ORIGINAL PAPER

Validation of a Western Blot for the detection of anti-*Trichinella* spp. antibodies in domestic pigs

C. F. Frey · M. E. Schuppers · K. Nöckler ·
A. Marinculić · E. Pozio · U. Kihm · B. Gottstein

Abstract Trichinellosis is a zoonotic disease in humans caused by *Trichinella* spp. According to international regulations and guidelines, serological surveillance can be used to demonstrate the absence of *Trichinella* spp. in a defined domestic pig population. Most enzyme-linked immunosorbent assay (ELISA) tests presently available do not yield 100% specificity, and therefore, a complementary test is needed to confirm the diagnosis of any initial ELISA seropositivity. The goal of the present study was to evaluate the sensitivity and specificity of a Western Blot assay based on somatic *Trichinella spiralis* muscle stage (L1) antigen using Bayesian modeling techniques. A total of 295 meat juice and serum samples from pigs negative for *Trichinella* larvae by artificial digestion, including 74 potentially cross-reactive sera of pigs with other nematode infections, and 93 meat juice samples from pigs infected with *Trichinella* larvae were included in the study. The diagnostic sensitivity and specificity of the Western Blot were ranged from 95.8% to 96.0% and from 99.5% to 99.6%, respectively. 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.

Frey and Schuppers contributed equally to this work.

This work was funded by the Swiss Federal Veterinary Office, grant number 1.06.03.

C. F. Frey (✉) · B. Gottstein
Vetsuisse Faculty, University of Bern, Institute of Parasitology,
P.O. Box 8466, 3001 Bern, Switzerland
e-mail: caroline.frey@ipa.unibe.ch

M. E. Schuppers · U. Kihm
SAFOSO,
Bremgartenstrasse 109A,
3012 Bern, Switzerland

K. Nöckler
Federal Institute for Risk Assessment,
Diedersdorfer Weg 1,
12277 Berlin, Germany

A. Marinculić
Faculty of Veterinary Medicine, University of Zagreb,
Heinzelova 55t, P.O. Box 466, 10000 Zagreb, Croatia

E. Pozio
Department of Infectious,
Parasitic and Immunomediated Diseases,
Istituto Superiore di Sanita,
Viale Regina Elena 299,
00161 Rome, Italy

Introduction

The nematode *Trichinella* spp. is the etiological agent of trichinellosis, a zoonotic disease (Dupouy-Camet and Bruschi 2007). Many carnivorous and omnivorous animal species may become infected with *Trichinella* spp., including domestic pigs. There is a general agreement that animals do not get sick following infection. However, the course of infection in humans includes disease that can range from subclinical to fatal (Kociecka 2000; Pozio et al. 2003; Dupouy-Camet and Bruschi 2007). In order to prevent disease in humans, susceptible animal species destined for human consumption must be tested for the presence of *Trichinella* spp. at slaughter or at game handling plants in the European Union (EU) and Switzerland (Anonymous 2005; European Commission 2005).

The method prescribed for routine testing of pigs is the artificial digestion method (European Commission 2005),

which is typically applied by pooling up to 100 samples of at least 1 g of diaphragm muscle tissue. The artificial digestion method is frequently considered to be the reference technique for detection of infected pigs. However, this test has limitations in terms of diagnostic and analytical sensitivity. Analytical sensitivity is defined as the smallest detectable amount of the specimen (World Organisation for Animal Health 2008a). The theoretical limit of detection for a 1 g sample is considered to be one larva per gram (LPG; Nöckler and Kapel 2007). However, the diagnostic sensitivity, i.e., the proportion of known infected reference animals that are tested positive (World Organisation for Animal Health 2008a) by artificial digestion, is below 100% when samples of less than 5 g are used (Forbes and Gajadhar 1999).

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 (European Commission 2005; World Organisation for Animal Health 2008b). Several serological techniques were developed so far for detection of antibodies against *Trichinella* spp. Especially, the enzyme-linked immunosorbent assay (ELISA) was regularly used for research purposes to evaluate the presence of anti-*Trichinella* antibodies in pig populations (Gamble et al. 1983, 2004; Nöckler et al. 1995, 2000). The diagnostic sensitivity and diagnostic specificity of such ELISAs do not yield 100% (Gamble et al. 2004); however, an advantage of ELISA tests is their higher analytical sensitivity in comparison to the routine artificial digestion method. ELISAs are able to detect antibodies in pigs with larval densities at least as low as 0.01 LPG (Gamble et al. 1983; World Organisation for Animal Health 2008a).

If the ELISA is considered to be used for routine serological surveillance purposes, a protocol should be developed to deal with samples reacting positively in the ELISA. ELISA-positive samples may be truly positive, when the sample originated from a truly infected pig. However, due to imperfect specificity of the ELISA, a seropositive reaction in ELISA should be subjected to a confirmatory test before a final decision is made.

Confirmation could be done by artificial digestion of a larger muscle sample. Artificial digestion of a larger muscle sample is currently considered the reference method not only for routine testing but also for confirmatory *Trichinella* testing of seropositive individuals. The OIE even recommends to use at least 100 g of tissue to confirm seropositive findings (World Organisation for Animal Health 2008a). However, collection and storage of such large samples may prove impractical under the conditions of routine serological surveillance. Confirmation of ELISA results by an appropriate second serological method may therefore be of high practical value.

Western Blot assays are already used for diagnostic investigations in other parasitic diseases like echinococcosis and cysticercosis in humans (Gekeler et al. 2002; Muller et al. 2007) or infections in cattle with *Neospora caninum* (Staubli et al. 2006) and *Besnoitia besnoiti* (Cortes et al. 2006). For serological testing in humans for *Trichinella* spp., commercialized Western Blot assays are routinely used as a confirmatory test to distinguish between patients with *Trichinella* infections and other helminth infections (Robert et al. 1996; Kociecka 2000; Yera et al. 2003). Western Blots have also been used for the detection of anti-*Trichinella* antibodies in animals, mostly in pigs (Marinculic et al. 1991; Nöckler et al. 1995; Ortega-Pierres et al. 1996) and horses (Yepez-Mulia et al. 1999; Pozio et al. 2002; Sofronic-Milosavljevic et al. 2005). The specificity of the Western Blot is considered to be very high (Robert et al. 1996; Yera et al. 2003), but little is known regarding the sensitivity of this method.

Before the Western Blot can be used as a confirmatory method in routine serological surveillance, it must be validated. As laid out above, no "gold standard" serological test is available for such a validation, and also the routine artificial digestion test cannot be considered to be a gold standard test. In a situation of validation without a true gold standard, appropriate statistical methods should be used to correct the estimates of sensitivity and specificity of the new test for the imperfect reference test (World Organisation for Animal Health 2008a). Sensitivity and specificity of both tests can be estimated reliably if both tests are applied in two populations with a different prevalence (Hui and Walter 1980; Johnson et al. 2001). The underlying assumption of this approach is that the sensitivity and specificity of the diagnostic tests are the same in both populations. By applying Bayesian techniques in the test validation, prior information about the sensitivity and specificity of the diagnostic test can be used to improve their posterior estimates (Branscum et al. 2005). Most models assume that the results of the different tests are independent from each other, conditional on the infection status (Hui and Walter 1980; Johnson et al. 2001). This assumption is valid when the Western Blot assay is compared with the artificial digestion technique because the first test is based on antibody detection, whereas the second is based on detection of the infecting agent. However, when the tests are conditionally dependent, the outcomes of the validation may be biased if the model is not corrected for this conditional dependence (Georgiadis et al. 2003; Branscum et al. 2005). This could be the case when the Western Blot assay is compared with the ELISA because both tests are based on antibody detection.

The goal of this study was to validate a Western Blot assay for the detection of anti-*Trichinella* antibodies in domestic pigs. Bayesian modeling techniques were used to

account for the absence of a gold standard test, as well as to correct for conditional dependence between serological tests.

Materials and methods

Test samples

The minimum required sample size was calculated using standard epidemiological techniques as 70 samples for each of the groups finishing pigs, free ranging pigs, adult pigs, potentially cross-reactive samples, and *Trichinella*-positive pigs based on an assumed sensitivity and specificity of the Western Blot of 99.9%. This would allow determination of sensitivity and specificity with a precision of 1% and a 99% confidence interval. No quantitative prior estimates for the sensitivity and specificity of the Western Blot were available; therefore, the assumption was based on personal judgment of the authors.

A total of 295 *Trichinella* larvae negative (221 meat juice and 74 serum samples) and 93 *Trichinella* larvae positive meat juice samples were included in the evaluation study (Table 1). In the period of January–March 2007, a total of 221 diaphragm tissue samples of 20 g each were collected at Swiss slaughterhouses from finishing pigs, free ranging pigs, and adult breeding pigs. The infection status of the pigs was determined at the slaughterhouse by routine pooled artificial digestion of 1 g (finishing pigs) or 2 g diaphragm tissue (adult pigs). All samples were negative for *Trichinella* larvae. Meat juice samples from these 221 pigs were used for the serological analysis. Additionally, 74 serum samples from *Trichinella*-negative pigs, which were known to be infected with other nematodes, were used. These samples had been collected within the framework of a study (porc'99) designed to monitor the health status of the Swiss pig population (Conzelmann 1999; Huber 1999; Hadorn et al. 2002). The coprological detection of intestinal helminth infections had been carried out with a conventional sedimentation and flotation technique (Bauer 2006).

Seventy-nine meat juice samples from naturally infected pigs from Italy and Croatia were included in the study. Larvae could be isolated from 60 naturally infected pigs and had been identified as *Trichinella spiralis* (58 samples) and *Trichinella britovi* (two samples) using a multiplex PCR (Zarlenga et al. 2001). Finally, 14 meat juice samples from experimentally infected pigs were included in the validation study. Pigs had been inoculated with *T. spiralis* (12 pigs), *Trichinella pseudospiralis* (one pig), or *T. britovi* (one pig). Additional details on the meat juice and serum samples used for this study are provided in Table 1.

ELISA

For the in-house ELISA, E/S antigen coated plates and positive and negative control sera of experimentally infected pigs were provided from BfR, Berlin, Germany (Gamble et al. 1983, 1988; Nöckler et al. 1995). Serum samples and controls were diluted 1:100 in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), pH 7.2. Meat juice samples were diluted 1:10 in PBS-T. Of each of the samples and controls, 50 µl was added per well. The plate was incubated for 30 min at 37°C and then washed three times with PBS-T. Peroxidase-labeled IgG-conjugate (50 µl; Sigma Cat No. A5670; diluted 1:4,000 in PBS-T) were added to each well, followed by incubation

Table 1 Description of the meat juice and serum samples from pigs used in this study

Infection status	Explanation	Number of samples	Origin of sample	Larval density in larvae per g (number of samples)	Infective species (number of samples)
Trichinella-negative	Finishing pigs	72	Meat juice	–	–
	Free ranging pigs	73		–	–
	Adult pigs	76		–	–
Trichinella-negative, other nematode infections	*Hyostrongylus*	21	Serum	–	–
	Ascaris	8		–	–
	Trichuris	32		–	–
	Strongyloides	1		–	–
	Multiple infection	12		–	–
Trichinella-positive	Naturally infected from Croatia	77	Meat juice	0.025–350 (72), unknown (5)	*T. spiralis* (58), unknown (19)
	Naturally infection from Italy	2		4–34 (2)	*T. britovi* (2)
	Experimentally infected	14		1.65–454 (14)	*T. spiralis* (12), *T. britovi* (1), *T. pseudospiralis* (1)

for 30 min at 37°C. After incubation, microtiter plates were washed three times with PBS-T. Then, 50 μl of freshly prepared developing solution was added, prepared by dissolving one ABTS (2,2′-azino-di (3-ethylbenzthiazoline-6-sulfonate di-ammonium) tablet (Roche, Switzerland) into 50 ml of ABTS buffer (Roche, Switzerland). After an incubation of 15 min at room temperature (RT), the absorbance values were read at 405 nm. The results were calculated as percentage positivity (PP) based on the $A_{405\ nm}$ of the positive control on the plate:

$$\frac{A_{405\ nm}\text{Sample}}{A_{405\ nm}\text{PositiveControl}} \times 100\% = X\%\text{PP}$$

The cut-off was set at 23% PP after ROC analysis (data not shown). Results obtained above or equal the cut-off of 23% PP were considered positive. Results obtained below the cut-off of 23% PP were negative.

Western Blot

In order to generate somatic antigens for the Western Blot, muscle larvae of *T. spiralis* were recovered by HCl-pepsin digestion from experimentally infected mice. These mice had been orally infected with 500 *T. spiralis* L1 at least 3 months before their euthanasia. The larvae were washed in PBS, supplemented with 0.2 mM of proteinase inhibitor phenyl methyl sulfonyl fluoride, freeze-thawed three times, and then sonicated (2×30 s, 70 W, 0°C). After centrifugation (1,000×*g*, 30 min, 4°C), the supernatant was separated on a 10% SDS-PAGE gel under reducing conditions (Staubli et al. 2006) and subsequently electrophoretically blotted on nitrocellulose membranes (pore size 0.45 μm; Whatman, Cat No. 10401196). The membranes were cut into 17 strips each. Positive and negative control sera of experimentally infected pigs were obtained from BfR,

Berlin, Germany. Meat juice samples were diluted 1:50 in PBS with 0.3% Tween 20 and 2% fish gelatine (PBS-T-G), serum samples were diluted 1:50 in PBS-T-G and 5% milk powder. Strips were incubated with 500 μl of the diluted samples for 2 h at RT, before the sample liquid was removed and the strips were washed five times with PBS-T pre-warmed to 45°C. Protein A-conjugate (Calbiochem, Switzerland Cat No. 539253) was diluted 1:10,000 in PBS-T, added to each strip, and incubated for 1 h at RT. Afterward, strips were washed four times with PBS-T and two times with PBS. Subsequently, the substrate was added (one tablet of 4-chloro-1-naphtol (Sigma Cat No. C6788) diluted in 10 ml methanol, 30 ml PBS, and 40 μl 30% H_2O_2) and incubated for 10 min at RT. The strips were washed with deionized water and dried with filter papers, and the banding pattern was interpreted in comparison with the positive and negative controls. A Western Blot was considered positive when the following banding pattern became apparent: Within a pattern of bands localizing between 35 and 65 kDa, specific bands localized at 47, 49, and 52 kDa, and two specific bands at 60 and 63 kDa had to appear qualitatively, independent of the banding staining intensity (see Fig. 1; Denkers et al. 1990; Robert et al. 1996; Özkoç et al. 2005).

Confirmatory artificial digestion

Individual artificial digestion tests were performed with at least 20 g of diaphragm per animal to be tested. The artificial digestion (magnetic stirrer) method was carried out according to the EU regulation (European Commission 2005).

Sequence of diagnostic tests

First, the infection status of all samples had been determined using artificial digestion. This infection status
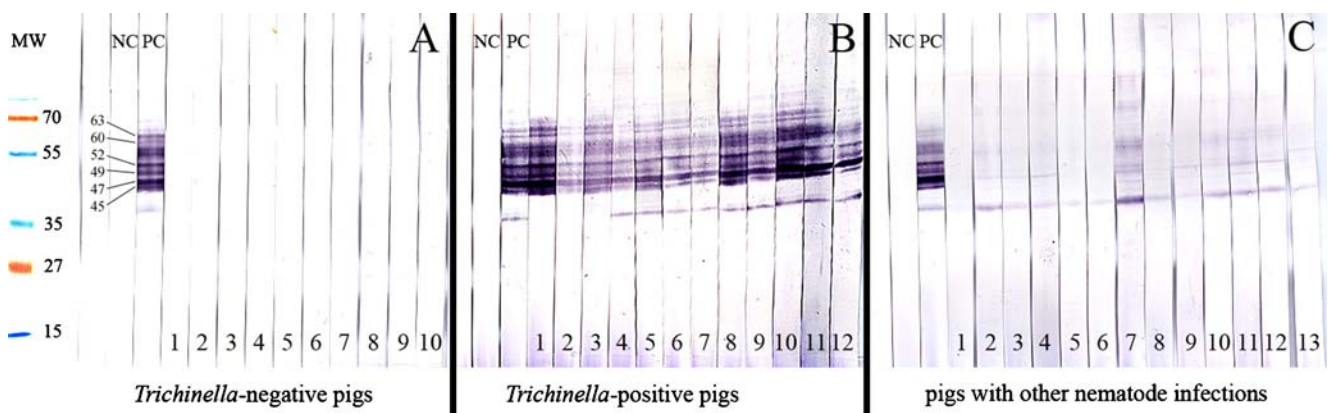

Fig. 1 Western Blot analysis of meat juice samples of *Trichinella*-negative pigs (**a**), *Trichinella*-positive pigs (**b**), and pigs with other nematode infections (**c**). *NC* negative control, *PC* positive control

was later used in Bayesian modeling to divide the samples into a non-infected population (no larvae detected by artificial digestion) and an infected population (larvae detected by artificial digestion; see also section below). Subsequently, all samples were tested in parallel by Western Blot and ELISA. Finally, an additional confirmatory artificial digestion was conducted for samples that were negative in routine artificial digestion of 1 or 2 g, but positive in at least one serological test.

Statistical analysis

Data were analyzed using NCSS 2007 (NCSS Statistical Software, Kaysville, Utah, USA), and the freeware WinEpiscope 2.0 (available through http://www.vetschools.co.uk/EpiVetNet/Epidemiological_analysis_software.htm) and WinBUGS 1.4 (available through http://www.epi.ucdavis.edu/diagnostictests/software.html). Beta distributions were established with the freeware BetaBuster.

In an initial step, the sensitivity and specificity of the Western Blot were calculated in NCSS 2007 using deterministic techniques based on the assumption that the artificial digestion technique acted as gold standard with a perfect sensitivity and specificity. In a second step, the sensitivity and specificity of the Western Blot were modeled against the artificial digestion test using Bayesian techniques in WinBUGS as described by Branscum et al. (2005). Here, the model for "2 independent tests—2 populations—no gold standard" was selected because it was considered that the artificial digestion test and the Western Blot were conditionally independent from each other. The parameterization for this model is given in Branscum et al. (2005). The appropriate programming code for this and other models can be found on the website given above.

In this model, the specificity of the artificial digestion method was still considered to be perfect, but the model allowed the sensitivity of the artificial digestion test to vary. The model was run with 100,000 iterations, including a burn-in phase of 2,000 iterations. The prior distribution for the sensitivity and specificity of the Western Blot had mode=0.90 with a tenth percentile=0.60, resulting in a Beta distribution with α=6.05 and β=1.56. The same prior distribution was selected for the sensitivity of the artificial digestion test. These prior distributions were selected because it was considered reasonable to assume that the sensitivity and specificity of the diagnostic tests would be well above 0.50, but the model still had enough freedom to vary. The prior distribution for the prevalence in the *Trichinella*-negative pig population had mode=0.02 with a 95th percentile=0.1 (Beta(1.84, 42.11)). The prior distribution for the prevalence in the *Trichinella*-positive pig population had mode=0.98 with a fifth percentile=0.90 (Beta(42.11, 1.84)). These prior

distributions were selected to stress that infection was considered completely absent from the first population and present in the second.

The sensitivity and specificity of the Western Blot were also modeled against the ELISA test. Here, a "2 dependent tests—2 populations—no gold standard" model was selected (Branscum et al. 2005). The model was also run with 100,000 iterations, including a burn-in phase of 2,000 iterations. The prior distributions for the sensitivity and specificity of the ELISA had mode=0.90 with a tenth percentile=0.60 (Beta(6.05, 1.56)). The prior distribution for the prevalence in the *Trichinella*-negative pig population had mode=0.02 with a 95th percentile=0.1 (Beta(1.84, 42.11)). The prior distribution for the prevalence in the *Trichinella*-positive pig population had mode=0.98 with a fifth percentile=0.90 (Beta(42.11, 1.84)). Prior distributions for the correlation parameters (λ, γ) had mode=0.90 with a fifth percentile=0.10 (Beta(1.32, 1.04)).

Alternative models were developed to assess the sensitivity of the two models regarding changes in the prior distributions. Prior distributions for the sensitivity and specificity of the diagnostic tests were narrowed, and the prior distributions for the prevalence were widened. Also, the models were adapted to specifically allow a zero prevalence in the *Trichinella*-negative population (τ<1). Finally, a "3 tests—1 population—no gold standard" model was selected (Branscum et al. 2005) in order to analyze the data as a single data set with three different diagnostic techniques. Prior distributions for the sensitivity and specificity of the diagnostic tests were as described above. The prior distribution for the prevalence had mode=0.25 with a 90th percentile=0.70 (Beta(1.45, 2.34)).

Results

The results of the Western Blot and ELISA tests are presented in Table 2. In the Western Blot, positive samples were characterized by a pattern of specific bands localizing between 35 and 65 kDa (Fig. 1). If the artificial digestion test was assumed to have a perfect sensitivity and specificity (deterministic model, Table 3), the sensitivity of the Western Blot was 96.8% and its specificity was 100.0%. Three digestion-positive samples in which no anti-*Trichinella* antibody was detected by the Western Blot originated from pigs naturally infected with *T. spiralis* with larval densities of 0.4, 1 and 32 LPG, respectively. The Western Blot did not detect anti-*Trichinella* antibodies in any of the samples from pigs with other nematode infections. Based on the same assumption of a perfect artificial digestion test (deterministic model, Table 3), the in-house ELISA test had a sensitivity of 97.8% and a specificity of 98.6%. Two digestion-positive samples in which no anti-*Trichinella* antibody was detected

Table 2 Results of the ELISA and Western Blot testing in reference to the artificial digestion method

Artificial digestion (%)		In-house ELISA (%)		Western Blot (%)	
Negative	295 (100)	Negative	291 (98.6)	Negative	291 (98.6)
				Positive	0 (0)
		Positive	4 (1.4)	Negative	4 (1.4)
				Positive	0 (0)
Positive	93 (100)	Negative	2 (2.2)	Negative	1 (1.1)
				Positive	1 (1.1)
		Positive	91 (97.8)	Negative	2 (2.1)
				Positive	89 (95.7)

by the in-house ELISA originated from pigs naturally infected with *T. spiralis* having a larval burden of 0.025 and 0.4 LPG, respectively. The sample containing 0.4 LPG had not been detected by Western Blot either. The ELISA reacted positively with four digestion-negative samples originating from breeding sows. For three of these samples, additional diaphragm tissue was available for a confirmatory artificial digestion test. In none of the samples, larvae were detected. The ELISA test classified all samples from pigs with other nematode infections as negative for anti-*Trichinella* antibodies.

Subsequently, the sensitivity and specificity of the Western Blot were modeled while allowing the sensitivity of the artificial digestion test to vary. Results are presented in Table 3. Various models were run to evaluate the sensitivity of the model to the selected parameters. The results showed that the sensitivity and specificity of the Western Blot were slightly overestimated under the assumption of a perfect artificial digestion test. The results also showed 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, implying that routine artificial digestion does

not detect all infected pigs. Under the assumption of imperfect sensitivity of the artificial digestion test, the sensitivity and specificity of the Western Blot were 95.8% and 99.6%, respectively, and the sensitivity of the artificial digestion test was 98.8%.

The level of agreement between the Western Blot and the ELISA was good; the Kappa value was 0.95 (CI, 0.85–1.00).

The sensitivity and specificity of the Western Blot were modeled against the results of the ELISA, assuming conditional dependence of the two tests (Table 4). The sensitivity and specificity of the Western Blot were 96.0% and 99.5%, respectively; the sensitivity and specificity of the ELISA were 97.4% and 98.3%.

The results of the 3 tests—1 population—no gold standard model were very similar to the results presented above (data not shown).

Discussion

The Western Blot validated in this study was methodically based on using antigens derived from mature whole muscle larvae of *T. spiralis* (crude worm extract/CWE). Several

Table 3 Median (95% probability interval) for the sensitivity and specificity of the Western Blot and artificial digestion

	Western Blot		Artificial digestion	
	Sensitivity	Specificity	Sensitivity	Specificity
Deterministic model	96.8 (90.9–98.9)	100.0 (98.7–100.0)	100.0	100.0
Basic model	95.8 (90.7–98.6)	99.6 (98.4–100.0)	98.8 (95.3–99.9)	100.0
Alternative model 1[a]	95.8 (90.6–98.6)	99.6 (98.4–100.0)	97.4 (94.3–99.2)	100.0
Alternative model 2[b]	95.6 (91.8–98.1)	98.9 (97.4–99.6)	98.8 (95.3–99.9)	100.0
Alternative model 3[c]	95.6 (91.8–98.0)	98.9 (97.4–99.6)	97.4 (94.3–99.1)	100.0
Alternative model 4[d]	95.8 (90.7–98.6)	99.6 (98.4–100.0)	98.8 (95.3–99.9)	100.0
Alternative model 5[e]	95.8 (90.7–98.6)	99.6 (98.4–100.0)	98.8 (95.3–99.9)	100.0

[a] Prior distribution for the sensitivity of the artificial digestion test: mode=0.95, tenth percentile=0.90

[b] Prior distribution for the sensitivity and specificity of the Western Blot: mode=0.95, tenth percentile=0.90

[c] Prior distribution for the sensitivity and specificity of the Western Blot and for the sensitivity of the artificial digestion test: mode=0.95, tenth percentile=0.90

[d] Prior distribution for the prevalence in the negative (positive) population: mode=0.50, 95th (fifth) percentile=0.95 (0.05)

[e] Probability that infection occurs in negative population (τ)=0.10

Table 4 Median (95% probability interval) for the sensitivity and specificity of the Western Blot and ELISA

	Western Blot		ELISA	
	Sensitivity	Specificity	Sensitivity	Specificity
Deterministic model	96.8 (90.9–98.9)	100.0 (98.7–100.0)	97.8 (92.5–99.4)	98.6 (96.6–99.5)
Basic model	96.0 (90.8–98.8)	99.5 (98.2–99.9)	97.4 (92.9–99.5)	98.3 (96.4–99.4)
Alternative model 1[a]	95.7 (90.6–98.7)	99.4 (98.1–99.9)	96.6 (93.0–98.7)	97.8 (95.9–99.0)
Alternative model 2[b]	96.0 (90.8–98.8)	99.5 (98.2–99.9)	97.3 (92.7–99.5)	98.3 (96.4–99.4)
Alternative model 3[c]	95.7 (90.6–98.6)	99.4 (98.0–99.9)	96.5 (93.0–98.7)	97.8 (95.9–99.0)
Alternative model 4[d]	96.1 (90.9–98.8)	99.5 (98.2–99.9)	97.4 (92.9–99.5)	98.3 (96.4–99.4)

[a] Prior distribution for the sensitivity and specificity of the ELISA: mode=0.95, tenth percentile=0.90

[b] Prior distribution for the prevalence in the negative (positive) population: mode=0.50, 95th (fifth) percentile=0.95 (0.05)

[c] Prior distribution for the sensitivity and specificity of the ELISA: mode=0.95, tenth percentile=0.90; prior distribution for the prevalence in the negative (positive) population: mode=0.50, 95th (fifth) percentile=0.95 (0.05)

[d] Probability that infection occurs in negative population (τ)=0.10

studies demonstrated that CWE is useful for Western blot analysis for anti-*Trichinella* antibodies (Marinculic et al. 1991; Nöckler et al. 1995; Kapel et al. 1998; Pozio et al. 2002). Although CWE may present a more complex banding pattern than E/S antigen and may thus be more difficult for determining the exact molecular mass of all bands that react specifically with anti-*Trichinella* antibodies, there are several arguments in favor of CWE. First, the dominant antigenic E/S components are part of CWE, as shown by Pozio et al. (2002). Gruden-Movsesijan et al. (2002) demonstrated three bands (45, 49, and 53 kDa) to range among the dominant E/S antigens; all three appear also in CWE. A similar banding profile was shown by Denkers et al. (1990) in a mouse model and by Özkoç et al. (2005) with human trichinellosis patients. The pattern of the major bands we demonstrated in the present paper matched also that revealed by Robert et al. (1996), Denkers et al. (1990), and Özkoç et al. (2005). Robert et al. (1996) were able to specifically discriminate between a true anti-*T. spiralis* humoral immune response in humans from potential cross-reactive sera due to autoimmune or other parasitic diseases based upon immunoreactivity to a 47–55 kDa banding pattern. Similar to our findings with pig sera, Robert et al. (1996) revealed some minor bands that cross-reacted with antibodies from sera originating from patients with other helminthic diseases. Thus, unspecific or cross-reactive banding activity can occur (Nöckler et al. 1995; Pozio et al. 2002), but can be distinguished upon the overall specific banding pattern characteristic for trichinellosis. E/S antigen that is also widely used for Western Blot (Nöckler et al. 1995; Pozio et al. 2002) is, however, more time consuming, laborious, and also more expensive in production when compared to CWE.

Three samples that were *Trichinella*-positive in the artificial digestion test were subsequently seronegative in Western Blot analysis. One of these samples was also negative in the ELISA. These three samples were obtained from pigs originating from various places, and all three specimens had been stored for an extended period of time prior to the present investigation. This is in contrast to the samples of negative pigs, which were all freshly collected in Swiss slaughterhouses and directly thereafter properly stored for a short time period prior to laboratory testing. Due to the extended storage period and multiple freezing and thawing processes prior to our laboratory testing, the quality of the *Trichinella*-positive serum samples may have degraded over time. The aspect of storage of diagnostic samples may require further respective investigations, as it may be an important parameter to standarization of serology, especially if samples are collected under rough field conditions such as slaughterhouses.

The estimated sensitivity and specificity of the Western Blot did not vary much between the deterministic and Bayesian approaches. However, the results of the Bayesian modeling provided a higher confidence in the results. If a new test is validated against an existing test that is erroneously assumed to be a gold standard, the estimated sensitivity and specificity of the new test will be biased (Hui and Walter 1980). Our Bayesian models were run under the assumption that the reference test (artificial digestion test or ELISA) was not a gold standard. The estimated sensitivity and specificity of the Western Blot were corrected for the imperfect sensitivity and specificity of the reference test and therefore provided a more accurate estimation.

The sensitivity analysis also showed only minor changes between the basic and alternative models. Adaptations in the prior distributions of the parameters did not cause large changes in the model outcomes. This demonstrated that the outcomes of the models were largely influenced by the data itself, i.e., by the results of the testing and not by the selected prior distributions for the parameters. This also increased the confidence in the results of the model.

The Bayesian models demonstrated that the sensitivity of the artificial digestion test was below 100%, and also the 95% probability interval did not include 100%. It is biologically plausible that the sensitivity of the routine artificial digestion test is not 100%, as is the case with most diagnostic tests. *Trichinella* larvae may not be distributed equally throughout the tissues (Nöckler and Kapel 2007), and a certain probability exists that the sample selected from an infected pig does not contain larvae. Assuming that larvae are randomly distributed following a Poisson distribution and considering a larval density of 1 LPG, the Poisson distribution demonstrates that there is a 0.7% probability that a 5 g sample does not contain any larvae at all. The maximum achievable diagnostic sensitivity of the artificial digestion using a 5 g sample would therefore be 99.3%. Using 1 g samples, even 36.8% of the samples would not contain any larvae. The maximum achievable diagnostic sensitivity will be further reduced when larvae are not randomly distributed. Furthermore, larvae may unwittingly be lost during the artificial digestion procedure, e.g., by not carefully rinsing the blender in the digestion beaker or by not allowing enough time for the sedimentation process.

It may be argued that the sensitivity of the artificial digestion test was similar to that of the Western Blot. However, it must be kept in mind that these estimates relate to the diagnostic sensitivity. In the case of the artificial digestion test, this is closely related to sample size, as discussed above. Also, the analytical sensitivity of the artificial digestion test is lower than that of serological tests, as demonstrated by the different limits of detection (Gamble et al. 1983; Nöckler and Kapel 2007; World Organisation for Animal Health 2008a). ELISA tests can detect antibodies in pigs with larval densities of at least 0.01 LPG (Gamble et al. 1983; World Organisation for Animal Health 2008a). This is a clear advantage if serological surveillance is used to demonstrate freedom from infection in a domestic pig population.

In a surveillance program for *Trichinella* spp. in domestic pigs, the test protocol that is used should provide sufficient guarantees that the number of false-positive and false-negative results is as low as possible. In an animal population that is considered to be free of infection, false-positive results may have consequences for the status of this animal population. False-negative results may have consequences for public health. For a serological surveillance of *Trichinella* infections in domestic pigs, the ELISA may be used as a screening test to investigate large numbers of samples. Samples that yield positive in the ELISA should be re-tested by Western Blot for confirmation of this result. Using the estimates from the basic model in Table 4, the sensitivity of such a test combination is 94.2% (95% probability interval/PI 88.3–97.8) and the specificity 99.8% (95% PI 98.9–100.0%). In a country like Switzerland,

where *Trichinella* spp. is considered to be absent from the pig population, the negative predictive value (the probability that a negative test result is truly negative) will be extremely close to 1, whereas the positive predictive value will be very low. Sero-positive results should therefore be followed-up by epidemiological investigations.

References

Anonymous (2005) Verordnung über das Schlachten und die Fleischkontrolle. Nr. 817.190. http://www.admin.ch/ch/d/sr/8/817.190.de.pdf, accessed 30 September 2008

Bauer C (2006) Parasitologische untersuchungsmethoden. In: Schnieder T (ed) Veterinärmedizinische parasitologie. Parey, Stuttgart, Germany, pp 87–91

Branscum AJ, Gardner IA, Johnson WO (2005) Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. Prev Vet Med 68:145–163

Conzelmann C (1999) "Schwein 99" - Ein Gesundheits- und Produktivitätsprofil der Schweine in der Schweiz. II. Teil: Ferkelmortalität. Universität Bern, Bern, Switzerland

Cortes HC, Nunes S, Reis Y, Staubli D, Vidal R, Sager H, Leitao A, Gottstein B (2006) Immunodiagnosis of *Besnoitia besnoiti* infection by ELISA and Western blot. Vet Parasitol 141:216–225

Denkers EY, Wassom DL, Krco CJ, Hayes CE (1990) The mouse antibody response to *Trichinella spiralis* defines a single, immunodominant epitope shared by multiple antigens. J Immunol 144:3152–3159

Dupouy-Camet J, Bruschi F (2007) Management and diagnosis of human trichinellosis. In: Dupouy-Camet J, Murrell KD (eds) FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis. OIE, Paris, pp 37–68

European Commission (2005) Regulation (EC) 2075/2005 of the European Parliament and of the Council of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat. Off J EC L338:60–82

Forbes LB, Gajadhar AA (1999) A validated *Trichinella* digestion assay and an associated sampling and quality assurance system for use in testing pork and horse meat. J Food Prot 62:1308–1313

Gamble HR, Anderson WR, Graham CE, Murrell KD (1983) Diagnosis of swine trichinosis by enzyme-linked immunosorbent assay (ELISA) using an excretory-secretory antigen. Vet Parasitol 13:349–361

Gamble HR, Rapic D, Marinculic A, Murrell KD (1988) Evaluation of excretory-secretory antigens for the serodiagnosis of swine trichinellosis. Vet Parasitol 30:131–137

Gamble HR, Pozio E, Bruschi F, Nöckler K, Kapel CMO, Gajadhar AA (2004) International Commission on Trichinellosis: recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. Parasite 11:3–13

Gekeler F, Eichenlaub S, Mendoza EG, Sotelo J, Hoelscher M, Loscher T (2002) Sensitivity and specificity of ELISA and immunoblot for diagnosing neurocysticercosis. Eur J Clin Microbiol Infect Dis 21:227–229

Georgiadis MP, Johnson WO, Gardner IA, Singh R (2003) Correlation-adjusted estimation of sensitivity and specificity of two diagnostic tests. Appl Statist 52:63–76

Gruden-Movsesijan A, Ilic N, Sofronic-Milosavljevic L (2002) Lectin-blot analyses of *Trichinella spiralis* muscle larvae excretory-secretory components. Parasitol Res 88:1004–1007

Hadorn D, Hauser R, Stark KD (2002) Epidemiologische Grundlagen und Resultate der Stichprobenuntersuchung 2001 in der schweizerischen Schweinepopulation. Schweiz Arch Tierheilkd 144:532–541

Huber R (1999) "Schwein 99" - Ein Gesundheits- und Produktivitätsprofil der Schweine in der Schweiz. I. Teil: Morbidität und Mortalität der Sauen. Universität Bern, Bern, Switzerland

Hui SL, Walter SD (1980) Estimating the error rates of diagnostic tests. Biometrics 36:167–171

Johnson WO, Gastwirth JL, Pearson LM (2001) Screening without a "gold standard": the Hui-Walter paradigm revisited. Am J Epidemiol 153:921–924

Kapel CM, Webster P, Lind P, Pozio E, Henriksen SA, Murrell KD, Nansen P (1998) *Trichinella spiralis*, *T. britovi*, and *T. nativa*: infectivity, larval distribution in muscle, and antibody response after experimental infection of pigs. Parasitol Res 84:264–271

Kociecka W (2000) Trichinellosis: human disease, diagnosis and treatment. Vet Parasitol 93:365–383

Marinculic A, Rapic D, Brglez J, Dzakula N, Stojiljkovic D (1991) Epidemiological survey of trichinellosis in Yugoslavia. Southeast Asian J Trop Med Public Health 22(Suppl):302–307

Muller N, Frei E, Nunez S, Gottstein B (2007) Improved serodiagnosis of alveolar echinococcosis of humans using an in vitro-produced Echinococcus multilocularis antigen. Parasitology 134:879–888

Nöckler K, Kapel CMO (2007) Detection and surveillance for *Trichinella*: meat inspection and hygiene, and legislation. In: Dupouy-Camet J, Murrell KD (eds) FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis. OIE, Paris, pp 69–97

Nöckler K, Voigt WP, Protz D, Miko A, Ziedler K (1995) Intravitale Diagnostik der Trichinellose beim Schwein mit dem indirekten ELISA. Berl Munch Tierarztl Wochenschr 108:167–174

Nöckler K, Pozio E, Voigt WP, Heidrich J (2000) Detection of *Trichinella* infection in food animals. Vet Parasitol 93:335–350

Ortega-Pierres MG, Yepez-Mulia L, Homan W, Gamble HR, Lim PL, Takahashi Y, Wassom DI, Appleton JA (1996) Workshop on a detailed characterization of *Trichinella spiralis* antigens: a platform for future studies on antigens and antibodies to this parasite. Parasite Immunol 18:273–284

Özkoç S, Bayram Delibas S, Akisü ç (2005) Use of the Western Blot assay in the diagnosis of trichinosis (in Turkish). Türkiye Parazitoloji Dergisi 29:26–30

Pozio E, Sofronic-Milosavljevic L, Gomez Morales MA, Boireau P, Nockler K (2002) Evaluation of ELISA and Western Blot analysis using three antigens to detect anti-Trichinella IgG in horses. Vet Parasitol 108:163–178

Pozio E, Gomez Morales MA, Dupouy-Camet J (2003) Clinical aspects, diagnosis and treatment of trichinellosis. Expert Rev Anti Infect Ther 1:471–482

Robert F, Weil B, Kassis N, Dupouy-Camet J (1996) Investigation of immunofluorescence cross-reactions against *Trichinella spiralis* by Western blot (immunoblot) analysis. Clin Diagn Lab Immunol 3:575–577

Sofronic-Milosavljevic L, Ilic N, Djordjevic M, Savic M, Gruden-Movsesijan A, Cuperlovic K, Murrell KD (2005) Anti-*Trichinella* antibodies detected in chronically infected horses by IFA and Western blot, but not by ELISA. Vet Parasitol 132:107–111

Staubli D, Nunez S, Sager H, Schares G, Gottstein B (2006) *Neospora caninum* immunoblotting improves serodiagnosis of bovine neosporosis. Parasitol Res 99:648–658

World Organisation for Animal Health (2008a) Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.1.16 Trichinellosis. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.16_TRICHINELLOSIS.pdf, accessed 30 September 2008

World Organisation for Animal Health (2008b) Terrestrial Animal Health Code 2008. Chapter 8.14 Trichinellosis. http://www.oie.int/eng/normes/mcode/en_chapitre_1.8.14.htm, accessed 30 September 2008

Yepez-Mulia L, Arriaga C, Viveros N, Adame A, Benitez E, Ortega-Pierres MG (1999) Detection of *Trichinella* infection in slaughter horses by ELISA and western blot analysis. Vet Parasitol 81:57–68

Yera H, Andiva S, Perret C, Limonne D, Boireau P, Dupouy-Camet J (2003) Development and evaluation of a Western blot kit for diagnosis of human trichinellosis. Clin Diagn Lab Immunol 10:793–796

Zarlenga DS, Chute MB, Martin A, Kapel CM (2001) A single, multiplex PCR for differentiating all species of *Trichinella*. Parasite 8:S24–S26