

DOI 10.2478/v10181-010-0006-3

Original article

Occurrence of leptospiral infections in swine population in Poland evaluated by ELISA and microscopic agglutination test

B. Wasiński, Z. Pejsak

Department of Swine Diseases of the National Veterinary Research Institute
Al. Partyzantow 57, 24-100 Pulawy, Poland

Abstract

Swine are one of significant reservoirs and sources of *Leptospira* infections for man. Serological screenings help to effectively control the epidemiological situation in swine herds and to prevent transmission of *Leptospira* from animals to man. The purpose of this study was to investigate, by the use of serological methods, the prevalence of infections caused by selected *Leptospira* serogroups in swine population in Poland.

A total of 7112 swine serum samples were examined. The samples were collected from January to October 2008 and came from 280 counties situated in all 16 provinces of Poland. All sera were examined preliminary by enzyme-linked immunosorbent assay (ELISA) using heat-stable antigenic preparation. The samples positive or doubtful in ELISA were investigated by microscopic agglutination test (MAT) with use of serovars Icterohaemorrhagiae, Pomona, Canicola, Sejroe, Tarassovi and Grippotyphosa.

Of the collected sera examined by ELISA 73 (1.02%) samples were positive, 85 (1.20%) – doubtful and 6954 – negative. Among ELISA-positive and doubtful sera 64 samples (coming from 14 provinces) were recognized in MAT as positive. Among MAT positive samples 42.19% of sera demonstrated titres with serovar Pomona, 32.81% – with Sejroe, 14.06% – with Icterohaemorrhagiae, 6.25% – with Tarassovi, 3.13% – with Grippotyphosa and 1.56% with Canicola.

Key words: leptospirosis, ELISA, MAT, pig

Introduction

Leptospirosis is a worldwide zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. Because of wide spectrum of clinical disease (involving multiorgan failure and high mortality) in humans and lack of the specific clinical symptoms it is difficult to make timely accurate diagnosis (Guerra 2009). One of important reservoirs and sources of

Leptospira infection for people are swine. The infection can be transmitted by the contact with infected urine, parts of placenta after parturition or during inspection of contaminated carcasses in abattoir (Faine 1994). The hazard of *Leptospira* transmission from pigs to man is additionally enlarged by frequently met lack of perceptible symptoms of the infection in swine. Diagnosis of leptospirosis in swine is difficult and usually based on laboratory methods. Isolation of lep-

tospire is time consuming (sometimes 10-25 weeks), laborious and not reliable. Molecular methods, although recently more available, are relatively expensive and need well-equipped laboratory facilities. In principle, for fast diagnosis of *Leptospira* infections serological methods are extensively used. They are also useful for evaluation of epidemiological situation in herds.

The purpose of the present study was to investigate, by the use of serological methods, the prevalence of infections caused by selected *Leptospira* serogroups in swine population in Poland.

Materials and Methods

Serum samples collected since January to the October of 2008 from 7 112 pigs were examined. Investigated animals came from randomly chosen farms located on the area of 280 counties situated in all 16 provinces (voivodeships) of Poland. Serum samples were examined preliminary by the enzyme-linked immunosorbent assay (ELISA) and in case of positive or doubtful results, finally by the microscopic agglutination test (MAT).

Cultures of reference strains of *Leptospira interrogans* serovar Icterohaemorrhagiae (strain RGA), *L. interrogans* serovar Pomona (strain Pomona), *L. interrogans* serovar Canicola (strain Hond Utrecht IV), *L. borgpetersenii* serovar Sejroe (strain M 84), *L. borgpetersenii* serovar Tarassovi (strain Perpelicin), and *L. kirschneri* serovar Grippotyphosa (strain Moskwa V) were used in the study. The strains were obtained from FAO/WHO Reference Laboratory for Leptospirosis of the Royal Tropical Institute in Amsterdam. *Leptospira* were cultivated in EMJH (Ellinghausen McCullough Johnson and Harris) liquid medium at 30°C in aerobic conditions. The strains were subcultured every 7 days. The cultures of mentioned six serovars are used in our laboratory for MAT during routine serological examinations (monitoring) of *Leptospira* infections in swine.

Heat-stable antigenic preparation used for ELISA was produced on the base of procedure proposed by Terpstra et al. (1980). Cultures of serovars Icterohaemorrhagiae, Sejroe and Pomona were used for preparation of the antigen. Equal volumes of mentioned serovars cultures (density 109 leptospire/ml) were centrifuged (10 000 x g for 15 min). The supernatant was removed and leptospire were washed by centrifugation (10 000 x g for 15 min) in physiological saline. After washing supernatant was removed and pellets were suspended in 1/3 of initial volume in Milli-Q-water. Suspensions of the three serovars were combined and inactivated with formalin (final concentration 0.5% v/v) for 60 min. After inactivation the suspension was heated in boiling water for 30 min.

and, after cooling, centrifuged (10 000 x g for 30 min). Obtained supernatant was used as the antigen for ELISA. Protein concentration in the antigen was determined by the use of Sigma protein assay kit. The antigen was stored at 4°C and was found to be stable for at least 6 months.

The microplates PolySorp (Nunc, Denmark) were used for the ELISA. Before coating of microplates the antigen was diluted in physiological saline to obtain protein concentration of 5 µg/ml. Into each well of microplate 100 µl of diluted antigen was introduced. Coating was led for 18 h at 37°C. After the incubation uncoated antigen was removed and microplates were dried at room temperature. Coated plates were stored in the dark place at room temperature. Period of the storage was found to be not shorter than 3 months.

Directly before use coated microplates were washed 4 times with phosphate buffered saline (PBS) pH 7.2 + 0.05% Tween 20 (PBST). During last two washings of the cycle, PBST remained in wells for 1 min. Test serum samples and controls diluted 1:100 in PBS + 0.5% bovine serum albumin (PBS+BSA) were introduced into appropriate wells in volume 100 µl and incubated for 60 min at 37°C. Each test serum was run in duplicate and each control was located into four wells. After the incubation sera were removed and microplates were washed as described above. Subsequently, all wells were filled with 100 µl of conjugate (horseradish peroxidase-conjugated rabbit anti-swine immunoglobulins, Dako, Denmark) diluted 1:10000 in PBS + BSA. Microplates with conjugate were incubated for 60 min at 37°C. After the incubation microplates were washed like above and chromogenic substrate solution, o-phenylenediamine (OPD) (Sigma, USA) 0.4 mg/ml dissolved in phosphate-citric buffer (pH 5.0) was introduced into the wells (100 µl per well). Microplates with substrate were incubated in darkness at room temperature for 15 min. Colour reaction was stopped with 4N H₂SO₄.

Plates were read in a photometric microplates reader (Labsystems Multiskan® RC) at the wavelength 490 nm. The results were interpreted on the base of S/P ratio (S/P ≥ 0.7 – positive, 0.7 > S/P ≥ 0.5 – doubtful, S/P < 0.5 – negative).

The samples positive or doubtful in ELISA were investigated by MAT carried out according to OIE Manual of Standards for Diagnostic Tests and Vaccines (Bolin 2008). Live cultures of the six mentioned above *Leptospira* serovars were used for the MAT. The serum samples were mixed with equal volume of each of the *Leptospira* serovars. Serum dilution (including added antigen) used during preliminary examination was 1:100. For samples reacting in the preliminary examination with one or more serovars, series of twofold dilutions were prepared to titre the end point – 50% agglutination. The samples with titres ≥ 100 were recognised positive. Sera with titres 100 and 200

were considered to be low positive ones and interpreted as possible symptom of starting or ending infection or as indication the exposure to *Leptospira*. Titres ≥ 400 were considered as highly positive and interpreted as indicating active infection.

Results

As it is presented in Table 1, in case of sera examined by ELISA 73 (1.02%) samples were positive, 85 (1.20%) – doubtful and 6954 (97.77%) samples – negative (Table 1). From the group of sera positive in ELISA 45.20% of samples were recognised in MAT as positive and 54.80% of samples – as negative. In the group of samples recognised in ELISA as doubtful 36.50% of sera was positive in MAT and 63.50% – negative.

Detected in ELISA positive or doubtful serum samples, recognised in MAT as positive, demonstrated antibodies reacting in MAT with serovars Pomona (42.19% of MAT positive samples), Sejroe (32.81%), Icterohaemorrhagiae (14.06%), Tarassovi (6.25%), Grippotyphosa (3.13%) and Canicola (1.56%) (Table 2). According to the data contained in

Table 1. Comparison of numbers/% of serum samples indicating positive or doubtful results in ELISA and recognised as positive or negative in MAT.

Result in ELISA	Result in MAT		Total
	positive	negative	
Positive	33 / 0.46	40 / 0.56	73 / 1.02
Doubtful	31 / 0.44	54 / 0.76	85 / 1.20
Total	64 / 0.90	94 / 1.32	158 / 2.22

Table 2 majority of serum samples positive or doubtful in ELISA and positive in MAT presented titres ≤ 200 .

Four samples demonstrating titres to more than one *Leptospira* serogroup. Two of them presented titres to serogroup Pomona and Grippotyphosa, one – to serogroups Canicola and Sejroe and one – to Sejroe and Pomona. First three of mentioned samples were recognized by ELISA as positive and the last one – as doubtful.

Distribution of MAT-positive results in groups of serum samples received from particular provinces is presented in the Table 3. Positive or doubtful results of ELISA confirmed by MAT were recorded in groups of sera coming from 14 provinces. The sera demonstrating titres to serogroup Pomona were found among samples from 8 voivodeships. In one province one sample present antibodies reacting with serovars Pomona and Sejroe. The sera from less numerous group, specific to serogroup Sejroe were found among samples from 9 provinces. Serum samples specific to serogroup Icterohaemorrhagiae were found in 3 voivodeships, samples specific to serogroup Grippotyphosa and Tarassovi – in 2 voivodeships and samples specific to serogroup Canicola – in 1 voivodeship. Additionally, in one voivodeship two samples demonstrating antibodies reacting with serovars Grippotyphosa and Pomona were found.

Discussion

Observed in the present study relatively low percentage of serum samples positive in MAT (0.9% of all examined samples) seems to indicate not high probability of *Leptospira* transmission from pigs to

Table 2. Number of positive or doubtful in ELISA serum samples demonstrating in MAT titres with different serovars.

Result in ELISA	Number of serum samples reacting in MAT with serovars						Titer in MAT
	Ictero.	Grippotyphosa	Sejroe	Tarassovi	Pomona	Canicola	
Positive	3	2	2	1	2		100
	1		3 (1)*		6	1	200
		(2)*	2		5		400
				1	1		800
					3		1600
					3		3200
Doubtful	3		6	2	1		100
	1		5		1 (1)*		200
	1				1		400
			3		2		800
					4		1600
				1		3200	

* – numbers in parentheses denote the samples demonstrating titres to mentioned serogroup as coagglutination apart of titer to another serogroup.

Table 3. Distribution of swine sera demonstrating positive and doubtful results in ELISA and positive MAT results in particular voivodeships.

Province	Number of examined serum samples	Number of serum samples positive in ELISA	Number of serum samples doubtful in ELISA	Number (%) of serum samples positive in MAT	Serogrup specificity of MAT positive samples
Dolnośląskie	364	7	6	5 (1.37)	Ictero., Gripo., Sejroe
Kujawsko-Pomorskie	586	6	3	5 (0.85)	Sejroe, (Sejroe)*, Canicola
Lubelskie	350	1	3	3 (0.86)	Sejroe, Pomona
Lubuskie	376	3	11	7 (1.86)	Sejroe, Pomona
Łódzkie	371	3	4	2 (0.54)	Sejroe
Małopolskie	411	0	4	1 (0.24)	Pomona
Mazowieckie	269	0	2	1 (0.37)	Sejroe
Opolskie	116	0	0	0	
Podkarpackie	902	23	11	14 (1.55)	Sejroe, Pomona, (Gripo.)
Podlaskie	385	2	5	4 (1.04)	Gripo., Tarassovi, Pomona
Pomorskie	295	13	7	5 (1.69)	Pomona
Śląskie	747	4	6	1 (1.34)	Pomona
Świętokrzyskie	166	0	0	0	
Warmińsko-Mazurskie	640	8	9	7 (1.09)	Ictero., Tarassovi, Pomona
Wielkopolskie	722	2	11	7 (0.97)	Ictero., Sejroe, (Pomona)
Zachodniopomorskie	412	1	3	2 (0.49)	Sejroe

* – serogrup names in parentheses denote samples in which the antibodies to mentioned serogrup are demonstrated together with titres to another serogrup.

man. Analyzing reasons of discrepancy between ELISA and MAT results it can be stated that one of them can be higher sensitivity of ELISA (Adler et al. 1980, Hartman et al. 1984, Ribotta et al. 2000). Another reason can be connected with detection by ELISA antibodies specific to other *Leptospira* serogroups then these, to which belong serovars used in MAT. The data obtained during described study indicate usefulness of ELISA as suitable method for preliminary serological monitoring of leptospirosis. High sensitivity of ELISA and the status of MAT as the standard method in serological diagnosis of leptospirosis needs confirmation of ELISA positive and doubtful results by MAT. Apart of it, MAT is still necessary for estimation of serogrup specificity and titer of detected antibodies.

Described serological investigation indicates domination of samples specific for serogroups Pomona and Sejroe. Results from the year 2008 confirmed data from previous years (Wasinski 2005, 2007), in which occurrence of animals presenting antibodies for mentioned two serogroups were most often registered. Domination of each of these serogroups was noted alternately in last following years. The percentage of swine demonstrating titres to other of mentioned serovars was usually considerably lower.

Serovar Pomona is typical pathogen of swine, occurrence of which is noted in herds all over the world (Bolt et al. 1995, Ellis 2006, Naito et al. 2007). Main reason of economic losses caused in swine husbandry by infections with this serovar are abortions.

Noted in our findings relatively high percentage of swine sera reacting with serovar Sejroe is an interesting phenomenon. In spite of high prevalence of swine demonstrating titres to serovar Sejroe, clinical symptoms of leptospirosis (including abortions) are not often observed in these animals. Infections with this serovar in swine were noted in Europe before (Brandis 1956, Combiesco et al. 1958, Ellis 2006). However, recently there are not many reports describing an occurrence of higher percentage of positive reactions with serovar Sejroe in serological screenings of swine herds in Europe and over the world (Wasiński 2005, 2007, Jung et al. 2009).

Few reports concerning application of ELISA for serological monitoring of leptospirosis in swine were published until now. Different types of antigens were used in described kits. In trials led by Waltman and Dave (1983), the antigen obtained from sonicated *Leptospira* cells enabled to attain relative ELISA sensitivity of 86.4% and specificity of 100%. The test was investigated with the group of 53 pigs. Mendoza and Prescott (1992) applied for ELISA the axial filament (AF) isolated from *Leptospira interrogans* serovar Canicola. Observed in this study correlation between AF-ELISA and ELISA with whole cell sonicated antigen obtained 0.97 ($p=0.0001$). In some ELISA sets selected lipoproteins of leptospiral outer envelope like LipL32 (Naito et al. 2006) or LipL41 (Theodoridis et al. 2005) were used as antigens. Preparation of this type of antigens needs however well-equipped laboratory. Heat-stable antigens, obtained from single

serovar, were used in ELISA kits detecting antileptospiral antibodies in human (Terpstra et al. 1980) and dog sera (Ribotta et al. 2000). Composed heat-stable antigenic preparation from four serovars (Icterohaemorrhagiae, Tarassovi, Pomona, Hardjo) was used in ELISA for examination of bovine serum (Tagliabue et al. 1994). Apart of simple preparation, important advantage of heat-extracted antigens is their long shelf life. The antigen used in presented home made ELISA, stored as a liquid at temp. 4°C, was found to be stable for at least 6 months. Stability of the antigen, after coating the microplates, stored at room temperature in darkness, was not shorter then 3 months.

The antigen used in our ELISA, prepared from cultures of serovars Icterohaemorrhagiae, Sejroe and Pomona, was able to detect in swine serum samples antibodies specific for mentioned serogroups and for serogroups Grippotyphosa, Tarassovi and Canicola. Serovars belonging to these six serogroups are most often the reasons of swine leptospirosis in Poland.

Developed ELISA seems to be convenient tool for preliminary serological monitoring of leptospirosis in swine herds. The use of MAT is necessary for confirmation of ELISA positive (or doubtful) results and for estimation of serogroup specificity and titres of antibodies in ELISA positive or doubtful serum samples.

Results of serological investigations performed with serum samples collected in the year 2008 confirmed trends in occurrence of particular *Leptospira* serogroups infections observed in Polish swine herds during last years in monitoring studies led by the use of MAT.

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