DETECTION OF *ISOSPORA SUIS* COPROANTIGENS USING INDIRECT SANDWICH ELISA – A PRELIMINARY STUDY

JACEK KARAMON, IRENA ZIOMKO, AND TOMASZ CENCEK

Department of Parasitology and Invasive Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland
J.Karamon@piwet.pulawy.pl

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Abstract

A preliminary study on the possibility for the detection of *Isospora suis* infection by using indirect sandwich ELISA for the detection of coproantigens was performed. The antigen was prepared from *I. suis* oocysts. IgG anti-*I. suis* for coating the plates were obtained from serum of immunised with the *I. suis* antigen rabbit. Secondary anti-*I. suis* antibodies were obtained from rats immunised with the *I. suis* antigen. Commercial rabbit anti-rat IgG conjugated to horseradish peroxidase was used as the conjugate and ABTS as the substrate. The results were read at 405 nm. After estimation of an optimal concentration of the components, the efficacy of indirect sandwich ELISA for *I. suis* antigen detection was examined. Diarrhoeal faeces of suckling piglets or water, both enriched by *I. suis* oocysts, were used as the examined samples. The oocysts were added to the samples in the following proportions: 300 000, 30 000 and 3 000 per 1 g of faeces or 1 ml of water. The backgrounds of the reaction were estimated by the examination of faecal samples or water without oocysts. Each sample was examined 6 times using the elaborated indirect sandwich ELISA. The highest mean OD values were obtained in the water samples containing 300 000 and 30 000 oocysts/ml (the results were significantly higher than the background reaction). However, a relatively high non-specific reaction was noticeable in the examination of faecal samples. Results significantly higher than the background reaction were obtained only in samples containing 300 000 oocysts/g. The developed test seems to create the possibility for the detection of *I. suis* antigens. However, its relatively low sensitivity and specificity at this stage exclude this test from putting it into practice.

Key words: *Isospora suis*, antigens, indirect sandwich ELISA, laboratory diagnosis.

Protozoal parasite *Isospora suis* (Biester, 1934) is one of main casual agents of diarrhoea in suckling piglets. During the recent 20 years, along with the development of the industrial swine production, the piglet isosporosis became an essential problem first in the U.S.A. and then in Europe and other continents (12). Problem of the piglet isosporosis also concerns Poland. Investigations carried out in recent years showed the presence of *I. suis* in piglets in 67% of Polish farms (7).

Isosporosis concerns only suckling piglets, most often in the 2nd and 3rd week of their life. The main symptom is diarrhoea – first watery, next pasty. The diarrhoeal faeces are mostly yellowish or greyish coloured. The dehydration and a decrease in weight gains can be observed in the infected piglets. As a result of a destructive action of *I. suis* on the intestinal epithelium during developmental stages, the intestinal villi are shortened and digestion and absorption of nutritional substances are disturbed (13).

The diagnosis of isosporosis is based on the detection of *I. suis* oocysts in faeces. However, there are some diagnostic difficulties connected with the course of the life cycle of the parasite. The symptoms of isosporosis are observed in the pre-patent period of the invasion when the patent forms, oocysts, are not shed. Moreover, properties of diarrhoeal faeces of sucking piglets that contain a lot of fat (from the mother’s milk) consequently make microscopic examinations difficult. For these reasons, many investigations try to improve the diagnostic methods for the detection of *I. suis* infection.

The aim of this preliminary study was to evaluate the possibility of the detection of *I. suis* infection by using indirect sandwich ELISA used for the detection of coproantigens.

Material and Methods

*Isospora suis* oocysts. The oocysts were used for the preparation of the *I. suis* antigen and for the enrichment of faecal samples in the analysis of sandwich ELISA sensitivity. They were obtained from faeces of experimentally infected piglets. The oocysts isolated from faeces were sporulated and stored in 2% potassium dichromate solution.

*Isospora suis* antigen. The antigen was prepared from *I. suis* oocysts suspended in 2% potassium
dichroma. Potassium dichromate was removed from the suspension by centrifugations (1 000 x g, 5 min, 3 times). Next, larger particles were removed by sieving with sieves with 0.06 and 0.03 mm meshes. Bacterial contamination was inactivated by mixing the oocyst suspension in 1% sodium hypochloride solution on magnetic stirrer for 1 h. Sodium hypochloride was removed by centrifugation (1 000 x g, 5 min, 3 times). Then, the suspension was filtered (filter mesh 0.01 mm). The oocysts obtained from the surface of filter were added to PBS (pH 7.2). The walls of oocysts and sporocysts and membranes of sporozoites were disrupted by vortexing oocyst suspension with glass beads and then by sonification (20 W, 2 x 60 s). Next, the suspension was centrifuged (8 500 x g, 30 min) and the obtained supernatant (antigen) was filtered through anti-bacterial filters (mesh 0.2 µm) and frozen (-20°C) in 0.5 ml tubes. Protein concentration in the antigen amounted to 1 mg/mL.

**Anti-Isospora suis sera and antibodies.** Anti-Isospora suis sera and antibodies were obtained by the immunisation of rabbits and rats. Two male 4-month-old white New Zealand rabbits and 6 male 3-month-old Wistar rats were used. No coccidial oocysts were detected by coproscopic examination in faeces of the animals before and during immunisation. The rabbit anti-I. suis serum was obtained by the immunisation of the rabbits with the I. suis antigen mixed with Freund adjuvant (i.m. injection). The immunisation was made 4 times at 10 d intervals. The first immunisation was made using 1 ml of the antigen mixed with 1 ml of the complete Freund adjuvant. Next, 3 immunisations were made using 0.5 ml of the antigen and 0.5 ml of incomplete Freund adjuvant.

The rabbit anti-I. suis IgG were separated from the serum by using 14% sodium sulphate and centrifugation (11,500 x g, 15 min, 3 times). The obtained pellet (IgG) was dissolved in PBS and divided into equal parts and deep frozen (-70°C). Protein concentration in the IgG solution amounted to 30 mg/mL.

The rat anti-I. suis serum was obtained by the immunisation of 3 rats according to the scheme used in the rabbit immunisation. The control consisted of 1 rabbit and 3 rats, which were injected intramuscularly with a sterile physiologic solution.

During immunisation, blood samples from rabbits were collected (from the ear marginal vein) at 10 d intervals, starting on the day of the first immunisation and obtained sera were stored at -20°C before examination.

The immunised and control animals were bled 6 d after the last injection of the antigen: rabbits by cutting the carotid artery, rats - by insertion of a needle into the heart. These operations were done under general anaesthesia evoked by ketamine. Obtained sera were frozen at -20°C. The efficacy of the immunisation was estimated by the control of I. suis antibody level in sera of the immunised and control animals by ELISA.

**Preparation of samples.** The samples for an indirect sandwich ELISA were either diarrhoeal faeces of suckling piglets or water, both enriched by I. suis oocysts. The faeces were obtained from 4 to 10-d-old suckling piglets free from I. suis invasion, as demonstrated in earlier examinations using Fülleborn flotation method and modified flotation method with using Percoll (6).

The faecal or water oocyst suspension was put into 2 ml Eppendorf-type tube with PBS/Tween (1:4). Next, the glass beads were added and the tube was vortexed for 30 min in order to disrupt I. suis oocysts. The sample was then centrifuged (10 000 x g, 10 min). The obtained supernatant was ready for the examination in indirect sandwich ELISA. The samples were prepared at the day of test performing.

**Indirect sandwich ELISA.** The general scheme of the elaborated test was shown on Fig. 1. Each well of polystyrene 96-well flat bottom plates (MaxiSorp, NUNC) was coated with 100 µl of rabbit I. suis IgG antibodies diluted in 10% fat-free milk in carbonate buffer (pH 9.6). The plates were incubated for 1 h at 37°C and overnight at 4°C. After overnight incubation, the plates were washed 3 times in PBS-Tween. All washes were performed using automatic microplate washer (BIO-RAD). Uncoated sites were blocked with 10% fat-free milk in carbonate buffer (pH 9.6) (200 µl per well). The plates were incubated at 37°C for 30 min and next the milk solution was removed (without washing). The prepared samples (100 µl) were added to wells. Four wells were used for each examined sample (2 wells for main test and 2 wells for control of non-specific binding of rat antibodies from control, non-immunised rat, with antigens contained in a sample). Samples with 300 000 I. suis oocysts were used as positive control (2 wells) and samples without I. suis oocysts - as negative control (2 wells). The plates were incubated at 37°C for 1 h. Then the samples were discarded and the plates were washed 3 times in PBS-Tween. Rat I. suis antibodies diluted in 10% fat-free milk in carbonate buffer (pH 9.6) were used as secondary antibodies (100 µl per well). The plates were incubated at 37°C for 1 h followed by 3 washes in PBS-Tween. Rabbit anti-rat IgG conjugated to horseradish peroxidase (SIGMA) was used as the conjugate. Conjugate was diluted in 10% fat-free milk in carbonate buffer (pH 9.6) and added (100 µl per well) and afterwards, the plates were incubated at 37°C for 1 h. After incubation, the plates were washed 3 times in PBS-Tween and once in PBS. Next, ABTS substrate (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diluted in citrate buffer (pH 4.0) was added (100 µl per well) and the plates were incubated at 37°C for 1 h. The resulting colour reaction was stopped by the incubation of the plates at 4°C for 10 min and read on microplate reader (Multiscan RC, Labsystems) at 405 nm.
Estimation of optimal concentration of indirect sandwich ELISA components. The suspension containing 300 000 oocysts per 1 ml of water was used as the positive sample and PBS-Tween as the negative sample. Optimal concentrations of components were determined by checkerboard titrations in two steps. First, in order to determine optimal dilution of rabbit \(I.\ suis\) antibodies, the plates were coated with rabbit anti-\(I.\ suis\) IgG in the following dilutions: 1:100, 1:200, 1:400, 1:800, 1:1 600, 1:3 200, and rat \(I.\ suis\) antibodies: 1:50, 1:100, 1:200. A conjugate was used in one dilution – 1:1 000. Second, in order to determine the optimal dilution of a conjugate and rat serum, the conjugate was used in the following dilutions: 1:500, 1:1 000, 1:2 000, 1:4 000 and rat \(I.\ suis\) antibodies: 1:50, 1:100, 1:200. Rabbit \(I.\ suis\) IgG antibodies coated on plates were used in one optimal dilution estimated in the first step of the standardisation.

Estimation of efficacy of indirect sandwich ELISA for \(I.\ suis\) antigen detection. The checkerboard titration of the components, showed that the optimal dilution of anti-\(I.\ suis\) rabbit IgG for coating the plates was 1:400, while rat serum should be diluted 1:100 and conjugate – 1:1 000. The results concerning the detection of \(I.\ suis\) antigen in water oocysts suspension by indirect sandwich ELISA were shown in Fig. 3. As it is seen from the Fig. 3, the highest mean OD was obtained in the sample containing the largest number of oocysts. The results were significantly higher from background of reaction (negative sample) in the examination of the sample containing 300 000 and 30 000 oocysts/ml. However, the difference between OD obtained in the sample with 30 000 oocysts/ml and negative sample was only 0.1. In the sample with 3 000 oocysts/ml OD value was close to OD obtained in the sample without oocysts.

Relatively high non-specific reaction was noticed in the examination of faecal samples (Fig. 4). The results (OD) were significantly higher from underground of reaction only in the examination of the sample containing 300 000 oocysts/ml and negative sample was only 0.1. In the sample with 3 000 oocysts/ml OD value was close to OD obtained in the sample without oocysts.

Statistical analysis. The t-Student test was used to compare the results (OD values). The differences were considered statistically significant when \(P<0.05\). The data were analysed by the use of the Microsoft Excel 2 000.

Results

The effectiveness of the immunisation. The level of \(I.\ suis\) antibodies in the immunised rabbit increased significantly after first antigen injection and after following two injections OD (optical density) values exceeded 1.00. In the day of bleeding of the rabbit, OD equalled 1.05. The OD values in serum of the control non-immunised rabbit were low for the entire period of immunisation and ranged from 0.04 to 0.09. Results concerning immunisation of rats also evidenced its effectiveness. The mean of OD values obtained from sera of the immunised rats equalled 0.58, while in control rats this value was low and equalled 0.11.
Fig. 3. Results of examination of water samples containing different numbers of *I. suis* oocysts by indirect sandwich ELISA.

*significantly different value with relation to negative sample (P<0.05)

Fig. 4. Results of the examination of faecal samples containing different numbers of *I. suis* oocysts by indirect sandwich ELISA.

*significantly different value with relation to negative sample (P<0.05)

Table 1

Results of examination of samples containing *I. suis* oocysts by indirect sandwich ELISA using serum of the non-immunised rat as the secondary antibodies

<table>
<thead>
<tr>
<th>Number of oocysts in 1 ml of suspension or 1 g of faeces</th>
<th>Mean OD values (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300 000</td>
</tr>
<tr>
<td>30 000</td>
<td>3 000</td>
</tr>
<tr>
<td>3 000</td>
<td>3 000</td>
</tr>
<tr>
<td>Suspension samples</td>
<td>0.065 (± 0.035)</td>
</tr>
<tr>
<td></td>
<td>0.192* (±0.034)</td>
</tr>
<tr>
<td></td>
<td>0.102 (±0.015)</td>
</tr>
<tr>
<td></td>
<td>0.081 (±0.051)</td>
</tr>
<tr>
<td>Faeces samples</td>
<td>0.563 (±0.040)</td>
</tr>
<tr>
<td></td>
<td>0.774* (±0.217)</td>
</tr>
<tr>
<td></td>
<td>0.575 (±0.103)</td>
</tr>
<tr>
<td></td>
<td>0.565 (±0.131)</td>
</tr>
</tbody>
</table>

*significantly different value with relation to negative sample (P<0.05)

Discussion

There were described many methods, which made the isosporosis diagnostics more effective. Most of them concerned different modifications of flotation method. Changes in the composition of the flotation solution in order to improve the visibility in microscopic examination were often proposed, e.g. the addition of phenol or sugar (4, 11). The detectability of isosporosis increased by using the phenomenon of autofluorescence of *I. suis* oocysts in UV light. Great hopes are given to investigations of Joachim (5) who applying the PCR technique obtained higher detectability than with flotation method.

The preliminary study, presented in this article, was the first published data concerning the use of a sandwich ELISA for the diagnosis of *I. suis* infection. The test was adopted by many researchers for the detection of many different parasite coproantigens. There were elaborated tests for the detection of coproantigens of numerous tapeworms, roundworms, trematodes (2, 3), and protozoal parasites (1, 10). However, among coccidian parasites, *Cryptosporidium* sp. is only the one species, that has been detected with the sandwich ELISA and results connected with these investigations were described and published (8-10).

Relatively low sensitivity was obtained in the developed sandwich ELISA for the diagnosis of *I. suis* infection. Significantly higher OD values were only
observed during the examination of samples containing 300 000 oocysts per 1 g of faeces or water. This low sensitivity is evident, particularly in comparison to flotation methods that make it possible to detect several times less oocysts. Probably, the use of monoclonal *I. suis* antibodies in the developed test might increase its sensitivity. But it must be stressed that the detectability obtained in a similar test for the detection of *Cryptosporidium* sp. infection was 500 000 oocysts per 1 ml of faeces (9). Next, the threshold of the detectability obtained by Lindergard (8) in indirect sandwich ELISA for soil examination was 10 000 *Cryptosporidium* sp. oocysts per 1 g of soil. In the case of *Cryptosporidium* such levels of sensitivity of the tests can be sufficient; therefore, on account of minute sizes of oocysts, the microscopic examination is very difficult and requires high oocyst concentration in the sample and a great deal of experience on behalf of the laboratory staff. However, in the case of isosporosis, routine microscopic methods, despite difficulties connected with properties of diarrhoeal piglet faeces, is characterised by higher sensitivity than the sandwich ELISA method elaborated by us.

High OD values obtained in the examination of faecal samples without oocysts showed a relatively strong non-specific reaction. Non-specific antibodies included in polyclonal rat's serum were responsible for this reaction. They were binding with some antigens contained in faeces, different than *I. suis* antigen. The proteolytic action of enzymes contained in faeces also negatively influenced the course of specific reaction of antibodies with antigens. In our study it also manifested because the difference between the OD value obtained in samples containing oocysts and background of reaction was smaller in examination of faeces than in examination of water suspension. Some authors tried to reduce unfavourable non-specific influence of faeces, among others, by boiling the samples. However, the effectiveness of such proceedings and their influence on the credibility of the examination were not completely explained (10). It is most probably that in the further investigations on sandwich ELISA for the detection of *I. suis* coproantigens in order to increase the specificity and sensitivity of the test, applying monoclonal antibodies will be essential.

To sum up, it is possible to state that the elaborated indirect sandwich ELISA gives the possibility for the detection of *I. suis* antigens and this preliminary study can be the solid basis for further more advanced investigations. However, relatively low sensitivity and specificity at this stage excludes this test from putting it into practice.

### References