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Evaluation of ELISA for the detection of *Trichinella* antibodies in swine: results from a ring trial

Evaluierung eines ELISA zum Nachweis von Trichinella-Antikörpern beim Schwein: Ergebnisse einer Laborvergleichsuntersuchung

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Summary

Regulation (EC) No 2075/2005 ensures official inspection of food of animal origin with specific rules on official controls for *Trichinella* in meat. Regarding certification of *Trichinella*-free farms, this regulation recommends serological methods for monitoring. During a ring trial 21 participants tested sera prepared by the German National Reference Laboratory for Trichinellosis and also field samples from their own sample collection. The provided commercial ELISA-kit demonstrated a very good diagnostic sensitivity and robustness. In conclusion the evaluated ELISA is a suitable method for monitoring purposes, also for serological examination of pigs determined for human consumption.

Keywords: ELISA, serology, pig, evaluation, ring trial

Zusammenfassung

Die EU-Verordnung 2075/2005 regelt die amtliche Überwachung von zum menschlichen Verzehr bestimmten Erzeugnissen tierischen Ursprungs mit spezifischen Vorschriften für die amtliche Überwachung auf Trichinen. Bezüglich der Zertifizierung von als *Trichinella*-frei anerkannten Betrieben sind nach der Verordnung serologische Verfahren für das Monitoring vorgesehen. Im Rahmen einer Laborvergleichsuntersuchung mit 21 Teilnehmern wurden sowohl Testseren aus dem Deutschen Nationalen Referenzlabor für Trichinellose als auch Feldproben aus den Teilnehmerlaboren getestet. Das den Teilnehmern zur Verfügung gestellte kommerzielle ELISA-Kit zeigte dabei eine sehr gute diagnostische Sensitivität und Robustheit. Damit ist die serologische Untersuchung von Schweinen, deren Fleisch für den menschlichen Verzehr vorgesehen ist, im Rahmen der Vorgaben der Verordnung eine für Monitoringzwecke geeignete Methode.

Schlüsselwörter: ELISA, Serologie, Schwein, Evaluierung, Laborvergleichsuntersuchung

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Introduction

In Germany trichinellosis is a rare food-borne disease caused by nematodes of the genus *Trichinella.* Depending on the infectious dose the course of disease ranges from mild to fatal. Humans are infected by consuming raw or insufficiently heated meat or raw sausages containing parasitic larvae.

Each pig is examined for *Trichinella* during slaughter in Germany. However *Trichinella* findings are very rare (less than 1 per 10 Mio.) and were exclusively traced to pigs which came from small farms and are kept outdoors. Most human infections are related to the consumption of meat products from high risk areas such as Eastern Europe.

According to the Regulation (EC) No. 2075/2005 different methods of artificial digestion have been approved for the detection of *Trichinella* in fresh meat, whereupon the magnetic stirrer method for pooled sample digestion is recommended as reference test. Serological tests such as the ELISA are useful for monitoring purposes and may be implemented in surveillance programs for farms or regions with a negligible risk of infection with *Trichinella.* ELISAs for the diagnosis of infection with *Trichinella* in swine are well established (Gamble et al. 1983, Nöckler et al. 1995).

To evaluate a new ELISA for serological detection of antibodies against *Trichinella* in pigs, a ring trial was organised by the Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR, Berlin, Germany) with 21 participants in September 2009.

Material and Methods

Participants

21 laboratories from eleven German Federal States (Baden-Württemberg, Bavaria, Berlin, Hesse, Mekklenburg-Western Pomerania, Lower Saxony, North Rhine-Westphalia, Saxony, Saxony-Anhalt, Schleswig-Holstein, Thuringia) participated in the ring trial.

After receiving the samples, the laboratories had to return the results in a filled form together with a copy of the ELISA protocol within five weeks.

Additionally a questionnaire was added to collect information about the participating laboratories, the handling of the ELISA kit and the procedure of ring trial.

ELISA

One kit of PIGTYPE® Trichinella Ab ELISA (Labor Diagnostik GmbH Leipzig, Leipzig, Germany) was provided to each participant. Each kit contained two pre-coated microtitre plates and all reagents needed for the test. The ELISA was performed as described in the manufacturer s manual, with the exception that all samples were tested in duplicate. Briefly the samples were diluted (meat juice samples 1:10, serum samples 1:100) and pipetted together with the ready-to-use controls into each well of the micro titre plate. Anti-IgG-HRP-conjugate and TMB substrate solution, respectively, were added to each well followed by incubation and several washing steps before addition of the next component. To stop the reaction a stopping solution was added and the optical density (OD) was measured in the spectrophotometer at 450 nm; using a reference wavelength (620–650 nm) was optional. To verify consistent coating of the micro titre plate, the results were interpreted for each well. Furthermore the participants were asked to repeat the test within 5 working days.

Testing panel

A testing panel consisting of 22 pig sera was designed and provided to each of the participants. Positive sera originated from experimentally infected pigs. All pigs were infected with Trichinella spiralis with doses ranging from 1 000 to 40 000 larvae with recovery rates in the diaphragm muscle varying from 61.97 up to 469 larvae per gram. For all pig sera used the titres had been determined previously by the in-house ELISA used at BfR.

Dilution series of two Trichinella-positive sera diluted in Trichinella-negative serum were included into the test panel. The test panel was pre-tested at the BfR with the PIGTYPE® Trichinella Ab ELISA before being sent to the participants. Expected ELISA results (negative/positive) were calculated from the optical density values (OD-values) as sample/positive (S/P) ratios according to the manufacturer s manual. The ratios were used for the evaluation of the results from the ring trial participants by means of statistical analysis (Tab. 1).

Field samples

Each participant was asked to additionally examine 22 field samples from the own collection (sera or meat juice samples from pigs or wild boars).

TABLE 1: *Serum titre for Trichinella antibodies (IgG) and the expected ELISA results calculated from the S/P ratio of pig sera in testing panel.*

serum identification	serum titre (BfR-Inhouse-ELISA)	expected ELISA result (evaluation)
1	>/= 1:1280	positive
2	1:10 dilution of serum 1	positive
3	1:2 dilution of serum 2	positive
4	1:2 dilution of serum 3	positive or negative
5	1:2 dilution of serum 4	negative
6	1:2 dilution of serum 5	negative
7	1:2 dilution of serum 6	negative
8	1:2 dilution of serum 7	negative
9	1:80	positive or negative
10	1:40	positive or negative
11	1:320	positive
12	1:160	positive
13	1:160	positive
14	1:10 dilution of serum 13	positive
15	1:2 dilution of serum 14	positive
16	1:2 dilution of serum 15	positive or negative
17	1:2 dilution of serum 16	negative
18	1:2 dilution of serum 17	negative
19	1:2 dilution of serum 18	negative
20	1:2 dilution of serum 19	negative
21	1:160	positive
22	>/= 1:1280	positive

Statistical analysis

Qualitative analysis

For each participant, ELISA results, expressed as "positive" or "negative", were compared with expected results to identify the number of false-positive and false-negative results.

Quantitative Analysis

The repeatability of the assay was analysed for the four OD-values of each serum (including positive and negative controls from the ELISA kit) and each laboratory by calculation of the variation coefficient (VC; VC = SD/MV_{OD}; SD = standard deviation of the four OD values; MV_{OD} = mean value of the four OD values).

Additionally for each laboratory the following parameters were calculated based on the S/P-ratio of the OD-values:

- z-scores (ISO 13528:2005; $z = (x - X) / \sigma$) – to determine the deviation of the laboratory mean (x) from the overall mean (X) scaled to a fixed desired reference standard deviation (σ). In the statistical analysis for the ring trial the reference standard deviation for all samples was set to a fixed value of 0.1. Results presented here refer to a complementary analysis of the true positive samples applying a reference standard deviation of 0.2*X.
- Mandel's k (DIN ISO 5725-2) – to analyse the variance of the laboratories measurements in comparison with the mean variance.
- Pearson correlation coefficient r – to evaluate the reproducibility of the test results in different laboratories in comparison to the results determined at BfR.

For each serum 84 OD-values and S/P-ratios (four measurements from each laboratory) were available for statistical analysis.

Results

Statistical analysis

For the qualitative analysis results are expressed as S/P (sample/positive) ratio and evaluated as positive ($S/P \geq 0.3$) or negative ($S/P < 0.3$).The ELISA results of 14 out of the 21 participating laboratories were in complete agreement with the expected results. Five laboratories had false-positive and two false-negative results. In most laboratories a single technician performed the test on two different days. In eight laboratories the tests were performed by two different technicians. One false-positive result could be attributed to the fact that two different persons performed the test. In all other laboratories with two technicians performing the ring trial, ELISA results could be reproduced.

Estimates for the diagnostic performance of tests are diagnostic sensitivity and diagnostic specificity. The diagnostic sensitivity was specified by OIE (2009) as the proportion of samples from known infected reference animals, which are tested positive, and the diagnostic specificity as the proportion of samples from known uninfected reference animals, which are tested negative in the assay, respectively. For this study borderline sera were not taken into account for the calculation of the diagnostic sensitivity and specificity. Thus, the overall diagnostic sensitivity and specificity was 98.93 % and 95.39 %, respectively (Tab. 2). For the borderline sera #4, #9, #10 and #16 the ratio of negative to positive results were 21/63, 1/83, 12/72 and 20/64, respectively. 83.93 % of the results of borderline sera were positive and 16.07 % negative.

TABLE 2: *Diagnostic sensitivity and specificity for ELISA calculated from the S/P ratios for pig sera from the testing panel of the ring trial. For each serum four OD-values have been taken into account except one result of a positive sample which could not be analyzed.*

	positive pig sera: 839	negative pig sera: 672
positive ELISA results	830	31
negative ELISA results	9	641
sensitivity/specificity	98.93 %	95.39 %

Variation coefficients (VC) assess the repeatability of an ELISA. A total number of 504 VCs were calculated from OD values of 24 sera (including the positive and negative control of the ELISA kit) of 21 laboratories. In this ring trial the VCs for the OD-values of serum samples ranged from 0.7-67 %. Out of 504 VCs, 31 (6.2 %) were between 20 % and 30 % and 34 (6.8 %) were >30 %. For the latter group with a VC >30 %, ten sera were *Trichinella* positive, 17 negative and seven borderline.

Further qualitative analysis was done with calculation of z-scores to determine the deviation of the laboratory mean from the overall mean. The results presented here refer to an analysis of only the true positive samples. Mandel's k was used to analyse the variance of the laboratory measurements in comparison with the mean variance. The distribution of z-scores and Mandel's k values over all laboratories is illustrated for the first test (Fig. 1 and 2; data for the second test are not shown). Horizontal lines depict the 95 and 99 % confidence intervals, respectively. Considering all data for each test there was one laboratory, which showed z-values outside the 99 % confidence interval. For Mandel's k this applies to eight laboratories in test one and to four laboratories in test two, respectively. All other laboratories did not show any value out of the range of tolerance.

Reproducibility is defined by OIE (2009) as the ability of an assay to provide consistent results, when aliquots of the same samples are tested in different laboratories, and estimated by correlation coefficients. The average Pearson correlation coefficient between the S/P-ratio of the OD-values obtained in each laboratory and the BfR reference measurements is 0.983±0.017 (mean ± standard deviation). Figure 3 graphically depicts the correlation between the ELISA results for examined pig sera of the participating laboratories compared to the ELISA results defined by the Reference Laboratory.

In several cases participants used other criteria for the evaluation than the recommended S/P-ratio, e. g. the OD-value-level. In some cases instead of taking the mean values for both the positive and negative control in duplicate as recommended by the manufacturer only one of the positive control results was used to calculate S/P-quotients.

Analysis of the field sample testing

The participants tested 212 serum, 33 plasma and 169 meat juice samples from pigs and 26 samples from wild boars. All field samples were serological negative except four samples from wild boars.

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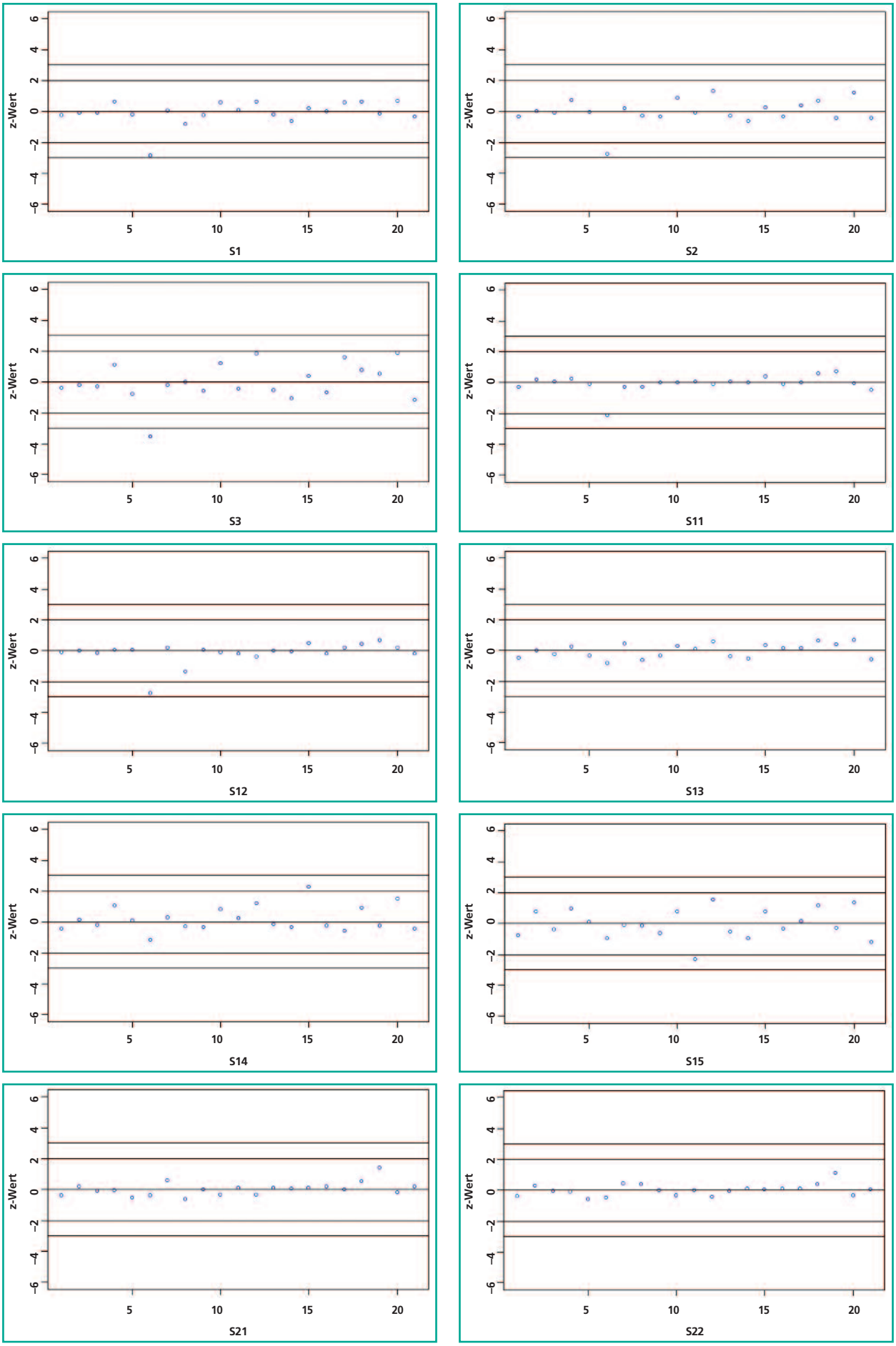

FIGURE 1: *Demonstration of the z-scores for the positive sera #1, #2, #3, #11, #12, #13, #14, #15, #21 and #22 for the first test. Horizontal lines show confidence intervals of 95 % and 99 %, respectively.*

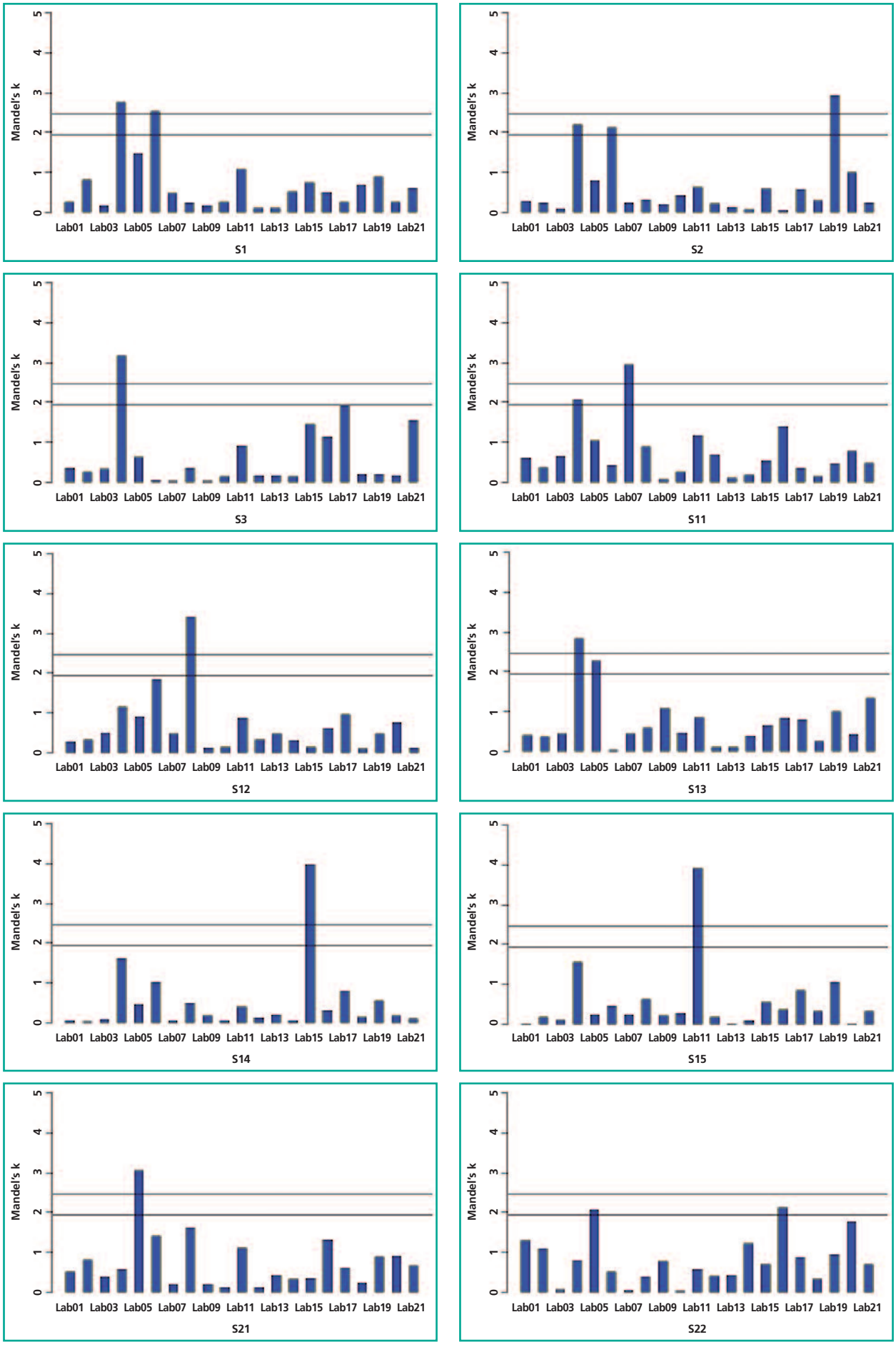

FIGURE 2: *Demonstration of the Mandel's k analysis for the positive sera #1, #2, #3, #11, #12, #13, #14, #15, #21 and #22 for the first test. Horizontal lines show confidence intervals of 95 % and 99 %, respectively.*

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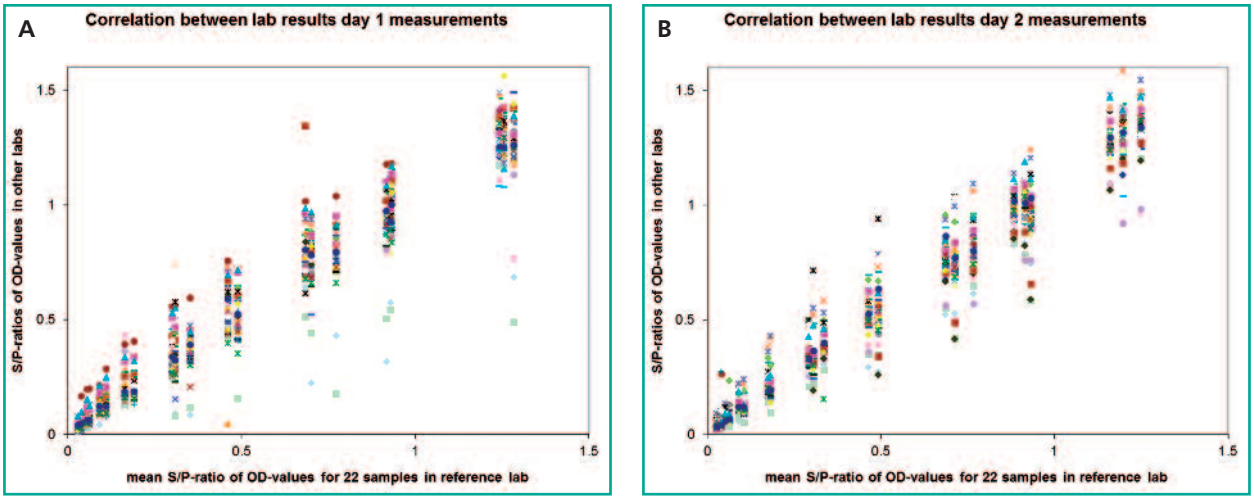

FIGURE 3: *Correlation for the calculated ELISA S/P ratio between participating laboratories and the reference laboratory measurements (A: first test, B: second test).*

Questionnaire

Eighteen of the participating laboratories were accredited. The laboratories vary in size, volume of serological services and sample material, respectively. There were also variations in the equipment used for testing (especially washing of micro titre plates). Five laboratories performed the tests manually, 13 laboratories used an ELISA-washer with automatic aspiration and one laboratory a washer without aspiration. Two laboratories used ELISA-washers without information concerning aspiration. Kit-package, handling of reagents, test procedure and rapidity were rated on average as sufficient. (The given choice was "insufficient", "sufficient" and "highly sufficient".)

Several deviations from the protocol were reported to the ring trial coordinator. In four cases the positive-control was interchanged with the negative-control on the plate, but this did not affect the calculation of the S/P-quotient. Once, the control sera were wrongly diluted. Also one participant diluted the washing buffer with demineralised instead of distilled water.

Another participant mentioned that the test would not be useful for automated application as sera must be strongly diluted (1:100) and manual performance would depend on very high concentration, because the coloured sample dilution buffer mimics the added serum. Concerning the procedure of the ring trial the participants considered the instructions to be helpful and the timeframe of approximately five weeks for testing adequate.

Conclusion

In Europe *Trichinella* infections in swine kept indoors have not been reported yet. However, free-ranging pigs and pigs kept in back yard conditions have an increased risk of infection with *Trichinella* since wild boars and other wildlife play an important role as *Trichinella* reservoirs. The negative ELISA results for field serum samples in this study seem to confirm that pigs are at low risk, while four of the 26 serum samples from wild boars were positive for *Trichinella* antibodies. However, results may be only indicative due to the small number of serum samples which were included from the field. Pally (2000) examined more than 16 000 wild boars from Mecklenburg-Western Pomerania by ELISA and found a *Trichinella*-seroprevalence of 1.38 %. During the same time period the prevalence in 292 460 wild boars tested with magnetic stirrer method for Germany was 0.003 % (German Federal Statistical Office, 2001–2007).

The main objective of the ring trial comprising 21 laboratories was to evaluate the PIGTYPE® Trichinella Ab ELISA regarding test accuracy and practical usage. Overall the ELISA results for laboratories demonstrate a good sensitivity and specificity of ELISA with 98.93 % and 95.39 %, respectively. Most of the borderline sera were identified as positive indicating that the diagnostic sensitivity of the evaluated ELISA was higher than the in-house ELISA.

Variation coefficients were used to assess the repeatability of the ELISA. According to OIE recommendations for test validation (2009), variation coefficients below 20 % show adequate repeatability in case of OD-values, while a variation coefficient above 30 % indicates too much variation within the assay. Because only 6.8 % of the sera showed a variation coefficient above 30 %, the repeatability of the ELISA was good for the participating laboratories.

Z-scores and Mandel's k were calculated to analyze the variability of the test results in more detail. Interpretation of these z-score analysis results must take into account that a laboratory with test results outside the calculated z-score confidence limits could still give correct qualitative diagnostic results. Nevertheless this analysis additionally demonstrated that the test results of both tests were reproduced by most laboratories. Two laboratories reported measurement values for at least one serum that were out of the 99 % confidence intervals. In both cases reasons should be identified. While for the ring trial report, which was sent to the participants, an extensive statistical analysis was performed on all sample measurements (including borderline and negative samples) this publication focuses on the test performance of the true positive samples. Taken together the PIGTYPE® Trichinella Ab showed a stable performance in both repeatability and reproducibility in this ring trial. Even for those laboratories where two technicians performed the tests, most obtained results were within the range of tolerance. The close correlation for S/P ratios between participants and the reference laboratory

also demonstrate a good performance of the ELISA.

By use of a questionnaire, participants were asked to give some information about their laboratory skills in order to better interpret possible problems during the performance of the test kit. However, participants showed a high compliance with the ELISA since the manual was clear and the test was easy to perform.

Altogether, results demonstrate that the evaluated ELISA is a suitable method for the serological *Trichinella* monitoring in pigs.

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+++ Nachrichten aus Forschung und Industrie +++

(Die Verantwortlichkeit für die Texte liegt ausschließlich bei den Instituten und werbenden Unternehmen.)

Sauerstoff hoch drei für sichere Lebensmittel

Experten diskutieren in Bremerhaven über Desinfektion mit Ozon

Wie kann Ozon effektiv zur Desinfektion von Oberflächen oder Lebensmitteln eingesetzt werden? Im Rahmen des sechsten Ozon-Workshops lädt das ttz Bremerhaven am 27. Oktober 2011 zum Wissens- und Erfahrungsaustausch ein. Erwartet werden Vertreter aus Wissenschaft, Forschung, Lebensmittelindustrie, Anlagenbau, Landwirtschaft und Politik.

Hochwertige, keimfreie und ökologisch einwandfreie Produkte herzustellen – das ist für Lebensmittelproduzenten und deren Zulieferer eine große Herausforderung. Die Desinfektion von Oberflächen und Lebensmitteln mit dem reaktionsfreudigen Ozon stellt in diesem Zusammenhang einen interessanten Weg zur umweltschonenden Qualitätssicherung dar. Der vom ttz Bremerhaven initiierte Workshop „Einsatz von Ozon zur Desinfektion in der Lebensmittelindustrie“ zeigt am 27. Oktober 2011 in Bremerhaven Wege auf, wie das Molekül nutzbar und eine optimale Produktqualität erreicht werden kann. Von 9.30 Uhr bis 16 Uhr wird über innovative Verfahren und Erfahrungen beim Einsatz von Ozon als Desinfektionsmittel diskutiert.

Aufgrund seiner starken Desinfektionswirkung ist Ozon, eine Erscheinungsform von Sauerstoff, bei korrekter Anwendung für eine schadstofffreie Desinfektion von Oberflächen und Lebensmitteln prädestiniert. Bei Kontakt mit Mikroorganismen oxidiert Ozon die Zellmembran von Bakterien, Viren, Pilzen, Sporen und Einzellern und leitet damit ihre Zerstörung ein. Als Reaktionsprodukt bleibt lediglich Sauerstoff zurück. Im Gegensatz zu umweltschädlichen Chemikalien, die üblicherweise in der Industrie eingesetzt werden, bleiben vom Ozon keine gefährlichen Rückstände an den Maschinen oder den Lebensmittelprodukten zurück.

Darüber hinaus bestehen weitere Vorteile: Weil bei der Desinfektion mit Ozon keine Rückstände bleiben, wird das Nachspülen der zu desinfizierenden Fläche mit Klarwasser, was bei herkömmlichen Desinfektionsverfahren nötig ist, überflüssig. So werden Wasser und Kosten gespart und darüber hinaus die

Ozon-CIP-Prototyp, entwickelt im Rahmen des von der EU geförderten Projektes OZONECIP. Foto: ttz/pr

Umwelt entlastet. Daher ist die Desinfektion mit Ozon in der Lebensmittel-Branche zukunftsweisend. Das ttz Bremerhaven möchte mit der Tagung die Entwicklung Ozon-basierter Desinfektionsverfahren fördern.

Ansprechpartnerin für fachliche und organisatorische Rückfragen ist die verantwortliche Projektleiterin Birte Ostwald, Tel.: +49 (0)471 9448 703, Fax: +49 (0)471 9448 722, E-Mail: bostwald@ttz-bremerhaven.de – die Teilnahmegebühr beträgt 290,– Euro.

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