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Original article

Reactivity of heat-stable *Leptospira* antigenic preparation used in enzyme-linked immunosorbent assay for detection of antibodies in swine serum

B. Wasiński, Z. PejsakDepartment of Swine Diseases, National Veterinary Research Institute
Al. Partyzantów 57, 24-100 Puławy, Poland

Abstract

Serology plays an important role in laboratory diagnosis of leptospirosis. Apart from the most often used microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA) seems to be useful especially in screenings of animal herds. The ELISA used for detection of antibodies against selected *Leptospira* serogroups in swine serum samples was investigated during the study. An essential element of this test is heat-stable antigenic preparation from cultures of *Leptospira interrogans* serovars Icterohaemorrhagiae, Pomona and *L. borgpetersenii* serovar Sejroe.

The aim of the present study was to identify and analyze ELISA heat-stable antigen fractions playing a role in the reaction with leptospiral antibodies indicated in swine serum.

Reactivity of the three-component antigenic preparation was compared in immunoblotting with reactivity of six heat-stable antigenic preparations made from the following single serovars: *L. interrogans* serovars Icterohaemorrhagiae, Pomona, Canicola, *L. borgpetersenii* serovars Sejroe, Tarassovi and *L. kirshneri* serovar Grippotyphosa. All antigenic preparations were submitted to SDS-PAGE and transferred to a nitrocellulose membrane using a semidry system. After the transfer, the membrane was incubated with diluted swine serum containing antibodies specific for one of the six above mentioned *Leptospira* serovars.

For the three-component antigenic preparation and antigens prepared from single serovars the immunoblot revealed reaction of sera with fractions of the 20-26 kDa region and around the 14.5 kDa region. The investigated heat-stable *Leptospira* antigenic preparation contains fractions demonstrating serogroup- and species-specificity. Fraction 20-26 kDa showed serogroup-specific activity, whereas the fraction around 14.5 kDa showed species-specific activity.

Key words: *Leptospira*, antigens, swine, ELISA

Introduction

Leptospirosis is a worldwide distributed zoonosis caused by spirochetes of the genus *Leptospira* (Vinetz 2001). It is a systemic disease, characterized in humans by a wide variety of symptoms ranging from a mild, influenza-like illness from which the patient may recover without medication, to the severe form (e.g. Weil's disease) with hepatic, renal, sometimes pulmonary failure and death. In domestic animals the disease is an important cause of abortions, stillbirths, infertility and agalactia (Guerra 2009). In swine, leptospirosis can demonstrate a range of clinical signs varying with the infecting serovar (Bolin 1994); however, the majority of cases are subclinical (Ellis 2006) and the only perceptible symptom can be abortions. Chronic infections and asymptomatic carrier states in swine can remain unrecognized for some time creating a significant possibility of *Leptospira* transmission to other animal species (e.g. serovar Pomona often transmitted from pigs to cattle) or to man (mainly serovar Icterohaemorrhagiae).

Leptospirosis can be a cause of major reproductive losses in swine breeding herds. Although the losses are reported from various countries located in all parts of the world, knowledge about the economic impact and incidence of the disease is confined to the intensive pig industries of the Northern Hemisphere and Argentina, Australia, Brazil and New Zealand (Ellis 2006).

The protean nature of leptospirosis means that the diagnosis often has to rely on the results of laboratory tests. Serological methods are most extensively used for fast diagnosis of *Leptospira* infections and for evaluation of the epidemiological situation in herds. Apart from the most often used but laborious, time-consuming, subjective microscopic agglutination test (MAT), which is hazardous for laboratory staff, the enzyme-linked immunosorbent assay (ELISA) seems to be useful especially in screenings of animal herds. Few reports concerning the use of ELISA for serological diagnosis of swine leptospirosis have appeared during the last few decades. In described ELISA sets for swine, various types of antigens such as the axial filament (Mendoza and Prescott 1992) or outer membrane protein LipL32 (Naito et al. 2007) were used. The ELISA presented in this paper contains a heat-stable antigenic preparation from cultures of *Leptospira interrogans* serovars Icterohaemorrhagiae and Pomona and *L. borgpetersenii* serovar Sejroe (Wasiński and Pejsak 2010). Apart from antibodies specific to the serogroups mentioned above, the antigenic preparation is able to detect antibodies reacting with serovars Tarassovi, Canicola and Grippotyphosa.

The aim of the present study was to identify and analyze ELISA heat-stable antigen fractions playing a role in reaction with leptospiral antibodies indicated in swine serum.

Materials and Methods

Leptospira serovars

Leptospira interrogans serovars Icterohaemorrhagiae (strain RGA), Pomona (strain Pomona), Canicola (strain Hond Utrecht), *Leptospira borgpetersenii* serovars Tarassovi (strain Perpelicin), Sejroe (strain M 84) and *Leptospira kirschneri* serovar Grippotyphosa (strain Moskwa V) were used in the study. The strains were obtained from the FAO/WHO Reference Laboratory for Leptospirosis of the Royal Tropical Institute in Amsterdam. They were cultivated in liquid EMJH medium (Ellinghausen and McCulloch 1965) at 30°C in aerobic conditions. The strains were subcultured every seven days.

Antigen preparations

The heat-stable antigen used in the ELISA was prepared on the basis of the method described by Terpstra et al. (Terpstra et al. 1980). Cultures of serovars Icterohaemorrhagiae, Sejroe and Pomona were used for preparation of the antigen. Equal volumes of these serovar cultures (density 10^9 leptospores/ml) were centrifuged (10 000 x g for 15 min). The supernatant was removed and the leptospores were suspended in initial volume in physiological saline and centrifuged (10 000 x g for 15 min). After washing the supernatant was removed and pellets were suspended in 1/3 of the 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). The supernatant was used as the antigen for ELISA.

Apart from the antigenic preparation described above, six heat-stable antigens were produced from each serovar. The six above-mentioned *Leptospira* serovars were used for preparation of the single antigens. The procedure for the single antigen preparation was identical to that described in the case of trivalent ELISA antigen with one exception: after washing of leptospores in physiological saline they were suspended in initial volume of Milli-Q-water (they were not concentrated to the 1/3 of the initial volume).

The protein concentration in the described antigenic preparations was measured according to the Lowry method (Lowry et al. 1951).

Immune sera

Antisera specific for the above mentioned six *Leptospira* serovars were obtained from immunized swine. Six gilts, seven months old, were used for immunization. The animals came from one litter and were maintained in an isolated piggery. The sow, from which came the animals used for immunization, was routinely vaccinated against swine erysipelas and parvovirus infections and was not vaccinated against leptospirosis. Gilts used for immunization were maintained without vaccination. One week before the start of immunization each of the gilts was located in an individual pen isolated from other animals. Three days before the first immunization, blood samples were collected from all animals. Serum samples were investigated by MAT for detection of antibodies reacting with mentioned above *Leptospira* serovars.

Each gilt was immunized by one serovar. Animals were inoculated intravenously into the marginal ear vein with five successive doses of one week-old cultures containing approximately 10^9 leptospores/ml. Doses of 1 ml, 2 ml, 4 ml, 6 ml and a further 6 ml were administered at 7-day intervals (Faine, 1982). Immune sera used in the study were collected one week after the last inoculation. The MAT titers of the collected immune sera were: serum from swine immunized by serovar Icterohaemorrhagiae – 1 : 800, swine immunized by Grippotyphosa – 1 : 400, swine immunized by Sejroe – 1 : 800, swine immunized by Tarassovi – 1 : 1600, swine immunized by Pomona – 1 : 800 and swine immunized by Canicola – 1 : 800.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The trivalent, heat-stable antigenic preparation used in ELISA and preparations obtained from single serovars were submitted to SDS-PAGE. To increase protein concentration in the investigated antigenic preparations all of them were lyophilized and subsequently resolved in a smaller volume of Milli-Q-water before the start of the procedure. The protein concentration in concentrated antigens ranged between 1.0 and 1.5 mg/ml. The SDS-PAGE was performed (Laemmli 1970) using a Mini Protean[®]II (Bio-Rad) set with 17% (w/v) polyacrylamide resolving gel and 4% (w/v) stacking gel. It was run for 70 min at 200

V (45 mA). The antigens were transferred thereafter from the gel to a nitrocellulose membrane using a semidry system in Trans Blot[®] SD (Bio-Rad) apparatus. The transfer was allowed for 35 min at 35 V (350 mA). After the transfer the membrane was blocked with 1% (v/v) bovine albumin for 18 h at 4°C. Thereafter, the membrane was washed three times (every time for 10 min) in TBS buffer containing 0.05% (v/v) Tween 20 (TBST) and incubated for 60 min at 37°C with diluted 1:100 swine serum containing antibodies specific for one of the six above-mentioned *Leptospira* serovars. After washing (as before) and incubation (60 min at 37°C) with alkaline phosphatase conjugated rabbit anti-swine immunoglobulin at 1:500 dilution, the reaction was visualized using 4-chloro-1-naphthol.

Results

The trivalent heat-stable antigenic preparation used for the ELISA reacted with all antisera specific for particular serovars. The results of the reactions with antisera prepared against serovars Pomona, Sejroe and Grippotyphosa are shown in Figs. 1, 2 and 3, respectively. The antisera used in the study showed on lanes with the three-component antigenic preparation and on lanes with antigens prepared from single serovars the reaction with fractions of 20-26 kDa region and around the 14.5 kDa region. The antigenic fraction 20-26 kDa was visible in each reaction of the three-component antigen with serum specific for par-

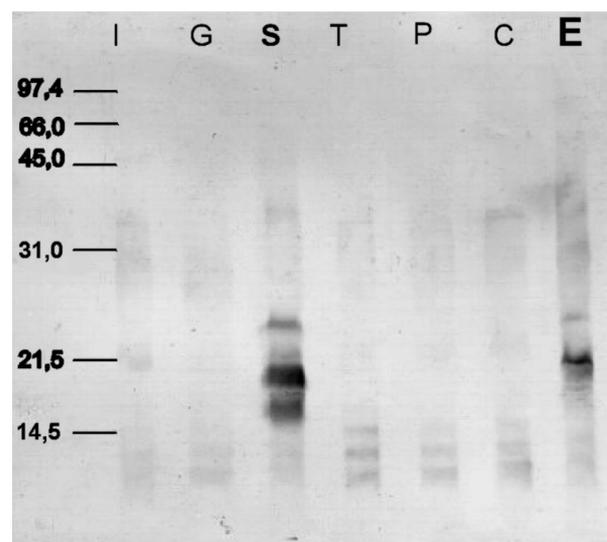


Fig. 1. Immunoblot of three-component antigen preparation (lane E) and antigens prepared from single serovars Icterohaemorrhagiae (lane I), Grippotyphosa, (lane G), Sejroe (lane S), Tarassovi (lane T), Pomona (lane P), Canicola (lane C) after reaction with swine antiserum to serovar Sejroe. Numbers on the left indicate molecular mass markers (kDa).

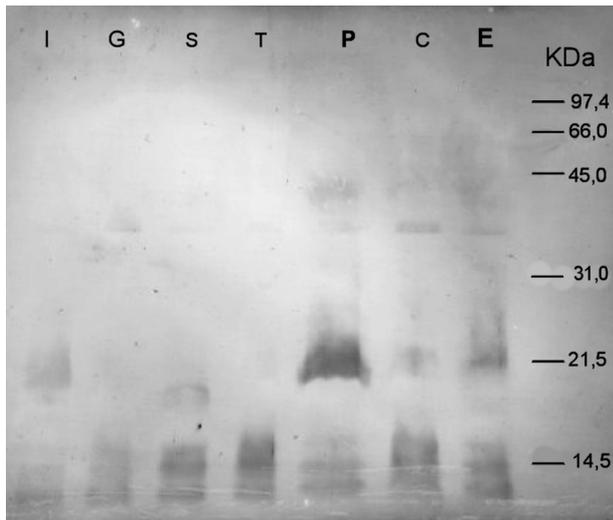


Fig. 2. Immunoblot of three-component antigen preparation (lane E) and antigens prepared from single serovars *Icterohaemorrhagiae* (lane I), *Grippotyphosa*, (lane G), *Sejroe* (lane S), *Tarassovi* (lane T), *Pomona* (lane P), *Canicola* (lane C) after reaction with swine antiserum to serovar *Pomona*. Numbers on the right indicate molecular mass markers (kDa).

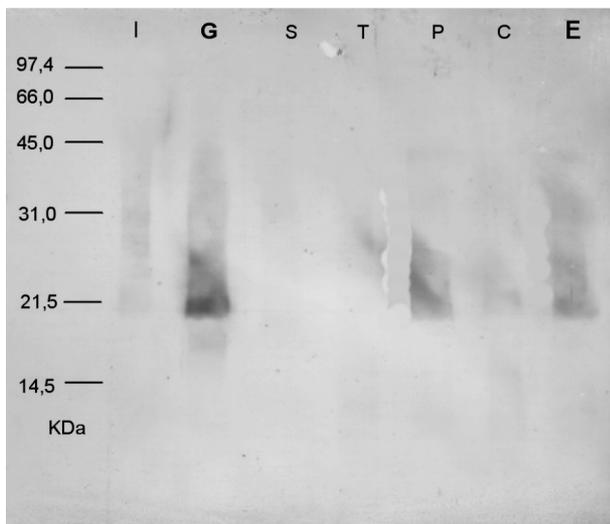


Fig. 3. Immunoblot of three-component antigen preparation (lane E) and antigens prepared from single serovars *Icterohaemorrhagiae* (lane I), *Grippotyphosa*, (lane G), *Sejroe* (lane S), *Tarassovi* (lane T), *Pomona* (lane P), *Canicola* (lane C) after reaction with swine antiserum to serovar *Grippotyphosa*. Numbers on the left indicate molecular mass markers (kDa).

ticular single serovars. Epitopes of this fraction were visible also on lanes with single serovar antigens specific for serum used in the reaction. In the majority of cases the reactions on lanes with antigens of serovars homologous for the antiserum used were stronger than the reaction with the trivalent antigenic preparation (Figs. 1, 2, 3 and data not shown). Only in the case of antiserum specific for serovars *Canicola* and

Icterohaemorrhagiae was the reaction of trivalent antigen as strong as in the case of the single antigen preparation specific for the serum used (data not shown).

The antiserum prepared against serovar *Sejroe* showed a prominent reaction against the 21 kDa band on lanes with preparation from serovar *Sejroe* and with trivalent ELISA antigenic preparation (Fig. 1). On both of the lanes the 25 kDa band was also visible. Apart from this, the antiserum reacted with low molecular mass antigens, around the 14.5 kDa region, in the case of all investigated antigenic preparations.

With the antiserum prepared against serovar *Pomona* the immunoblots showed (Fig. 2) a prominent reaction with antigens of the 20-26 kDa region in the trivalent antigenic preparation and in the preparation from serovar *Pomona*. A slight reaction with antigens from this region was visible also in preparations from serovars *Icterohaemorrhagiae* and *Canicola*. Apart from this, the reaction with antigens from the region around the 14.5 kDa was observable in all antigenic preparations.

In the case of antiserum prepared against serovar *Grippotyphosa* a reaction with low mass antigens, around the 14.5 kDa region was mildly visible (Fig. 3). Clearly visible reactions with antigens of the 20-26 kDa region were observed in the trivalent antigenic preparation and in preparations from serovars *Grippotyphosa* and *Pomona*.

The antisera prepared against serovars *Icterohaemorrhagiae*, *Tarassovi* and *Canicola* exhibited (data not shown) similar types of reactions with immunoblots of the investigated heat-stable antigenic preparations as described above. Antiserum prepared against serovar *Icterohaemorrhagiae* showed a reaction against antigens of the 20-26 kDa region in lanes with an homologous antigen and trivalent antigenic preparation. Antiserum against serovar *Tarassovi* reacted with antigens of the 20-26 kDa region on lanes with the trivalent antigenic preparation and preparations of serovars *Tarassovi* and *Sejroe*. A reaction with antigens around the 14.5 kDa region was visible on lanes with the trivalent and all single antigenic preparations.

Immunoblots of the heat-stable trivalent antigenic preparation and preparations from serovars *Canicola*, *Icterohaemorrhagiae* and *Pomona*, using antiserum prepared against serovar *Canicola*, showed a prominent reaction against the 20-26 kDa region. A slight reaction against antigens of this region was observable also in the case of preparations of serovars *Grippotyphosa* and *Tarassovi*. Apart from this, antiserum prepared against serovar *Canicola* showed a reaction against antigens, around the 14.5 kDa region in case of all investigated antigenic preparations.

Discussion

Heat-stable antigenic preparations were used previously in ELISA for detection of anti-leptospiral antibodies in humans (Terpstra et al. 1980) and in some species of animals (Tagliabue et al. 1994, Ribotta et al. 2000). However, according to the authors' knowledge, until now there were no investigations concerning reactivity of this kind of antigenic preparation with swine serum. The main elements of the profiles exhibited by immunoblotted trivalent and single antigenic preparations with antisera prepared against six pathogenic *Leptospira* serovars were fractions of the 20-26 kDa region and around the 14.5 kDa region. The antigenic zone ranging from 20 to 26 kDa seems to be engaged mainly in serovar or serogroup specific reactions. The antigenic zone around 14.5 kDa was detectable in all antigenic preparations during reactions with all antisera used in the study.

Ribotta et al. (2000) observed a similar immunoblot profile (19-27 kDa region and around 14.4 kDa region) of heat-stable antigenic preparation from the serovar Pomona with homologous rabbit antiserum. In the case of preparations from the serovars Bratislava, Autumnalis, Icterohaemorrhagiae, Hardjo and Grippotyphosa bands around 14.4 kDa were detected (Ribotta et al. 2000). Reactions of immunoblotted heat-stable antigenic preparation from the serovar Pomona with sera from dogs infected with *Leptospira* indicated the presence of serogroup specific antibodies against the 18-25 kDa region and not serogroup specific antibodies against antigens around the 14 kDa region (Ribotta et al. 2000). The slight variation between ranges of molecular weights presented by the region engaged in serovar or serogroup specific reaction (Ribotta et al. 2000) and in the present study can be explained by differences in results of analysis. However the 20-26 kDa region observed in this study seems most likely to correspond to the 19-27 kDa or 18-25 kDa region from Ribotta et al. (2000). Another cause of these divergences may be differences in quantity of proteins. The variation may be also a consequence of the use for the reactions of sera from various species of animals.

Serovar or serogroup specificity of the antigenic zone from 20 to 26 kDa was observed also by Gitton et al. (1992) in reactions of immunoblotted SDS extracts from cells of different *Leptospira* serovars with homologous rabbit antisera. Similar reactions were found by Kelson et al. (1988) with flagellar preparations from serovars Pomona and Hardjo. However, probably because of contamination of these preparations by LPS, the reactions of this antigenic zone with heterologous rabbit antisera could hardly be detected.

The crude nature of the investigated antigenic preparation and technical limitations of method used in the present study did not allow antigenic fractions responsible for serovar or serogroup specific reaction to be more precisely indicated. However, the intensity of the reaction inside the 20-26 kDa region (Fig. 1. lanes P, E; Fig. 2. lanes S, E and Fig. 3. lanes G, P, E) and some data obtained by other, investigators cited below, seems to indicate the role of the circa 21.5 kDa fraction as crucial for the reaction. This seems to concern various mammal species.

Sonrier et al. (2001) investigated phenol, chloroform/methanol/water extracts obtained from serovars Icterohaemorrhagiae and Canicola. Immunoblot of the extracts with antiserum against serovar Icterohaemorrhagiae revealed the 21.5 kDa band and, much more clearly, the 14 kDa band. Extract prepared from serovar Canicola, however, induced protection against Icterohaemorrhagiae challenge (Sonrier et al. 2001).

Cullen et al. (2003) described a surface-exposed outer membrane lipoprotein LipL21, one of abundant, conserved leptospiral outer membrane proteins. LipL21 was found in virulent and culture-attenuated strains of pathogenic serovars and not detected in saprophytic serovars (Cullen et al. 2003). Recombinant LipL21 reacted with sera from hamsters challenged with host- and culture-derived leptospires. LipL22 antigen, designated p22, reacted with sera from hamsters challenged with culture-derived leptospires but did not react with sera from hamsters challenged with host-derived leptospires (Barnett et al. 1999). However, antigen p22 reacted with pooled human serum from leptospirosis patients (Guerreiro et al. 2001). On the basis of two-dimensional electrophoresis results Cullen et al. (2003) suggested that p22 and LipL21 are the same.

The heat stability of the glycolipid antigen (molecular size 23 to 30 kDa), designated as *Leptospira* protective antigen (PAG) was investigated by Masuzawa et al. (1990). PAG was extracted from *L. interrogans* serovar Lai lyophilized whole cells with a chloroform-methanol-water solution. The heat stability of the extract was compared with that of whole-cell (WC) antigen. A band of 23 to 30 kDa visible in untreated PAG and WC shifted to position with a molecular size of ca. 20 kDa after heat treatment of PAG at 80°C for 30 min and WC at 100°C for 30 min. Agglutinating antibody-inducing activities, inhibition activities in ELISA and opsonin-inducing activities of PAG and WC were reduced by heat treatment. The immunogenicity and antigenicity of the PAG present in WC were however more stable than extracted PAG. The authors also concluded that coexistence of other cellular components with PAG might protect and stabilise PAG from the heat treatment (Masuzawa et al. 1990).

Oliveira et al. (2008) investigated immune response to two recombinant proteins of *Leptospira interrogans* serovar Copenhageni, designated MPL17 and MPL21. The proteins were evaluated by ELISA against sera from patients with confirmed early and convalescent phases of leptospirosis. The authors presumed that presenting higher specificity (MPL17) and detecting in ELISA IgM antibodies (MPL21) may constitute together a useful pair of leptospiral antigens.

Lin et al. (2010) characterized B- and T-cell combined epitopes of the outer membrane lipoprotein LipL21 and showed that the epitopes of LipL21 (designated LipL21₉₇₋₁₁₂ and LipL21₁₇₆₋₁₈₄) had similar reactive abilities to specific antibodies contained in sera from rabbits infected by *L. interrogans* and in sera from rabbits immunized by recombinant LipL21.

The weaker reaction of trivalent antigen observed in the present investigation in comparison with single antigens is a phenomenon which also appears in the case of other combined ELISA antigens. However, trials with single serovar antigens performed during elaboration of this ELISA showed insufficient sensitivity of the assay in reactions with heterologous antisera. Therefore, finally combined antigen was used for the test.

The present report describes results of preliminary investigations concerning the reactivity of a crude heat-stable antigenic preparation used in ELISA for detection of antibodies specific for selected serogroups of *Leptospira* in swine serum samples. Further studies are required for more precise identification of the following antigens and epitopes engaged in reaction with sera from swine infected by leptospire. The results of these investigations may create a potential base for elaboration of ELISA with the use of recombinant antigen. They may also be useful for vaccine preparation.

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