = 100%

Discussion & conclusions

The SensiVet™ Paratub System, which combines a specific MAP enrichment to RT-PCR, increases by 6.9 folds the number of animals detected as carrier of MAP. To the opposite of the usual methods, it allows the detection of young infected animals (the youngest was 16 months). A second sampling has been processed on one of the three herds after eight months. It shows a concordance of 90% between the results of the two samplings, with only 3 previously neg animals becoming POS and one POS becoming neg, all of them with low bacterial shedding. Considering the consistency of these results, it seems the phenomenon of MAP food transit is very limited, even with the high sensitivity of the method.

Based on this study, the SensiVet™ Paratub System enables new efficient protocols for disease control of Bovine paratuberculosis.

References

1. Collins, M. T., S. J. Wells, K. R. Petrini, J. E. Collins, R. D. Schultz, and R. H. Whitlock. 2005. Evaluation of five antibody detection tests for bovine paratuberculosis. *Clin. Diagn. Lab. Immunol.* 12:685-692.
2. Speer, C. A., M. C. Scott, J. P. Bannantine, W. R. Waters, Y. Mori, R. H. Whitlock, and S. Eda. 2006. A novel enzyme-linked immunosorbent assay for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infections (Johne's disease) in cattle. *Clin. Vaccine Immunol.* 13:535-540.
3. D. L. Clark, Jr., J. J. Kozickowski, R. P. Radcliff, R. A. Carlson and J. L. E. Ellingson 2008 Detection of *Mycobacterium avium* Subspecies *paratuberculosis*: Comparing Fecal Culture Versus Serum Enzyme-Linked Immunosorbent Assay and Direct Fecal Polymerase Chain Reaction. *J. Dairy Sci.* 2008. 91:2620-2627.

PROFICIENCY TESTING (PT) FOR LABORATORIES PERFORMING CHEMICAL ANALYSIS OF MEAT AND MEAT PRODUCTS ORGANISED BY THE NATIONAL VETERINARY RESEARCH INSTITUTE IN PULAWY

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Proficiency testing, z-score, chemical analysis

Introduction

The aim of this study is the qualification of laboratories with help of interlaboratory proficiency testing (PT) in range of analysed substances. Results of analyses should be credible with previously determined standard uncertainty of measurement. The harmonisation of correct results of chemical analysis is secure by the validation of analytic methods, standard uncertainty for individual measurement or for the method of analysis, using certified reference materials and participation in proficiency testing (PT). Information on the precision and accuracy of the results are to be taken into consideration in the design of the assay. In case of lack of reference materials or reference certified materials, participation in PT is the only method for confirming the technical competences of the laboratory..

Materials & methods

Meat sterilised cans were used for analysis. Every participant received sample together with instruction of transportation as well as a document which allows to introduce the sample in a quality system of laboratory. In this sample laboratory personnel should analyse at least one of parameters as sodium nitrate, salt (NaCl) by Mohr method, water, fat, phosphorus, proteins, ash, hydroxyproline, and starch. Deadline of realisation of analyses was appointed by organizer. The homogeneity of material was estimated on the basis investigation of eight random chosen samples/cans from designed to analysis, applying the criterion of the homogeneity $S_s < 0,3 \sigma$.

Statistical calculations and homogeneity assessments have been conducted in accordance with the principles stipulated in ISO 13528:2005 "Statistical methods for use in proficiency testing by inter-laboratory comparisons".

In the proficiency test 31 laboratories participated (official veterinary labs as well as private) and 43 analysts. Laboratory code numbers were known only for laboratory and organizer. The participants of PT applied the standardized method or such, which they use during normal analysis, after their validation.

Reference values for each analysed parameter were calculated with a help of algorithm A, according to Standard PN-ISO 57255: 2000, based on results from participating laboratories. The calculated reference values as well as standard deviations made it possible to calculate z-score according to the formula:

$$z = \frac{x - X^*}{s^*}$$

where,

- s* - standard deviation for proficiency assessment
- X* - assigned value
- x - participant result

For the purposes of performance assessment for a single round, z scores are interpreted as follows:

| | |
|----------------------------------|-----------------------|
| $ z \leq 2.00$ | satisfactory result |
| $2.00 < z \text{ and } < 3.00$ | questionable result |
| $ z \geq 3.00$ | unsatisfactory result |

Results

The z-score for the majority of analyses was situated in range of $|z| \leq 2.00$ and only in few cases the z value offends $|z| \geq 3.00$. Among sent 270 results of analysis, 252 results had z-score below $|z|$ and nine results were classified as unsatisfactory with z-score above 3,00

Table 1: Results of statistical calculation for tested samples

| | NaNO ₃ , [mg/kg] | NaCl, [g/100g] | Nitrogen, [g/100g] | Water, [g/100] | Fat, [g/100g] | Phosphorus, [mg/kg] | Ash, [g/100g] | Starch, [g/100g] | Hydroxyproline [g/100g] |
|---------------------------------|-----------------------------|----------------|--------------------|----------------|---------------|---------------------|---------------|------------------|-------------------------|
| Reference value | 11,77 | 1,75 | 2,27 | 63,2 | 18,87 | 2999,1 | 2,14 | 1,2 | 0,167 |
| Standard uncertainty | 0,42 | 0,01 | 0,01 | 0,30 | 0,3 | 20,12 | 0,01 | 0,09 | 0,00 |
| Std | 2,2 | 0,07 | 0,08 | 1,7 | 1,8 | 113,82 | 0,03 | 0,28 | 0,012 |
| Coefficient of variation cv (%) | 18,69 | 4,00 | 3,52 | 2,69 | 9,6 | 3,80 | 1,40 | 1,40 | 7,19 |

Table 2: The number of results for respective parameters, which z score carried out $|z| > 2$

| z - score | NaNO ₃ , [mg/kg] | NaCl, [g/100g] | Nitrogen, [g/100g] | Water, [g/100] | Fat, [g/100g] | Phosphorus, [mg/kg] | Ash, [g/100g] | Starch, [g/100g] | Hydroxyproline, [g/100g] | NaNO ₃ , [mg/kg] |
|-------------------|-----------------------------|----------------|--------------------|----------------|---------------|---------------------|---------------|------------------|--------------------------|-----------------------------|
| Number of results | 27 | 28 | 8 | 35 | 33 | 34 | 32 | 25 | 10 | 20 |
| $2 < z < 3$ | 3 | 2 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 0 |
| $ z \geq 3$ | 3 | 0 | 0 | 1 | 0 | 0 | 2 | 3 | 0 | 0 |

Discussion & conclusions

In a case of receiving questionable results ($2.00 < |z|$ and < 3.00), laboratory should start with preventive action. Responsible person should tracing the analytic procedure, review analytical records, the state used reagents or discuss with analyst executing analysis to aim at detection and the elimination of analytic shortcomings or mistakes. Obtainment $|z| \geq 3.00$ is effective with nonconformities and introducing corrective action. The procedure of corrective action should start with an investigation to determine root cause(s) of the problem. Corrective action shall be to a degree appropriate to the magnitude and the risk of the problem. After introduction and the realising of corrective actions additional audit and/or participating in the next PT should confirm in this way "practical" effectiveness of these procedures is recommend

References

- European Standard EN ISO/IEC 17025:2005
- Proficiency Testing Report No 1/2009 – National Veterinary Research Institute in Pulawy
- Myszewski J.M.2005. *Schemat międzylaboratoryjnych badań biegłości. Part E. OBJW Zetom*
- Guide to Quality in Analytical Chemistry. 2002, CITAC/ EURACHEM GUIDE
- ISO/IEC Guide 43-2:1997, Proficiency testing by inter-laboratory comparisons – Part 2: Selection and use of proficiency testing schemes by laboratory accreditation bodies.

IMPROVED VALIDATION METHOD FOR (Q)PCR ASSAYS

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Validation (q)PCR assays, thermocycler calibration, ISO 17025, molecular diagnostics

Introduction

Standard and real-time thermocyclers are currently perceived by the end user as black box systems that are assumed to perform adequately as specified by the thermocycler manufacturer. When combined with validated veterinary diagnostic kits it is assumed that validation of the assay, as required by the ISO 17025:2005 norm (1) is adequately covered. In this study the temperature performance of over 10.000 individual thermocyclers worldw ide, new and used, has been evaluated. The data of this study put a critical note at the currently used validation methods of kit manufacturers as substance spreads in thermal performance were observed between different brands and models of thermocyclers, but also w ithin the same brand and model.

Materials & methods

10.454 thermocyclers, both normal and real-time with both standard and fast blocks, have been calibrated using the 16 sensor dynamic MTAS system (CYCLERtest, The Netherlands). All calibrations were performed directly in the block without plastics or reaction mix, in the most basic block mode of the thermocycler to allow all measurements to be comparable to each other and to exclude the impact of non controlled variables. For open systems the heated lid was turned off. In closed systems the heated lid could not be turned off and therefore w as taken into the measurement.

All thermocyclers w ere measured w ith an identical temperature protocol, being 30°C for 60 s, 95°C for 180 s, 30°C for 60 s, 90°C for 180 s, 50°C for 180 s, 70°C for 180s, 60°C for 180s and 30°C for 60 s to allow comparability. The number of sensors used and the layout w ere block type defined. The parameters w hich have been measured are accuracy, uniformity, heating rate, cooling rate, overshoot, undershoot and plateau time. In the results and discussion section only accuracy and uniformity will be discussed, as we consider the other parameters outside the scope of this poster, but not less important.

Results

The thermocycler calibrations show that there is a large spread in both the accuracy and uniformity between different brands (figure 1, dots of different colors) and betw een different models of the same brand (figure 1, dots of the same color).

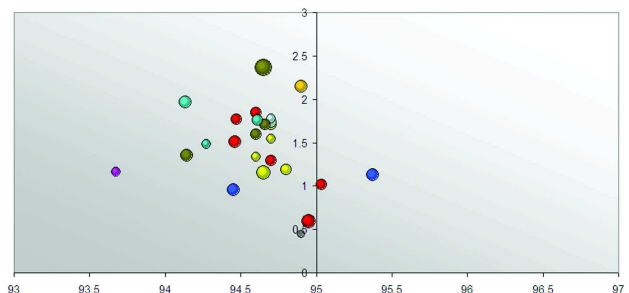


Figure 1: Average accuracy and non-uniformity of different models and brands of thermocyclers (dots of the same color are thermocyclers of the same brand)

Furthermore, when the calibration data are compared on individual serial number level (figure 2) also large spreads in both accuracy and uniformity are found.

In figure 2 it can be observed that the performance of model A and B of the same brand of thermocycler have little overlap in both accuracy and uniformity.

In case of an example kit optimized for 95C denaturation and robust enough handle 2C non uniformity and still give a reliable and reproducible results in all 96 w ells, 8% of model A does not give a reliable result in all 96 w ells.

When the same kit is used on model B it only gives a reliable result in all 96 w ells in 6% of the thermocyclers.

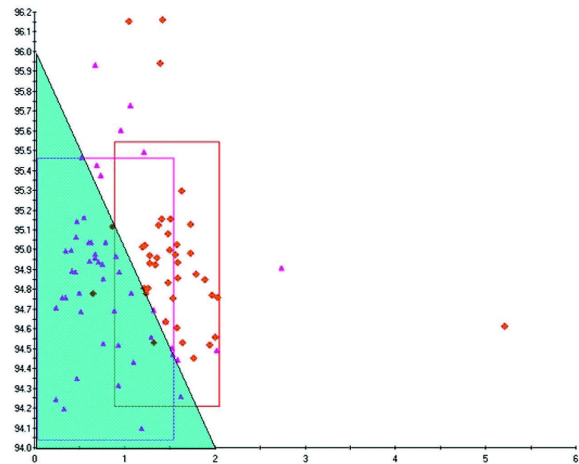


Figure 2. Individual accuracy and non-uniformity of thermocyclers of model A (triangles) and model B (diamonds) of the same brand versus example dynamic range of a kit (large triangle). The rectangles represent the 95% boundaries.

Discussion & conclusions

When comparing the accuracy and uniformity of different brands, models and also individual serial numbers of thermocyclers substantial differences can be observed. Practically this leads to situations that (q)PCRs are functional on a particular thermocycler, on which they have been optimized, but may not necessarily function on another thermocycler, even of the same brand and model.

The ISO 17025:2005 norm (1) requires individual assay validation. Often validated kits are bought by veterinary diagnostic labs, in combination w ith a particular cycler, hoping to ensure the reliability and reproducibility of a particular assay. Typically kit manufacturers validated their kits on small sample populations of thermocyclers (< 5-10 individual thermocyclers). These small sample populations are not representative for the total populations of the different thermocyclers. The risk for the end user is to buy a kit in combination w ith a thermocycler w ith the purpose to obtain reliable and reproducible data and end up w ith incorrect data due to the thermocycler variability as is currently exists in the field.

Taken the current thermocycler variability into account, we recommend both kit manufacturers and accredited laboratories to determine the thermal boundaries of an assay and qualify thermocyclers as being suitable to run a particular assay on. More information on how to determine thermal boundaries can be obtained from the author.

References

1. ISO 17205:2005, IDT – General requirements for the competence of testing and calibration laboratories

PRELIMINARY VALIDATION OF THE ID SCREEN RIFT MULTI-SPECIES ELISA

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Rift Valley Fever, ELISA, serology, diagnostics

Introduction

Rift Valley fever (RVF) is a vector-borne viral zoonosis transmitted by mosquitoes that infects a wide range of vertebrate hosts, including cattle, sheep and goats.

In livestock, infection is characterised by high rates of abortion and neonatal mortality.

The disease, which may cause severe illness or death in humans, is endemic in many countries of sub-Saharan Africa and in Egypt. In 2000, outbreaks occurred for the first time outside of Africa in Yemen and Saudi Arabia, raising fears that the virus will emerge in new areas.

RVF diagnosis may be obtained through tissue culture, PCR, histopathology, and serology. Seroneutralization is the prescribed serological test for international trade, but it may only be performed with live virus, raising biosecurity questions in non-endemic areas. In contrast, the ELISA method does not pose such risks, and is therefore particularly suited to antibody surveillance in disease-free countries.

IDVET has developed the ID Screen Rift Multi-Species ELISA. This ELISA is easy-to-use and detects anti-nucleoprotein antibodies in ruminants, horses, cats, dogs and humans.

This study evaluates the test's specificity, analytical sensitivity, and sensitivity, including correlation with the virus neutralization test (VNT).

Materials & methods

ID Screen RVF Multi-species ELISA was used according to the manufacturer's instructions.

The virus neutralization test (VNT) was performed according to OIE specifications (1).

Specificity on negative populations: 356 bovine sera, 88 cat and 74 dog sera from disease-free European populations were tested, as well as 81 European human sera never having been exposed to the virus.

Sensitivity: 40 cattle sera, collected in Djibouti and Mayotte in 2008, were tested using the ID Screen test. These sera were declared positive by VNT.

Analytical sensitivity: As no international standard exists for RVF serodiagnosis, analytical sensitivity was tested using an internal standard (pool of positive sera) developed by IDVET. Using this internal standard, IDVET is able to guarantee that the kit's detectability remains constant between batches.

Results

Specificity: all sera tested were correctly identified by the ID Screen test. Specificity was found to be 100% (CI95: 99.38%-100%).

Sensitivity: all sera which had tested positive by VNT were found positive by the ID Screen test. Sensitivity was measured to be 100% (CI95: 91.24%-100%).

Analytical sensitivity: the internal standard was consistently found positive up to a dilution of 1/8.

Discussion & conclusions

The ID Screen Rift Multi-Species direct ELISA is particularly easy-to-use (ready-to-use components and results in 60 minutes).

In addition, the direct method is particularly effective at detecting IgM antibodies, allowing for the early-detection of RVF-infected animals.

Preliminary validation data indicate:

- ▷ 100% specificity on the cattle, cat, dog and human sera tested.
- ▷ 100% sensitivity on the cattle sera tested. (Given the difficulty inherent in obtaining positive sera from other

species, IDVET welcomes scientific collaborations— please do not hesitate to contact idvet.info@id-vet.com.)

Acknowledgements

IDVET would like to thank **Catherine Cêtre-Sossah** (CIRAD - Département Systèmes Biologiques UPR "Contrôle des Maladies Animales Exotiques et Emergentes" - Groupe Virologie - TA A-15/G - Campus International de Baillarguet, Montpellier, France), for her collaboration on this study and in particular, for her work with virus neutralization testing.

References

1. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Fifth Edition, 2004. Chapter 2.1.8.*

VALIDATION OF THE THE ID SCREEN VISNA MAEDI INDIRECT ELISA: SPECIFICITY ON BTV-8 VACCINATED POPULATIONS AND DETECTION OF SEROCONVERSION

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Visna Maedi, ELISA, BTV vaccine

Introduction

Maedi Visna is an economically important persistent lentivirus infection of sheep. Although most infections are subclinical, a minority of animals develops progressive, untreatable disease symptoms including dyspnea (maedi) or neurologic signs (visna). ELISA testing is a valuable tool for the surveillance of infection by the Maedi Visna virus (MVV).

Further to the massive Bluetongue vaccination campaign in 2008, however, it was observed by a number of users and reference laboratories (AFSSA, FLI) that some commercial ELISA detected non-specific visna seroconversion in BTV-8 vaccinated animals. The ID VET indirect ELISA, ID Screen[®] Maedi Visna Indirect, is a peptide-based assay. The aim of this study was to evaluate the specificity and sensitivity of the test, and to verify that the assay's performance is not affected by BTV-8 vaccination.

Materials & methods

ID Screen[®] Maedi Visna Indirect ELISA uses a panel of peptides from the Maedi Visna virus (MVV) GAG and surface proteins as antigen, and an anti-sheep IgG peroxidase as conjugate. The test was performed according to the manufacturer's instructions.

Specificity on non vaccinated negative populations: 365 negative serum samples from disease-free herds in France (Aveyron) were tested.

Specificity on BTV-8 vaccinated negative animals: Sera from 198 Merial BTV-8 vaccinated sheep from three different herds were tested by the ID Screen[®] Maedi Visna Indirect ELISA at 0 and 50 days post-vaccination (dpv). In addition, 4 hyperimmunised sheep having received 4 doses (day 0, 15, 50 and 70) of the Merial BTV-2,4 vaccine were tested at 104 dpv.

Sensitivity: 10 sheep, experimentally-infected with two different MVV strains were tested using different commercial ELISA tests at day 8, 15, 28, 42, 58, 71, 84, 99 post infection. This study was conducted by Dr. Feliziani, IZS Perugia.

Results

Specificity on negative populations: 362 samples gave negative results, one gave a positive result and two gave doubtful results. The observed specificity was 99,18% (95CI 97,61-99,72%).

Specificity on BTV-8 vaccinated animals: All sera which were MVV-negative before BTV-8 vaccination remained negative 50 dpv when tested with the ID Screen[®] test. None of the hyperimmunised sheep were detected as positive by the ID Screen[®] Maedi Visna test 104 dpv.

Sensitivity:

At day 99 after infection ID Screen[®] ELISA detected 8/10 experimentally infected sheep when the other commercial ELISA detected only 3 and 4 animals respectively. None of the ELISA kits detected seroconversion in the 2 "missed" sera at day 99 post infection suggesting a very slow seroconversion on these animals.

Discussion & conclusions

The peptide-based ID Screen[®] Maedi Visna Indirect ELISA shows excellent specificity on disease-free populations and unlike other commercially available kits, does not detect non-specific seroconversion following BTV-8 vaccination. In addition, experimental infection data shows that the ID Screen ELISA detects seroconversion earlier than other commercial ELISAs.

VALIDATION OF THE ID SCREEN EQUINE INFECTIOUS ANEMIA INDIRECT ELISA

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Equine Infectious Anemia, ELISA, serology diagnostics

Introduction

Equine infectious anemia (EIA) is an infectious and potentially fatal viral disease caused by the equine infectious anemia virus (EIAV). A notifiable disease in most countries, serology is often used to control and monitor EIA.

This study evaluates the performance of the commercial ELISA developed by ID VET (www.id-vet.com), ID Screen® Equine Infectious Anemia Early Detection, for the detection of anti-EIAV antibodies in horse sera.

Materials & methods

The ID Screen® Equine Infectious Anemia Early Detection ELISA uses NP (Gene GAG) recombinant antigen and a NP antigen-peroxidase conjugate.

It makes use of the presence of two Fab (on IgG) or ten Fab (on IgM): one Fab binds the serum antibodies to the microplate, and the other Fab binds a peroxidase antigen used as conjugate.

This method detects both IgM and IgG antibodies, and therefore allows earlier testing of animals as well as long-term disease surveillance.

The test was performed according to the manufacturer's instructions.

AGID (Agar Gel ImmunoDiffusion): As per OIE specifications (1).

Specificity: 440 Coogins-negative samples kindly provided by the Laboratoire Départemental Frank Duncombe, Calvados, France, were tested.

Sensitivity: 24 AGID positive sera from France, Italy, the Czech Republic and the USA were tested.

Analytical sensitivity was tested by analysing serial dilutions of the OIE international serum standard (2).

Results

Specificity on negative populations: All 440 samples tested gave negative results.

The observed specificity was 100% (95CI: 99,8-100,00%).

Sensitivity: All 24 AGID-positive sera tested were found positive by the ID Screen® test, giving a measured sensitivity of 100%.

Analytical sensitivity: The serum standard was found positive when diluted 1/8. (The OIE requires that this standard be found positive when tested pure.)

Discussion & conclusions

The ID Screen® Equine Infectious Anemia ELISA shows excellent specificity and sensitivity:

- Specificity of the ID Screen® ELISA was evaluated at 100%(95CI: 99,8-100,00%).
- The kit correctly identified all 24 AGID-positive samples as positive.
- Analytical sensitivity goes beyond OIE standardization requirements.

This ELISA is an effective, reliable and rapid tool for the specific detection of anti-EIAV antibodies in horse sera.

Acknowledgements

IDVET would like to thank Mr. PTEL from the Laboratoire Départemental Frank Duncombe, Calvados, France for kindly providing the negative sera tested in this study.

References

1. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Fifth Edition. 2004. Chapter 2.5.4: Equine Infectious Anemia.*
2. *OIE International Reference Equine Infectious Anemia (Ecole Nationale Vétérinaire, 7 avenue du Général de Gaulle, 94704 Maisons-Alfort Cedex, France).*