EVALUATION OF THE COUNTERIMMUNOELECTROPHORESIS REACTION AS A SERODIAGNOSIS TEST FOR SWINE LEPTOSPIROSIS

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ABSTRACT

The Counterimmunoelectrophoresis reaction (CIE) was tested as a genus specific test for swine leptospirosis diagnosis using three soluble extracts obtained from *Leptospira* sp, serovars pomona, icterohaemorrhagiae and patoc. The extracts were treated with hot Triton X-100 and applied to serum samples of animals divided in three groups: Group 1, 10 swines experimentally infected with the Pomona strain; Group 2, 50 naturally infected swines and Group 3, 10 swines control. These animals were serologically evaluated by CIE and by the Microscopic Agglutination Test (MAT), the WHO reference technique. Groups 1 and 3 were monitored during a period of 93 days after infection (a.i.). Group 1 serum convertion took place around the 10th day a.i. by MAT but ocurred earlier by CIE using any antigen. When CIE was carried out with the homologous antigen to the experimental infection, the results were consistent with MAT, but not when the heterologous antigens were used. Groups 1 and 3 showed distinct results: in Group 3, diferences between results of CIE accomplished with any three antigen extracts were not significant, indicating lack of dependence on the serovar responsible for the outbreak. Although being safe, fast, easy to perform, inexpensive and ideal for analysis of large number of samples, CIE revealed limited genus specificity, which is not convenient for field screening tests. The advantage of CIE is the capability to detect antibodies earlier than MAT.

Key words: Counterimmunoelectrophoresis, swine leptospirosis, seradiagnosis

INTRODUCTION

In swine leptospirosis, abortion is the main clinical evidence. In severe outbreaks, it may be responsible for 100% of birth losses, affecting economically the property (4,6). The current leptospirosis diagnosis is based on a serological reaction using the Microscopic Aglutination Test (MAT) which is the World Health Organization (WHO) reference test (2,3). As MAT takes long time to reveal the humoral antibodies, presents high contamination risks to the operator and is time-consuming for screening of whole herds, some more modern, faster and preferably genera specific methods have been investigated for the detection of leptospiral antibodies.

Based on the wide use of Counterimmunoelectrophoresis (CIE) for the diagnosis of human infectious diseases (1,8,9,10,12,13, 16,17), Terpstra *et al.* (14), Myers (7) and Yasuda (16) used this technique for the diagnosis of human leptospirosis. The advantage mentioned by these authors is that CIE is able to detect acute infection earlier than MAT, employing only one genus specific antigen.

The purpose of the present paper was to evaluate the applicability and sensitivity of a CIE test for use in swine leptospirosis serodiagnosis, compared to MAT.

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MATERIALS AND METHODS

1. Animal Groups
Group 1: Experimentally infected animals

Ten 70-day old pigs, weighing about 160 Kg, clinically healthy and serologically negative by MAT were experimentally inoculated through the intraperitonial route with 4 ml of hamster liver suspension (1% w/v), previously inoculated with the 7th passage of a Pomona strain culture containing 30-40 leptospires/microscopic field (200 x). Serum samples were collected one day before the inoculation and on alternated days between the 3rd and 15th days, every 3 days between the 15th and 39th days and weekly between the 39th and 93rd days after infection (a.i.).

Group 2: Naturally infected animals

Fifty serum samples were collected from swines involved in an outbreak of leptospirosis characterized by weakness, lack of appetite, fever and jaundice 34 females aborted in the last third of gestation, six of them died and seven had stillbirth.

Group 3: Control group

Ten swines with the same characteristics as those in Group 1 were used as a negative control group. They were kept under strict hygiene conditions and proper herd management. The serum samples were collected on the first, 44th and 92nd days of the experiment.

2. Microscopic Agglutination Test (MAT)

MAT was carried out according to the recommendations of WHO (2,15), using the following living *Leptospira* sp serovars as antigens: australis, autumnalis, castelonis, bataviae, butembo, canicola, whitcombi, cynopteri, grippotyphosa, hebdomadis, icterohaemorrhagiae, copenhageni, javanica, panama, pomona, pyrogenes, wolffi, hardjo, shermani, tarassovi, patoc. The strains were grown in EMJH (Difco Laboratories-USA) containing 10% EMJH Enrichment (Difco Laboratories-USA), incubated at 30ºC for 10 days. Serological reaction at a 1:100 dilution or higher was considered positive, when 50% of the leptospires were agglutinated. The titer was the highest dilution where 50% of the leptospires still agglutinated.

3. CIE Reaction

Three *Leptospira* sp serovars were chosen to be used as antigen extract in the CIE test: pomona (Pomona strain), icterohaemorrhagiae (RGA strain) and patoc (Patoc-1 strain). RGA and Patoc-1 strains were selected because they are the most prevalent serovars in swines and can be transmitted to humans, playing an important role in public health. Patoc is the most studied serovar of *L. biflexa* used in several genus specific tests, presenting good agreement with MAT results for human leptospirosis diagnosis. The Pomona, RGA and Patoc-1 strains were grown in EMJH containing 10% EMJH Enrichment

incubated at 30ºC for 10 days. The antigen extract from these strains was obtained as described by Myers (7), with some modifications. Briefly, each strain was grown in 250ml of medium and harvested by centrifugation at 11,000 x g for 15 min. The pellet was treated with 10 ml Triton X-100 (4%) in 0.01 M PBS, pH 7.2 and heated in a water bath for 4 h at 50ºC, with periodic shaking. This suspension was centrifuged at 11,000 x g for 20 min.The supernatant was preserved with sodium azide (1%). The antigens were titrated by doubling dilutions in PBS against a positive swine serum reactive by MAT to pomona (51,200), butembo (6,400), autumnalis (1,600), icterohaemorrhagiae (400), pyrogenes (400), hardjo (200), wolffi (100) and patoc serovars (100). They were also titrated against a MAT negative serum sample, collected from an apparently healthy swine. The working antigen consisted of 1:4 dilution. Glass slides (75 x 50 mm) were covered with 8 ml of 0.9% melted agarose, type II (Sigma-Aldrich Co-USA) in 0.05 M Tris HCl buffer, pH 7.5. After gelling at room temperature, the slides were placed at 4ºC for a few minutes. A template was placed under each slide and three double parallel rows of three wells each (2 mm diameter) were drilled, each pair separated by a distance of 5 mm. The distance between each row was 5 mm. In the first stage, the wells corresponding to the anode were filled with undiluted sera and a constant eletric current (6 mA/slide) was applied to each side of the slide for 30 min, at a potential difference of 10 V, with the same buffer. Connecting filter-paper was placed on both ends of the slide. Subsequently, the cathode side of slide was filled with the antigen, and the run was continued for aditional 90 minutes. After in a wet chamber for 24 hours, the slides were washed with PBS buffer pH 7.2, stained with Starch Schwartzs 10B at 0.5% w/v in acetic acid 1% for 5 min. and destained with acetic acid 5%. The positive sera showed one or more precipitation bands between the cathode and anode wells. In the second stage, the serum samples were diluted in PBS buffer, pH 7.2 in serial two fold up to 1:32 and submitted to the process described above. The end point titer was the highest dilution presenting one or more bands. The antigen and serum specificity reaction were investigated against PBS buffer. Positive and negative control serum samples were included in each run.

RESULTS

Before inoculation, all animals in Group 1 were negative by MAT. After inoculation, none of the animals showed any clinical signs of leptospirosis, but became reactive to pomona serovar (Table 1). The serum convertion took place around the 10th day (sd ± 1) a.i. and the titers ranged from 100 to 6,400 by MAT (X = 1,600, sd ± 1,117.1) reaching the peak on 22.7 days (sd ± 6.9days.). On the 93rd day a.i. 50% of Group 1 swines were negative.

By CIE, no unespecific reaction was observed in the three extract antigens or in negative and positive control serum samples.

Table 1. Microscopic Agglutination Test (MAT) titers using *Leptospira* sp serovar pomona as antigen on serum samples from swines experimentally infected with Pomona strain (Group 1). São Paulo, 2000.

D.A.I.	Animal Identification										X	sd
	1	2	3	4	5	6	7	8	9	10		
0	N	N	N	N	N	N	N	N	N	N	0	0
3	N	N	N	N	N	N	N	N	N	N	0	0
5	N	N	N	N	N	N	N	N	N	N	0	0
7	N	N	N	N	N	N	N	N	N	N	0	0
9	100	100	400	N	N	N	200	N	200	N	100	126.5
11	200	200	400	400	100	400	200	400	1600	1600	550	535.2
13	800	1600	800	400	400	400	200	400	3200	1600	980	878.4
15	1600	1600	800	800	400	400	400	400	3200	1600	1120	854.2
18	1600	1600	3200	800	400	400	400	400	3200	1600	1360	1046.1
21	3200	1600	800	800	400	3200	400	400	3200	1600	1560	1151.7
24	1600	3200	800	800	3200	800	400	400	3200	1600	1600	1117.1
27	1600	800	800	800	400	800	400	100	6400	1600	1370	1737.8
30	1600	800	800	800	800	800	400	N	3200	3200	1240	1050.0
33	800	800	200	800	800	800	400	N	3200	800	860	829.7
36	800	800	100	1600	800	800	400	N	3200	400	890	881.4
39	800	800	100	1600	800	800	200	N	1600	400	710	533.8
44	800	800	100	800	800	400	100	N	1600	400	580	457.8
51	400	800	100	200	200	400	100	N	400	400	300	219.1
58	400	100	100	200	100	200	100	N	200	400	180	124.9
65	400	100	100	200	N	200	N	N	200	200	140	120.0
72	200	100	100	200	N	100	N	N	200	200	110	83.1
79	200	100	N	200	N	100	N	N	200	200	100	89.4
86	200	100	N	200	N	100	N	N	200	100	90	83.1
93	100	100	N	100	N	N	N	N	100	100	50	50.0

D.A.I. = Day after infection; N = negative; X = MAT titers geometric mean, expressed as the reciprocal of the serum dilution; sd = standart deviation.

The results of undiluted serum samples from Group 1, submitted to CIE using Pomona extract as antigen are shown in Table 2. The precipitating antibodies could be detected in 5.1 days (sd ± 1.9 days) a.i.. Around the 7th day a.i., 90% of the samples were positive, while by MAT they were still negative. On the 9th day a.i., positivity of CIE reached 100% and 50% by MAT. Only from the 11th to 13th days a.i., CIE and MAT were equivalent. Positive and negative irregular responses by CIE was observed from the 19.6th days (sd ± 5.9 days) a.i. to the end of the experimental period.

The precipitating antibody titers did not correlate with agglutinating ones. From the 5th to 9th day a.i., the results by CIE with Pomona extract after the sera dilution were similar to those with undiluted sera (Table 3). CIE showed 100% positive reactions from the 9th to 27th day a.i., the same as MAT. The positive result increase was observed during the period when serologic responses by CIE with undiluted sera were irregular, prior to the peak of agglutinating antibodies.

The CIE precipitating antibodies could be first detected by RGA extract in undiluted serum samples of four animals only, but they were irregular until the end of the experimental period. Even after dilution, the results disagreed with those by MAT. Similarly to the RGA extract, the Patoc-1 extract didn't present regular precipitating bands and was also in desagreement with MAT.

Statistic Cochran Q test (12) showed disagreement between MAT and CIE responses with Pomona, RGA and Patoc-1 extracts, with undiluted and diluted Group 1 sera, respectively (Qui Square = 65.85 and 19.5; critic Q Square = 7.82; p ≤ 0.05). Through the correlation of the multiple squares study; the sensitivity increase obtained with the sera dilution was significant (Qui Square = 38.2; critic Qui Square = 3.84; p ≤ 0.05).

Among 50 serum samples of Group 3, 48 (96%) were positive by MAT to *Leptospira* sp, serovars pomona 50% (24/48) and icterohaemorrhagiae 39.6% (19/48). Titers ranged from 100 to 51,200, and five samples were positive to both serovars at the same titer. By CIE, 40/50 samples were positive to the RGA, 33/50 to the Pomona and 39/50 to the Patoc-1 extracts. Only two serum samples were negative by MAT. One of them was positive to RGA and Patoc-1 extracts, but negative to Pomona extract by

Table 2. Counterimmunoelectrophoresis (CIE) results using Pomona strain (pomona serovar) extract as antigen on undiluted serum samples from swines experimentally infected with Pomona strain. São Paulo (Group 1), 2000.

D.A .I.	Animal Identification									
	1	2	3	4	5	6	7	8	9	10
0	N	N	N	N	N	N	+	N	N	N
3	N	N	N	N	N	N	+	N	N	N
5	+	+	+	+	+	N	+	N	N	+
7	+	+	+	+	+	+	+	+	+	N
9	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	N	+	+	+	+
18	N	+	N	+	N	+	+	+	+	N
21	N	+	N	+	+	N	N	+	+	N
24	+	N	+	+	N	+	+	+	+	+
27	+	+	+	+	N	+	+	+	+	+
30	N	N	N	N	N	N	N	N	+	+
33	+	+	N	N	N	N	N	N	N	N
36	+	N	N	N	N	N	+	N	+	+
39	+	N	+	+	N	N	+	N	N	+
44	+	N	N	+	N	+	N	N	N	N
51	+	+	+	+	+	N	+	+	+	+
58	+	+	N	+	N	+	+	+	N	+
65	+	+	N	+	N	N	+	+	+	N
72	+	N	+	N	N	N	+	N	N	+
79	N	N	+	+	N	+	N	N	N	N
86	N	N	+	+	N	+	N	N	N	N
93	N	N	N	+	N	+	N	N	N	+

D.A.I. = Day after infection; N = negative; + = positive.

CIE. The other serum sample was negative to the three extracts. In this group, the Cochran Q test showed agreement among the CIE responses to any three antigen extracts (Qui Square = 5.21; critic Qui Square = 5.99, p ≤ 0.05).

The sensitivity, specificity, agreement, positive and negative false and positive and negative preditive values (5) for Group 1 and Group 3, comparing CIE and MAT results, are presented in Tables 4 and 5.

All the control group serum samples were negative by MAT with all serovars listed. Three animals reacted weakly to Pomona extract on the 44th day by CIE, but all the others were negative to the three antigens during all experimental period.

DISCUSSION

The CIE using homologous antigen to experimental infection showed earlier responses than MAT, but presented irregular positive and negative responses during the experimental period. As these inconsistent and variable responses were observed in the experimental period prior to the peak of agglutinins and at the end of the experiment (93 days), the possible occurrence of prozone phenomenon was raised. As CIE is a semi-quantitative test, this hippotesis can not be proved. But, when the sera were submitted to dilution and titer evaluation, these irregular responses almost disappeared, reaching results equivalent to MAT. As Yasuda (16) had already stated, no correlation between MAT and CIE test titers was observed either. These results were not in agreement with Terpstra (14) who showed ascendent titers in CIE, presenting discrepancy only with weakly reactive sera, even though a limited number of paired samples was evaluated. The statistic analysis of the results obtained by CIE test with Pomona extract in Group 1 diluted serum samples and by MAT with pomona serovar showed significant agreement between them and any

Table 3. Counterimmunoelectrophoresis (CIE) titers using Pomona strain (pomona serovar) extract as antigen on diluted serum samples from swines experimentally infected with Pomona strain (Group1) São Paulo, 2000.

D.A .I.	Animal Identification										X	sd
	1	2	3	4	5	6	7	8	9	10		
0	N	N	N	N	N	N	1	N	N	N	0.1	0.3
3	N	N	N	N	N	N	1	N	N	N	0.1	0.3
5	8	1	4	1	2	N	4	N	N	1	2.1	2.4
7	8	4	8	1	1	4	8	4	4	N	4.2	2.8
9	8	8	2	1	1	4	4	1	8	8	3.0	4.5
11	8	8	4	1	4	2	4	4	2	8	4.5	2.5
13	4	8	8	1	2	2	8	1	2	2	3.8	2.8
15	4	2	2	1	8	2	4	4	8	2	3.7	2.4
18	8	4	8	1	2	1	2	1	8	16	5.1	4.6
21	8	4	16	1	2	2	8	4	8	4	5.7	4.2
24	8	4	8	1	4	2	2	4	8	8	4.9	2.7
27	8	2	4	1	4	1	8	4	16	8	5.6	4.3
30	8	4	2	N	N	N	4	N	8	8	3.4	3.4
33	4	8	8	1	2	2	8	N	4	8	4.5	3.1
36	4	4	4	N	2	2	8	N	8	8	4.0	3.0
39	4	2	4	2	2	2	8	N	8	4	3.6	2.5
44	4	2	4	2	N	2	4	N	8	4	3.0	2.2
51	4	4	4	2	2	N	8	1	4	2	3.1	2.1
58	8	2	8	1	N	2	8	1	4	2	3.6	3.0
65	4	2	8	1	N	N	8	1	4	N	2.8	3.0
72	8	2	8	N	N	2	2	N	4	1	2.7	2.9
79	N	N	8	1	2	2	N	N	N	N	1.3	2.4
86	N	N	4	1	N	2	N	N	N	N	0.7	1.3
93	N	N	N	1	N	1	N	N	N	1	0.3	0.3

D.A.I. = Day after infection; N = negative; X = CIE titers geometric mean, expressed as the reciprocal of the serum dilution; sd = standart deviation.

Table 4. Serologic evaluation by CIE, taking MAT as reference, applied on Group 1 diluted (D) and undiluted (UD) serum samples from swines experimentally infected with Pomona strain. São Paulo, 2000.

	Pomona (%)		Icterohaemorrhagiae (%)		Patoc-1 (%)	
	ND	D	ND	D	ND	D
rS	62.7	87.6	50.3	63.9	41.4	65.1
rE	56.3	54.9	69.0	49.3	69.0	46.5
A	60.8	77.9	55.8	59.6	49.6	59.6
+F	43.7	45.1	31.0	50.7	31.0	53.5
-F	37.3	12.4	49.7	36.1	58.6	34.9
+PV	77.4	82.2	79.4	75.0	76.1	74.3
-PV	38.8	65.0	36.8	36.4	33.1	35.9

rS: relative sensitivity; rE: relative especificity; A: agreement; F+/-: false positive/negative results; PV+/-: positive/negative preditive values.

discrepancy was considered casual. The serum dilution effect over the relative sensitivity was statistically significant, but with the occurrence of some loss of specificity.

When CIE was carried out with the homologous antigen to the experimental infecction, consistent results with MAT were obtained, but not when the two heterologous antigens were used.

In Group 3, the diferences among the results of the CIE accomplished with all three antigen extracts were not significant, indicating lack of dependence on the serovar responsible for the outbreak. As only two serum samples were negative by MAT, the discrepancy between the specificity of two tests could be casual or due to the CIE capacity to detect antibodies earlier than MAT.

Some factors could explain the different results in the two animal groups. The difference between primary and secondary antibody responses and the maturation level of the antibody production cells, which are dependent on the antigen dose the animal was exposed to, are certainly (11).

Although being safe, fast, easy to perform; inexpensive and ideal for analysis of large number of samples (1), CIE revealed limited genus specificity, which is not convenient for field screening tests. Other types of antigen extracts should be tested for this purpose. However, CIE is advantageous because is able to detect antibodies earlier than MAT.

Table 5. Serologic evaluation by CIE, taking MAT as reference, applied on Group 3 serum samples from naturally infected swines . São Paulo, 2000.

Pomona (%)		Icterohaemorrhagie (%)	Patoc-1 (%)
rS	68.8	81.2	79.2
rE	100	50	50
A	70	80	78
+F	0	50	50
-F	31.2	18.8	21
+PV	100	97.5	97.4
-PV	11.8	10	9.1

rS: relative sensitivity; rE: relative especificity; A: agreement; F+/-: false positive/negative results; PV+/-: positive/negative preditive values.

RESUMO

Avaliação da reação de contraimunoeletroforese como teste sorológico para diagnóstico da leptospirose suína

Avaliou-se a reação de contraimunoeletroforese (CIE) como teste gênero-específico para diagnóstico da leptospirose suína, usando-se três extratos solúveis de *Leptospira* sp, sorovares pomona, icterohaemorrhagiae e patoc, obtidos pelo tratamento com Triton X-100 a quente e aplicados a amostras de soro de suínos subdivididos em três grupos: Grupo 1, 10 suínos experimentalmente infectados com estirpe Pomona; Grupo 2, 50 suínos naturalmente infectados e Grupo 3, controle. As amostras de soros foram submetidas à reação de CIE e os resultados comparados aos da Soroaglutinação Microscópica (SAM), técnica de referência pela WHO. Os Grupos 1 e 3 foram monitorados por 93 dias após a inoculação (p.i.). Pela SAM a soroconversão do Grupo 1 ocorreu por volta do 10º dia p.i., enquanto pela CIE, empregando-se qualquer extrato antigênico, foi anterior à SAM. Quando a CIE foi realizada frente a antigeno homólogo à infecção, seus resultados foram equivalentes aos da SAM, não se verificando o mesmo frente aos antígenos heterólogos. Neste aspecto, os Grupos 1 e 3 mostraram comportamento diferente pois não houve diferença significativa entre os resultados da CIE frente aos três antígenos, o que poderia significar serem independentes do sorovar responsável pelo surto ou infectante. Embora a CIE seja segura, rápida, de fácil execução, de baixo custo e ideal para análise em grande escala de amostras, revelou-se de limitada capacidade gênero-específica, o que não é desejavel para testes de triagem de campo; mas poderia ser útil na detecção precoce de resposta sorológica em relação à SAM.

Palavras-chave: Contraimunoeletroforese, leptospirose suína, sorodiagnóstico

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