

COMPARISON OF THREE ANTIGEN PREPARATIONS TO DETECT TRICHINELLOSIS IN LIVE SWINE USING IGG-ELISA

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Abstract. A swine infected with *Trichinella spiralis* is a source of transmission to human through consumption of raw or improperly cooked pork. Detection of larvae is suitable for carcasses, so that pigs in households or farms can be examined serologically for trichinellosis. This study compared antigens, crude (CAg), excretory-secretory (ESAg) and surface (SAg), for their potential use in IgG-ELISA. Serum samples were collected from 5 experimentally infected swine with *T. spiralis* (pTs), 147 positive cases of 9 other parasitic infections, 12 mixed infections of other parasites, and 35 normal controls. At the same 100% sensitivity, specificity of tests was in a range of 98-77%. ESAg was the best source of antigen with specificity of 98.3% at cut-off value of 0.439. False positives included coccidiasis (1/86) and mixed infections (2/39). For CAg, trichuriasis (2/11), coccidiasis (5/86), and mixed infections (8/39) gave cross-reactions and some of these samples had OD values far above cut-off value of 0.332. Cross-reactions of SAg were *Oesophagostomum* spp-like GI-nematode infection (1/1), unidentified GI-nematode infections (2/3), trichuriasis (5/11), coccidiasis (29/86) and mixed infections (4/39). Thus, ESAg has the highest potential in serodiagnosis, with antibody to *T. spiralis* in pigs being detected at the earliest 16 day post-infection. However, crude antigen demonstrated a good specificity at 91.8%, and this antigen has a potential to be used as a detection of choice for swine trichinellosis, but the antigen preparation must be improved for higher specificity.

Key words: *Trichinella spiralis* muscle larvae, swine trichinellosis, crude and ES antigens, IgG-ELISA

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INTRODUCTION

Trichinellosis is one of the food-borne parasitic zoonoses, which causes infection in humans mainly due to *Trichinella spiralis*. The main sources of human infection are pork and pork products, game meat

and horse meat, contaminated with infective larvae (Bruschi and Murrell, 2002). Trichinellosis continues to be a public health concern throughout the world. The prevalence of swine trichinellosis and the incidence of human trichinellosis appear to be greater in developing countries (Murrell and Pozio, 2000). In developed countries, meat inspection at slaughterhouses and the eradication of this infection from commercial pig farms have been carried out for prevention of trichinellosis (Pozio, 2000).

In Thailand, the main causative agent has been identified as *T. spiralis* with low incidence of *T. pseudospiralis* (Jongwutiwes *et al*, 1998) and *T. papuae* (Kusolsuk *et al*, 2006). In 2008, there were about 44 cases of trichinellosis from the north region of Thailand (The Annual Epidemiological Surveillance Report, 2008). The main source of infection in Thailand has been hilltribe pigs, wild boars, jackals and black bears (Suriyanon and Khunklin, 1972). As *Trichinella* infection in food animals, especially in pork, is the cause of trichinellosis in humans, therefore, this disease has relevant socioeconomic and sanitary implications, having a considerable impact on the international commerce of animals in many countries, principally that of pigs and their products (Todd, 1989; Robert and Murrell, 1993).

Two main methods are recommended for the diagnosis of *Trichinella* in food animals: (1) direct detection of first-stage larvae encysted in striated muscle tissue, and (2) indirect detection by testing for specific antibodies (OIE, 2004a). In Thailand, a sero-test for swine trichinellosis has not hitherto been developed and commercial test kits are not reliable for swine trichinellosis (OIE, 2008a; Frey *et al*, 2009; Vu *et al*, 2010) or human trichinellosis (Akisu *et al*, 2005). Researchers

should also evaluate the performance of a commercial test kit prior to use, by using selected negative and positive reference samples (OIE, 2008b), including serum samples of other parasitic infections for more evaluation of test in different regional laboratories. Therefore, this study evaluated IgG-ELISA method for the detection of *Trichinella* antibodies using crude, excretory-secretory and surface antigens, prepared from *T. spiralis* larvae from muscle.

MATERIALS AND METHODS

T. spiralis collection and maintenance

T. spiralis larvae from muscle were collected by artificial gastric digestion [1% (w/v) pepsin in 1% (v/v) hydrochloric acid] from infected mice (ICR), obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Then, 7-8 weeks old laboratory mice were inoculated by oral route with 100 infective larvae using stomach tube.

Infected mice were euthanatized after 2 months of infection, and the skin, head, tail, tarsus, carpus and visceral organs except diaphragm were removed. The remaining carcass was minced and digested as described above. The infective larvae were collected using the Baerman's technique (Justus and Morakote, 1981), washed with normal saline solution, followed by distilled water (DW) as the final wash. Larvae were kept at -70°C until used to prepare crude and surface antigens, or were cultured to obtain excretory-secretory products.

Animal sera collection

Serum samples were obtained from the Parasitology Section, National Institute of Animal Health, Department

of Livestock Development, Bangkok, Thailand. Five experimental swine were used in the study and were infected with 1,000 *T. spiralis* larvae (Nockler *et al*, 2005). Blood samples were collected for 24 consecutive periods as follows: on days 14 and 7 pre-inoculation (blood collection 1 and 2), inoculation day (blood collection 3) and days 3, 6, 9, 12, 16, 20, 24, 28, 33, 38, 43, 48, 55, 62, 69, 76, 83, 90, 97, 104, 111 post-inoculation (pi blood collection 4-24, respectively). Each sample was allowed to clot at room temperature and serum was stored at -70°C until used.

Swine fecal and/or intestinal samples were used for diagnosis by smear, flotation and/or scraping techniques for recovery of eggs, larvae, or other parasitic agents. Living/sacrificed pigs were used as source of blood collection. In case of fecal samples, flotation technique was used as a principle method for detection of nematode eggs and coccidian oocysts. For piglets, ≤ 3 months old, *Cryptosporidium* oocyst detection was also conducted by using smear technique, followed by modified Ziehl-Neelsen staining (OIE, 2004a). *Balantidium coli* in intestine was detected using flotation and scraping techniques. A sacrificed pig suspected of any parasitic infections was subjected to fecal and blood sample collection. Intestine of pig was examined for parasite infection by scraping technique followed by microscopy. One hundred and forty-seven swine sera were obtained as heterologous sera (21 infections) and were kept at -70°C until used.

Normal controls were selected from 183 sera that showed negative results by smear, flotation or scraping technique. To eliminate cases of previous infections with other parasites, all 183 sera were immuno-screened again using IgG-ELISA as follows: crude somatic antigen at 1 $\mu\text{g}/\text{ml}$, serum at 1:400 dilution and conjugate

antibody at 1:2,000 dilution. Thirty-five sera with the lowest OD values following statistical determination were selected as healthy control sera, and were kept at -70°C until used.

Antigen preparations

Crude somatic antigen (CAg) was prepared from frozen muscle/infective larvae of *T. spiralis*. In brief, frozen larvae were macerated with alumina in distilled water at 0-2°C and sonicated with Sonicator® XL 2020 (Microtip™ probe No. 418, Tip Dia. 1/16", Ultrasonic Processor XL, Farmingdale, NY) at 1 minute intervals for 8 minutes. The homogenate of larvae was centrifuged at 13,000g at 4°C for 1 hour. The protein content in supernatant was determined by Coomassie Blue Plus Protein Assay (Pierce Chemical, Rockford, IL) and sample stored at -70°C until used.

Excretory-secretory antigen (ESAg) was prepared from infective larvae following the procedures of Mahannop *et al* (1992) and OIE Manual (2004a,b) with some modifications. In short, living larvae were washed under a stereo-microscope 3 times, 20 minutes each, with Hank's Balance Salt Solution (HBSS) containing penicillin (200 units/ml) and streptomycin (200 units/ml), and then maintained in RPMI 1640 supplemented with 10 mM HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid), 2 mM glutamine, and penicillin (100 units/ml)/streptomycin (100 $\mu\text{g}/\text{ml}$) and gentamicin (50 mg/ml). Approximately 2,000 larvae/ml of medium were maintained for 18-20 hours at 37°C in a candle jar. Culture media were centrifuged at 13,000g at 4°C for 1 hour, and the supernatant was dialyzed and concentrated using Amicon filtration under pressure through a PM 5 membrane (cut-off 5 kDa). The protein content of ES products was determined by Pierce assay and then stored at -70°C until used.

Surface antigen (SAg) of frozen infective larvae was prepared as described by Cox *et al* (1981) with some modifications. In brief, 0.5 ml aliquot of larvae was washed 3 times in PBS at 4°C with a final wash with buffer (10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethanesulfonyl fluoride). Larvae were ground and centrifuged at 4,000g for 4 minutes. The pellet was washed 3 times with the same buffer, then suspended in ST buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and heated for 3 minutes at 100°C (AccuBlock™ Digital, Labnet, Woodbridge, NJ). After 1 hour of incubation at room temperature on a rocking platform, cuticles were sedimented at 13,000g, washed with DW 3 times, macerated with alumina in DW and sonicated at 0-2°C (Sonicator® XL 2020, Microtip™ probes No. 418, Tip Dia. 1/16", Farmingdale, NY) at 1 minute intervals for 5 minutes. Cuticle homogenate was centrifuged at 13,000g at 4°C for 1 hour (Hermle Z323 K, Labnet). Following protein content determination by Pierce assay, supernatant was kept at -70°C until used.

Indirect ELISA

Antibody response was determined by IgG-ELISA (Dekumyoy *et al*, 1998) under the following conditions: 0.5 µg/ml CAg, serum diluted 1:400 and conjugated antibody diluted 1:8,000; 1 µg/ml ESAg, serum diluted 1:800 and conjugated antibody diluted 1:4,000; and 0.02 µg/ml SAg, serum diluted 1:400 and conjugated antibody diluted 1:4,000. In brief, each well of microtiter plates (Nunc, Roskilde, Denmark) was coated with 50 µl/well of antigen (diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6). A 70 µl/well aliquot of blocking solution (0.5% BSA in PBS, pH 7.4, 0.02% NaN₃) was added and solution incubated for 1 hour. Then,

50 µl/well aliquot of serum diluted in diluent solution (PBS-Tween 20 containing 0.02% NaN₃, 0.008% bromphenol blue) was added, followed by 50 µl/well aliquot of conjugate (peroxidase-labeled affinity purified antibody to swine IgG (KLP, Gaithersburg, MD). Immune reactions were visualized by adding 50 µl/well aliquot of substrate [ABTS; 2, 2-azino-di-(3-ethylbenzothiazoline sulfonate) with H₂O₂] and incubating for 30 minutes. The reaction was stopped by the addition of 70 µl of 1% SDS. Optical density at 405 nm was measured in each well using an automated microplate reader (Sunrise™ absorbance reader, TECAN, Salzburg, Austria).

Ethical clearance

The study protocol was approved by the Animal Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (project No. FTM-ACUC 014/2007) and the National Institute of Animal Health, Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand [project No. EA 001/49 (R)].

RESULTS

Antibody response pattern of experimentally *T. spiralis*-infected swine

ELISA was positive for all five experimentally *T. spiralis* infected swine, and antibody response was quite different for each antigen. In the case of crude antigen, antibody levels started to increase on day 20 after inoculation and plateaued after pi day ranging from 24 to 106 (Fig 1A). For ES antigen, ELISA OD increased after pi day 16 and reached a lower plateau level on pi day ranging from 24 to 33 (Fig 1B). As regards surface antigen, there was fluctuation in antibody response

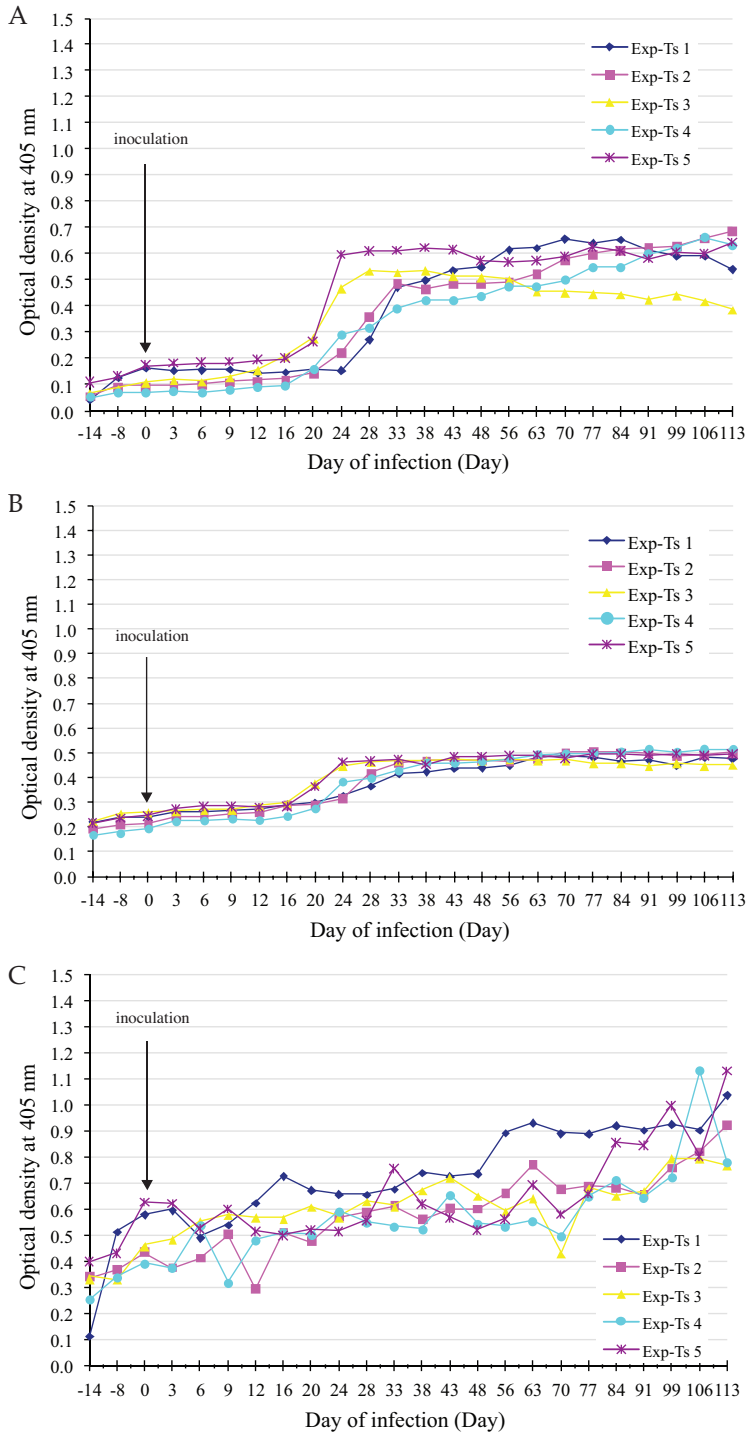


Fig 1—Antibodies response of five experimental swine infected with *T. spiralis* detected by IgG-ELISA using crude (A), excretory-secretory (B) and surface (C) antigens. Animals were infected with 1,000 larvae.

(Fig 1C) but a gradual rise in ELISA OD values were observed starting on pi day 9, and no plateau was reached.

Antibody response in domesticated swine

As crude antigen of muscle larvae could react with serum antibodies from other parasitic infections, a cut-off value (0.332, mean OD + 3SD) was determined from 35 sera of healthy controls. IgG-ELISA analysis, sensitivity, specificity, positive and negative predictive value was 100, 91.8, 25.0% and 100%, respectively. Cross-reaction of CAG-ELISA showed false positives with 15 cases (10%) of 8 parasitic infections (total of 21 infections, Tables 1 and 2). Coccidiasis gave highest cross-reactive samples (5 cases) but their OD values were not much higher than cut-off value. There were high antibody titers against crude antigen of muscle larvae, *Oesophagostomum* spp-like GI-nematode and ascariasis (1/1 case), *Oesophagostomum* spp-like GI-nematode and coccidiasis (1/7 cases), and mixed infection with unidentified GI-nematode and coccidiasis (2/3 cases) (Fig 2A).

Using ES antigen, evaluation of ELISA was

Table 1

Positivity of three serum groups using IgG-ELISA against *T. spiralis* antigens based on their cut-off values.

	CAg		ESAg		SAg	
	No.	% positive	No.	% positive	No.	% positive
Trichinellosis (<i>n</i> = 5)	5	100	5	100	5	100
Other diseases (<i>n</i> = 147)	15	10.2	3	2.0	41	27.9
Normal controls (<i>n</i> = 35)	0	0	0	0	0	0
Cut-off value	0.332		0.439		0.768	

determined by cut-off value 0.439 (mean OD+ 2SD). Sensitivity, specificity, positive and negative predictive value was 100, 98.3, 62.5 and 100%, respectively. Only 3 cases of other parasitic infections, namely, coccidiasis, mixed infection with *Oesophagostomum* spp-like GI-nematode and ascariasis were observed (Tables 1 and 2, Fig 2B).

Using surface antigen, sensitivity, specificity, positive and negative predictive value was 100, 77.5, 10.9 and 100%, respectively at cut-off value of 0.768 (mean OD +2SD). This antigen gave high OD values for all sera of trichinellosis but also other parasitic infections, especially coccidiasis (41 cases; 27.9%) (Tables 1 and 2, Fig 2C).

DISCUSSION

ELISA has widely used in immunodiagnosis of *Trichinella* infection of both swine and humans (Gamble *et al*, 1983; Mahannop *et al*, 1992; Moller *et al*, 2005; Korinkova *et al*, 2008). The suitable ELISA condition for each antigen is determined by a checkerboard titration. As the source of antigen is one important factor that affects the sensitivity and specificity of ELISA test, three kinds of antigens in

our study were provided from *T. spiralis* infective muscle larvae to examine *T. spiralis*-IgG antibodies in swine using indirect ELISA.

An observation of antibody against 1,000 larvae in our study, ELISA could detect IgG from 5 infected swine within 20 and 16 days pi using crude and ES antigens, respectively. Antibody response against *T. spiralis* muscle larval crude antigen was faster than against ES antigen. However, ELISA OD values showed irregular increase of antibody response with surface antigen. Nockler *et al* (1995) reported an increase of sero-positivity using ES antigen between 4 and 5 weeks of post-inoculation in pigs infected with 500 larvae of *T. spiralis* per animal. In addition, Nockler *et al* (2005) showed that in experimentally *T. spiralis* infected swine sero-conversion occurs from 30 and 40 dpi in SPF and Iberian pigs, respectively indicating different antibody response between different strains. Another study found the antibody response to *T. spiralis* using ELISA with ES antigen varies from day 21 to 31 pi. Most anti- *T. spiralis* response is due to an anti-fecundity reaction affecting the numbers of newborn larvae as early as 7 dpi in immunized animals, whereas it is observed after 3

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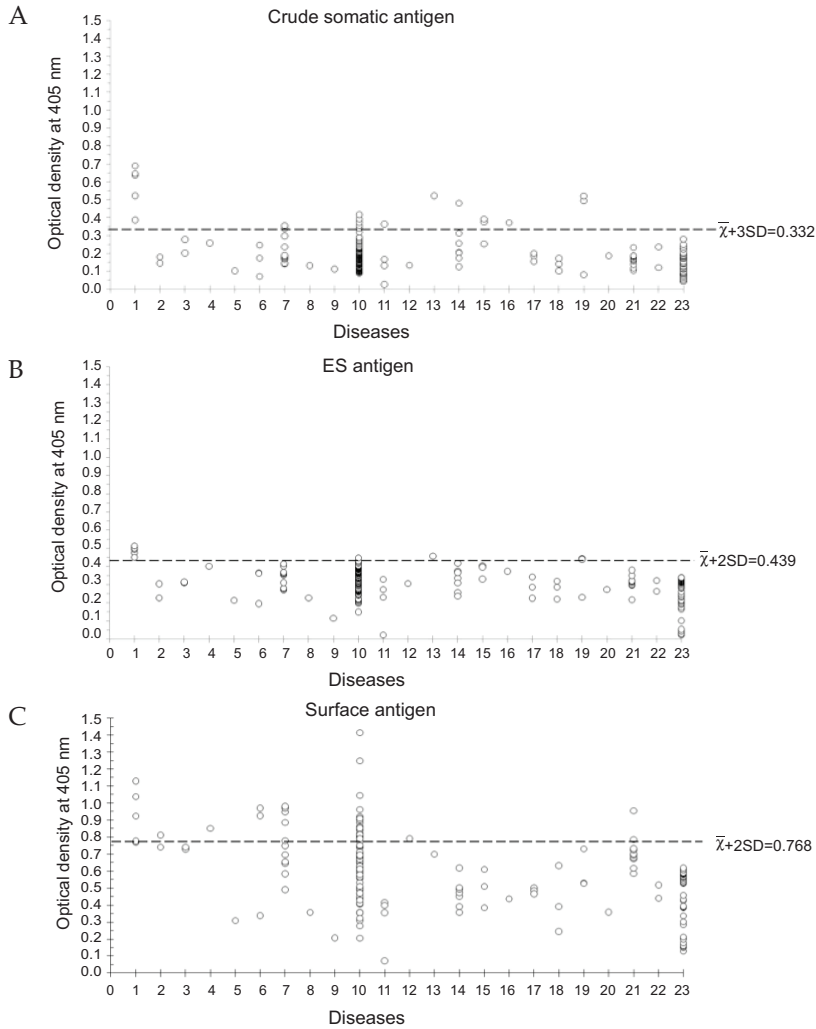


Fig 2—Scatter patterns of ELISA absorbance values using crude (A), ES (B) and surface (C) antigens against serum samples with various diseases and negative serum control. 1, trichinellosis ($n=5$); 2, strongyloidiasis ($n=1$); 3, ascariasis ($n=2$); 4, *Oesophagostomum* spp-like GI-nematode infections ($n=1$); 5, *Hyostrongylus* spp-like GI-nematode infections ($n=1$); 6, unidentified GI-nematode infections ($n=3$); 7, trichuriasis ($n=11$); 8, cryptosporidiasis ($n=1$); 9, balantidiasis ($n=1$); 10, coccidiasis ($n=86$); 11, mixed infection of strongyloidiasis and coccidiasis ($n=4$); 12, mixed infection of cestode and ascariasis ($n=1$); 13, mixed infection of *Oesophagostomum* spp-like GI-nematode and ascariasis ($n=1$); 14, mixed infection of *Oesophagostomum* spp-like GI-nematode and coccidiasis ($n=7$); 15, mixed infection of *Oesophagostomum* spp-like GI-nematode, strongyloidiasis and coccidiasis ($n=3$); 16, mixed infection of *Oesophagostomum* spp-like GI-nematode, strongyloidiasis, cryptosporidiasis and coccidiasis ($n=1$); 17, mixed infection of *Hyostrongylus* spp-like GI-nematode, ascariasis and coccidiasis ($n=3$); 18, mixed infection of unidentified GI-nematode and ascariasis ($n=3$); 19, mixed infection of unidentified GI-nematode and coccidiasis ($n=3$); 20, mixed infection of unidentified GI-nematode, ascariasis and coccidiasis ($n=1$); 21, mixed infection of trichuriasis and coccidiasis ($n=10$); 22, mixed infection of cryptosporidiasis and coccidiasis ($n=2$); 23, negative control ($n=35$).

Table 2
False positive cases of other parasitic infections and negative groups using IgG-ELISA against *T. spiralis* antigens.

Parasitic infection	Cross-reactivity (%)		
	CAG (at 0.332)	ESAg (at 0.439)	SAG (at 0.768)
Strongyloidiasis (<i>n</i> = 2)	-	-	-
Ascariasis (<i>n</i> = 2)	-	-	-
<i>Oesophagostomum</i> spp-like GI-nematode infections (<i>n</i> = 1)	-	-	100% (1/1)
<i>Hyostromylus</i> spp-like GI-nematode infections (<i>n</i> = 1)	-	-	-
Unidentified GI-nematode infections (<i>n</i> = 3)	-	-	67% (2/3)
Trichuriasis (<i>n</i> = 11)	18% (2/11)	-	45.5% (5/11)
Cryptosporidiasis (<i>n</i> = 1)	-	-	-
Balantidiasis (<i>n</i> = 1)	-	-	-
Coccidiasis (<i>n</i> = 86)	6% (5/86)	1% (1/86)	34% (29/86)
Mixed infection of strongyloidiasis and coccidiasis (<i>n</i> = 4)	25% (1/4)	-	25% (1/4)
Mixed infection of cestode and ascariasis (<i>n</i> = 1)	-	-	100% (1/1)
Mixed infection of <i>Oesophagostomum</i> spp-like GI-nematode and ascariasis (<i>n</i> = 1)	100% (1/1)	100% (1/1)	-
Mixed infection of <i>Oesophagostomum</i> spp-like GI-nematode and coccidiasis (<i>n</i> = 7)	14% (1/7)	-	-
Mixed infection of <i>Oesophagostomum</i> spp-like GI-nematode, strongyloidiasis and coccidiasis (<i>n</i> = 3)	67% (2/3)	-	-
Mixed infection of <i>Oesophagostomum</i> spp-like GI-nematode, strongyloidiasis, cryptosporidiasis and coccidiasis (<i>n</i> = 1)	100% (1/1)	-	-
Mixed infection of <i>Hyostromylus</i> spp-like GI-nematode, ascariasis and coccidiasis (<i>n</i> = 3)	-	-	-
Mixed infection of unidentified GI-nematode and ascariasis (<i>n</i> = 3)	-	-	-
Mixed infection of unidentified GI-nematode and coccidiasis (<i>n</i> = 3)	67% (2/3)	33% (1/3)	-
Mixed infection of unidentified GI-nematode ascariasis and coccidiasis (<i>n</i> = 1)	-	-	-
Mixed infection of trichuriasis and coccidiasis (<i>n</i> = 10)	-	-	20% (2/10)
Mixed infection of cryptosporidiasis and coccidiasis (<i>n</i> = 2)	-	-	-
Negative (<i>n</i> = 35)	-	-	-

weeks in naive pigs (Korinkova *et al*, 2008). This effect (antibody-dependent cell cytotoxicity) directed against newborn larvae has been observed with passive transfer of immunized pig serum. It induces a reduction in the parasite bur-

den without affecting worm fecundity (Marti and Murrell, 1986). With ingestion of high amounts of *T. spiralis* larvae, anti-*Trichinella* IgG can be detected in 2-3 weeks post infection. Conversely, there is a delayed antibody response for several

weeks if swine receives a low infection dose, such as 5-7 and 4-5 weeks of post infections with 100 and 500 larvae per swine, respectively. Such results are due to a lack of the kinetics of antibody responses in animals mildly or moderately infected with *T. spiralis* or infected with sylvatic *Trichinella* species (Gottstein *et al*, 2009).

When considering antigenicity among crude somatic, ES, and surface antigens, although crude somatic antigen is easier to prepare and the protein yield is higher than other antigens, the preparation contains numerous antigenic molecules to antibodies against *T. spiralis* and also other cross-reactive molecules to antibodies to other parasites. Somatic antigens of muscle larvae have yielded poor specificity in ELISA due to such cross-reaction (Taylor *et al*, 1980; Arriaga *et al*, 1989). When using crude antigens in our study, serum antibody response against *Trichinella* larvae of naturally infected individual pigs were dispersed. This may be attributed to several different antigenic determinants in CAg, which can react with these antibodies. Arriaga *et al* (1989), using experimentally infected pigs with individual parasites, *Trichuris suis* and *Ascaris suum*, showed that there are antibodies producing high ELISA OD values with crude antigen of muscle larvae.

A more specific antigen, such as ES product or metabolic products, consists of a group of structurally related glycoproteins. The quality of ES antigen depends on the methods of cultivation of muscle larvae (Gamble *et al*, 1983, 1988). The specificity of ELISA is improved by utilizing ES antigen (Gamble *et al*, 2004). However, results of various studies showed a variety of specificity of ELISA using ES antigens with serum samples of pigs, which can range from 90.6 to 99.6% (Murrell *et al*, 1986; Oliver *et al*, 1989; Van der Leek *et al*,

1992; Nockler *et al*, 2004). In our study, ES antigen was analyzed for the most suitable conditions of ELISA resulting in 100% sensitivity. Only 3 cases from 3 other parasitic infections gave false positives with OD values not far from the cut-off value. Swine antibodies from natural infections with *Ascaris* spp and *Trichuris* spp in our study showed true negative to ESAg consistent with the study of Arriaga *et al* (1989).

Using surface antigen, there was cross-reaction with 41 from 147 samples (7 of 21 diseases). Surprisingly, 29 samples of coccidiosis gave false positives with high ELISA OD values and also the highest value among other parasitic infections. When surface antigens were analyzed by SDS-PAGE using ProteoSilver™ Silver staining, 4-5 sharp bands and less defined bands (from 31 kDa to <14.4 kDa) were observed. Western blotting with pooled positive sera gave reactions with these bands (unpublished data). The less defined band may be glycoproteins or lipoproteins as the muscle larvae were grown in mammalian host. In addition, components of surface structure are changed in molting and within one stage, extract of radiolabeled iodine surface antigens of newborn larvae of *T. spiralis* shows structural changes in 6-18 hours. These components are immunogenic to sera (Jungery *et al*, 1983). Changes of surface components also occur during the developmental stages of larvae. The first and second stages of *Onchocerca lienalis* carry mannose or glucose/glucosamine residues on the surface and the third stage contains decreasing amounts of these saccharides, which are replaced by galactose/galactosamine residues (Ham *et al*, 1988). The changes in surface components would stimulate the host immune responses (Ham *et al*, 1988; Georgieva and

Mizinska-Boevska, 1999). In general, the complete encystment of larvae in striated muscle takes about 4-5 weeks but in our study, the muscle larvae were obtained at the age of 8 weeks of post-infection. It is possible that the fluctuation levels of OD values are due to antibodies against those changing antigens during development of newborn to muscle larvae, including individual immune responses of pigs to those antigens.

In summary, ESAg yielded better results than crude and surface antigens. When ELISA tests were determined with their sensitivity at the same level, ESAg gives the highest specificity (98.3%) among the three antigens. However, OD values of all swine trichinellosis sera to ESAg were close to cut-off value. Crude antigen had high antigenic response to antibodies against *T. spiralis* muscle larvae and give higher ELISA OD values far from cut-off value. Also, it is easy to prepare and gave 100% sensitivity and 91.8% specificity. Therefore, ES and crude antigens should be improved by eliminating cross-reactive antigens or purifying more reactive antigens to antibodies against *T. spiralis*.

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