Isolation and Identification of *Haemophilus parasuis* and Establishment of its Detection Method of Indirect ELISA

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Abstract: To provide the evidence for detection and epidemical investigation of *Haemophilus parasuis* by means of isolation and identification of *Haemophilus parasuis* and study on its detection method of ELISA. From the pathological specimens collected from several scaled-up pig farms, the strain of *Haemophilus parasuis* was isolated identified by means of observation of cultural characteristics and morphology and structure, Biochemical tests, Antibiotic Susceptibility tests, PCR identification and serovar identification. Subsequently, indirect ELISA Method of *Haemophilus parasuis* antibody for detection was studied by utilizing the isolated strain bacteria of treated with ultrasonication as the antigen with preparation of positive serum, optimization of concentration of the coating antigen, concentration of the secondary antibody and reaction time and Susceptibility test, Specificity test and Repeatability test. A strain of *Haemophilus parasuis* serovar 5, named as SCHPS5 which is sensitive to cephalosporin IV, cefotaxime, sulbactam, furaxone, chloromycetin. The detection sensitivity of the target antibody in indirect ELISA Method based on this isolate can reach over 1:10000 and this detection method has high specificity and high repeatability. This study has provided scientific evidence and a reliable antibody detection method for prevention and treatment of the disease resulting from *Haemophilus parasuis*.

Key words: *Haemophilus parasuis*, isolation and identification, indirect ELISA, serum, bacteria

INTRODUCTION

Haemophilus parasuis disease is multiple pig serositis and arthritis caused by *Haemophilus parasuis* whose main symptoms are fever, cough, respiratory distress, maransis, claudication, ataxia and coarse and disorderly hair. Pathological changes in anatomic examination exhibit as pleuritis, pneumonia, pericarditis, peritonitis, arthritis and meningitis (Straw *et al.*, 1998). In addition, *Haemophilus parasuis* may cause hematosepsis and probably lead to sequela, i.e., abortion in female pigs and chronic claudication in male pigs.

This disease was reported by Glasser for the first time in 1910 and so was also called Glasser's disease which exhibits global transmission and has become one of bacterial diseases which influence on the global pig-feeding industry (Oliveira *et al.*, 2001; Oliveria and Pijioan, 2004). In China the pathogen was isolated from some scaled-up pig farms. *Haemophilus parasuis* only infects pigs, those at the age of 2 weeks to 4 months are susceptible and usually those at the age of 5-8 weeks after ablactation are ready to have this disease. The morbidity

is generally 10-15% and if more seriously the mortality can reach 50% (Oliveira, 2003). Invasion of *Haemophilus parasuis* may result in general diseases with high morbidity and mortality. In combined feeding of different droves or introduction of boars, presence of is a thorny problem (Zhou *et al.*, 2009). In 2011 infection of *Haemophilus parasuis* broke out in several scaled-up pig farms in Sichuan and pig death occurred. In this study, routine isolation and identification of the pathological specimens submitted to examine was carried out and the method of indirect ELISA for detection of *Haemophilus parasuis* was established preliminarily by utilizing the isolated strain of as the antigen, by means of optimization of reaction conditions, Susceptibility test, Specificity test and Repeatability test.

MATERIALS AND METHODS

Pathological specimens: The lung, joint fluid, brain tissue and pericardial effusion and serum from pigs with this disease delivered for examination by several pig farm in Sichuan.

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Micro-biochemical test tubes and paper discs for Antibiotic Susceptibility test, bought from Hangzhou Microbiology Reagent Corp., Ltd. newborn cattle serum, Nicotinamide Adenine Dinucleotide (NAD), secondary enzyme-labeled rabbit anti-pig IgG-HRP, bought from Chengdu Kebite Biotech Corp., Ltd. and 96-well ELISA plate, BSA, filtering units, bought from Ya'an Rui-Jin-Te Corporation.

Preparation of media: Chocolate-agar medium, fresh-blood-agar medium, Maconkey medium, basic agar medium, trypsin and soybean agar, trypsin and soybean broth and so on were prepared in accordance with the related standards.

Design of primers: The PCR primers for identification of *Haemophilus parasuis* described by Li *et al.* (2011) were adopted to send to Sangon Biotech (Shanghai) Co., Ltd. for synthesis. The obtained primers were diluted according to the directions and stored for use at -20°C.

Isolation culture of bacteria: In accordance with the methods described by Li *et al.* (2011), the collected pathological specimens were treated; basic agar medium, chocolate agar medium and fresh blood agar medium were prepared and the strain of *Haemophilus parasuis* was isolated. Those clones suspected to be Hps ones were inoculated on the TSA medium to perform purification culture and staining and microscopic examination until all the clones and bacterial morphology were consistent.

Biochemical test: The single clone was inoculated in the Micro-biochemical test tubes containing sucrose, phenylalanine, maltose, sorbol, mannitol, urase, citrate, lysine, ornithine, glucose, gluconic acid, respectively. To each biochemical test tube 1 µL cattle serum and 1 µL NAD (factor V) were added to culture for 24-48 h at 37°C. The results of biochemical reactions were observed and according to the results the suspected clones were taken out to carry out satellite phenomenon visualization.

PCR detection: PCR was undertaken by utilizing the designed primers. The PCR reaction system (50 µL) contained: 2×PreMIX, 25 µL; upstream and downstream primers, 1.5 µL each; pure liquid culture template, 1 µL; sterilized double distilled water, complemented up to 50 µL. Reaction conditions: pre-denaturing at 94°C for 4 min; denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, extending at 72°C for 45 sec, 30 cycles in total; finally extending at 72°C for 10 min. Electrophoresis of the PCR product with 1% agarose was performed to observe, the remaining product was stored at 4°C.

Antibiotic Susceptibility test: At first, 400 µL pure culture in the TSM culturing liquor was pipetted to spread on the TSA plate containing NAD and serum. Then, paper discs for Susceptibility test of 30 common antibiotics including penicillin, streptomycin and so on were stuck horizontally to the TSA plate to culture at 37°C for 24 h and finally the result was observed.

Animal test: Randomly 10 mice were divided into two groups, 5 mice for each and the first group was test group and the second group was control group of normal saline. With sterilized PBS buffer the pure cultured bacteria were washed off from the TSA plate and the obtained culture was diluted to the bacteria concentration of 1.0×10^9 cfu mL^{-1}. The bacteria solution of 0.3 mL was injected intraperitoneally into each mouse and in the control group the same amount of normal saline was injected intraperitoneally. The mice were segregated and fed to observe death phenomenon. Died mice were dissected and tissue bacteria smears were made to conduct Gram staining for microscopic examination. The cardiac blood was collected from died mice to isolate and culture the target bacterium.

Serovar identification: The isolated strain obtained through purification culture was washed off the TSA plate to prepare bacteria suspension in normal saline. Detection was carried out according to the method described in the directions of Standard Serum kit of *Haemophilus parasuis*.

Preparation of serum
Preparation of antigen (culture of the strain): The pure culture strain was picked up to streak on the TSA plate. After culture the single clone was picked up to inoculate in the liquid TSB medium and then culture on a shaker at 37°C for 16 h. Suitable amount of the bacteria solution was taken to inoculate in the fresh TSB medium in a ration of 1:100.

Inactivation and aseptic examination of bacteria: To the total liquid TSB culture 0.4% formaldehyde to inactivate and then the inactivated culture was cultured in an oven at 37°C for 24 h. Finally, the bacteria solution was taken to streak on the TSA plate to culture at 37°C for 24-48 h and then the result was observed.

Emulsification of the antigen: The above bacteria solution was centrifuged at 3000 r min^{-1} for 20 min and the supernatant was discarded. It was washed 3 times with sterilized 0.1 mol L^{-1} PBS (pH 7.4) and the bacteria content was adjusted to 1×10^9 cfu mL^{-1}. The bacteria solution was mixed with complete and incomplete Freund's

adjuvant at the same amount till oil in water emulsion was formed. If the emulsion was not laminated it would be suitable for use.

Immunization procedure: In the first immunization, the mixing emulsion between complete Freund's adjuvant and the bacteria solution was used to immunize 4 piglets at the age of about 2 weeks by means of subcutaneous injection of 3 mL at multiple sites in the neck for each piglet. The second and third immunizations were performed on the 14th day and on the 28th day, respectively by both adopting the mixing emulsion of incomplete Freund's adjuvant and the same method as that in the first immunization. About 1 week after the third immunization, 3 mL living bacteria solution was injected at the auricular vein of the piglets to enhance the immunization. About 1 week later blood sample was selected at the auricular vein. The antibody was detected with agar diffusion test and if notable precipitate was formed blood sample was collected from the heart of the rabbits. The collected sample was stored with the same method as the negative serum.

Preparation of negative serum: The piglets was fed for 3 days to ensure that they were normal. Blood sample was collected at the auricular vein. The collected blood sample was placed in an oven at 37°C for 1 h and then was placed in a refrigerator at 4°C overnight to isolate the serum. The isolated serum was centrifuged at 5000 r min^{-1} for 15 min and the supernatant was pipetted into different centrifuge tubes for storing at -20°C.

Preparation of coating antigen: Firstly the strain was streaked on the TSA plate to culture at 37°C for 24 h. Then, the single clone was picked up to streak again on two TSA plates to culture at 37°C for 16 h. At the same time, the single clone was picked up to inoculate in the liquid TSB medium to culture on a shaker at 200 r min^{-1} at 37°C for 16 h. The bacteria solution in a ration of 1:100 was taken to inoculate in the fresh TSM medium to conduct scaled-up culture for 16 h. Then, the clones on the plate was washed off with sterilized PBS buffer to add into the liquid medium. The bacteria solution was centrifuged at 3000 r min^{-1} for 20 min and the supernatant was discarded; sterilized 0.1 mol L^{-1} PBS buffer (pH 7.4) was added to mix well by shaking, the bacteria solution was centrifuged at 3000 r min^{-1} for 20 min and the supernatant was discarded; the above steps were repeated several times to wash the bacteria solution. Finally, suitable amount of PBS in a volume ratio of 1:10 between bacteria solution and PBS and the supernatant was taken to perform filter with a filtering unit. The filtered antigen solution was detected with a nucleotide-protein

detector to determine the antigen concentration, then merthiolate in the final concentration of 0.1 g L^{-1} was added to the antigen solution for storing at -20°C.

Manipulation of indirect ELISA Method: The ultrosonicated antigen was diluted with the coating liquor at a ratio of 1:100 and 50 µL of the diluted antigen solution was added to each well and the ELISA plate was incubated at 37°C for 2 h and then transferred into a refrigerator at 4°C overnight for antigen-coating. On the next day each well was washed 3 times for 3 min for each with PBS buffer and padded dry; then the blocking buffer containing BSA was added, 50 µL per well and the plate was incubated at 37°C for 1 h and then taken out for padding dry. The serum diluted with PBS buffer in a ratio of 1:60, 50 µL per well and meanwhile the negative and positive serum control wells were set up. The plate was incubated at 37°C for 1 h and then taken out for padding dry. The rabbit anti-pig IgG-HRP was diluted in a ratio of 1:5000 and the diluted solution was added, 50 µL per well. The plate was incubated at 37°C for 1 h and then taken out for padding dry. The substrate color development solution of OPD-H_2O_2 prepared newly was added and the color was developed in the dark at 37°C for 15 min. The stop solution of 2 mol L^{-1} H_2SO_4 was added, 50 µL per well and then the $D_{490\ mm}$ value was measured in a ELISA reader, the P/N value was calculated and P/N$\geq$2 was judged as positive reaction.

Determination of ELISA reaction conditions
Determination of optimal antigen-coating concentration and optimal serum dilution ratio: Optimal antigen-coating concentration and optimal serum dilution ratio were determined with Matrix Titration Method. The ultrsonicated antigen was diluted in ratios of 1:50, 1:100, 1:200, 1:400 and 1:800, respectively and added to the wells in line A-E of the 96 well ELISA plate.

The negative serum and the positive serum were diluted in ratios of 1:40, 1:80, 1:160, 1:320 and 1:640 and added to the wells in row 1-5 and row 7-11. The manipulation of indirect ELISA was repeated twice and the average P/N values were calculated. The antigen concentration and the serum dilution ratio corresponding to the maximum P/N value were determined as the optimal ones.

Determination of optimal dilution ratio of the secondary enzyme-labeled antibody: The secondary antibody IgG was diluted in ratios of 1:2000, 1:3000, 1:4000, 1:5000, 1:6000, respectively. The P/N values were calculated in accordance with ELISA manipulation procedure and the dilution ratio corresponding to the maximum P/N value was determined as the optimal one.

Determination of optimal coating time: The antigen was diluted in a ratio of 1:100 for coating. The test contains 4 groups: at 37°C for 1 h and then at 4°C overnight; at 37°C for 2 h and then at 4°C overnight; at 37°C for 2 h and t at 4°C overnight. The known negative serum and the known positive serum were used to carry out indirect ELISA manipulation procedure in two groups for several times and the D_{490mm} values were measured.

Determination of blocking conditions: Under the determined conditions and with the determined manipulation, PBST containing 1 mL L^{-1} BSA and the one containing 5 mL L^{-1} degreased milk were used, respectively as the blocking solution were added 100 μL/per well. Blocking was performed at 37°C for 30 min, 1 h, 1.5 h, respectively. The known negative serum and the known positive serum were used to carry out indirect ELISA manipulation procedure in two groups for several times and the D_{490mm} values were measured to determine the optimal blocking conditions.

Determination of optimal antigen-antibody action time: The D_{490mm} values were measured under the conditions of antigen-antibody action time of 30, 60 and 90 min, respectively. For every time point 2 wells were used to perform indirect ELISA manipulation and the average was calculated. The binding time for which the D_{490mm} value of the positive serum approaches to 1 and the P/N value reach the maximum was determined as the optimal antigen-antibody action time.

Determination of secondary enzyme-labeled antibody action time: The secondary enzyme-labeled antibody IgG was diluted in a ratio of 1:5000 and under action time of 30, 60 and 90 min at 37°C, for every time point 2 wells were used to perform indirect ELISA manipulation and the average was calculated. The binding time, for which the D_{490mm} value of the positive serum approaches to 1 and the P/N value reaches the maximum was determined as the optimal secondary enzyme-labeled antibody action time.

Determination of optimal substrate color-developing time: Under the above optimized conditions ELISA test was performed. After adding the substrate the reaction acts in the dark at 37°C for 10, 15, 20 and 30 min, respectively and then was stopped. For every time point 2 wells were used to perform indirect ELISA manipulation and the average was calculated. The binding time, for which the D_{490mm} value of the positive serum approaches to 1 and the P/N value reaches the maximum was determined as the optimal substrate color-developing time.

Determination of negative and positive reaction standards: About 20 serum samples detected to be negative with Clinical Indirect Coagulation test were detected with indirect ELISA. For each serum sample 2 wells were used and the average of 20 serum samples was calculated. Then, the average $\bar{X}$ of S/N and the Standard Deviation (SD) were calculated. The calculation formula to determine the threshold between negative and positive reactions was $\bar{X}+2SD$.

Sensitivity test: The optimal coating antigen was selected, based on the optimized conditions in determination of optimal antigen-coating concentration and optimal serum dilution ratio and then the serum was diluted in ratios of 1:10, 1:100, 1:1000, 1:10000 and 1:100000 to determine the ELISA Method sensitivity.

Specificity test: The positive serum samples of Swine pasteurellosis, *Haemophilus parasuis* serovar 4, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* serovar 5 prepared and stored by the laboratory were used as the control to perform indirect ELISA detection.

Repeatability test: About 10 positive serum samples were taken to measure the D_{490mm} values at different time under the same conditions thus manipulation was repeated three times.

Clinical application: With the established indirect ELISA Method clinical pig serum samples to be detected were detected and the D_{490mm} values were measured.

RESULTS AND DISCUSSION

Isolation culture of bacteria: The bacteria did not grow on the basic agar plate or the Maconey medium did not grow well on the fresh blood agar plate and the chocolate plate and grew well on the TSA plate and NAD fresh blood agar plate.

After culturing for 24 h on the TSA plate, the obtained clones exhibited pinpoint-shaped round, gray white, semitransparent and 0.5-1 mm in diameter. With microscopic examination of Gram-stained sample the bacteria was determined to be Gram negative. Morphology of the bacteria was multiple and exhibited filament, short rod and even ball in shape.

Result of Biochemical test: The Biochemical test result of the isolated strain was shown in Table 1. According the Biochemical test results, the suspected clones were picked up to inoculated on the fresh blood agar plate and

hence, the obvious satellite phenomenon occurred. Near staphylococcus lawn the clones to be tested grew well but far from it none of the clone grew.

PCR detection: By PCR amplification with purified isolated bacteria as the template, through 10 g L^{-1} agarose gel electrophoresis it was found in the gel imaging system that there existed a specific band in size of about 791 bp, the specific PCR product was consistent with the expected length of the amplified fragment (Fig. 1).

Antibiotic Susceptibility test: Referring to the antimicrobial drug susceptibility test standard of WS/T 125-1999 Paper Disc method, the test was carried out in Clinical Trial Standard Institute (CLSI) and the detailed results were shown in Table 2. The results revealed that the isolated strain was tolerable to only gentamycin, enrofloxacin, compound sulfamethoxazole and penicilin and highly susceptible to cephalosporin IV, cefotaxime, sulbactam, furaxone and chloromycetin.

Animal test: The mice in the challenge group were all dead while in the control group the mice were all normal. The blood sample from the heart of the mice was taken to streak on the TSA plate for culturing, the same strain was isolated which suggested that this bacteria may have strong virulence to mice. The PCR-amplified product fragment was consistent with that from the isolated strain.
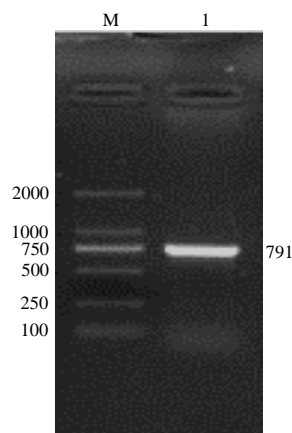

Fig. 1: PCR results from isolates M: Maker DL 2000; 1: Isolates

Table 1: Result of Biochemical test

Pilot projects	Results	Pilot projects	Results
Sucrose	+	Lactose	-
Xylose	-	Indole	-
Ornithine	+	Glucose	+
Catalase	+	Mannitol	-
Maltose	+	VP	-
H$_2$S	+	Indole	-
Nitrate reduction	+	-	-

+ represents the positive result; - represents the negative result

Identification of serovar: According to the manipulation described in 2.6 serovar of the isolated strain was identified. The result showed that isolated strain has stronger coagulation reaction with the positive serum of Hps serovar 5, in which white laminar coagulate was formed but no coagulation reaction with other standard positive serum samples and normal saline.

Determination of optimal ELISA reaction conditions
Determination of optimal antigen-coating concentration and optimal serum dilution ratio: The results of the Matrix Titration Method showed when the ultrasonicated antigen was diluted in a ratio of 1:100 and the serum was diluted in a ratio of 1:160, the P/N value was the maximum. The detailed results were shown in Table 3.

Determination of optimal dilution ratio of the secondary enzyme-labeled antibody: The test results of different

Table 2: Result of Drug Sensitive test

Drugs	Inhibit ory circle (mm)	Sensibility
Gentamicin	04	Resistance
Cephalexin	24	Hypersensitive
Bactrim	08	Resistance
Cefotaxime	34	Hypersensitive
Kanamycin	18	Mid-range
Sulbactam	26	Hypersensitive
Furaxone	20	Hypersensitive
Chloramphenicol	30	Hypersensitive
Enrofloxacin base	02	Resistance
Florfenicol	26	Hypersensitive
Amikacin sulphate	15	Mid-range
Cefradine	22	Hypersensitive
Cefazolin	20	Hypersensitive
Fortum	20	Hypersensitive
Tetracycline	14	Mid-range
Polymyxin b	14	Mid-range
Rocephin	23	Hypersensitive
Cefalotin	35	Hypersensitive
Penicillin	07	Resistance
Streptomycin	10	Mid-range

Table 3: Determination of the optimal coating antigen concentration and the best dilution of serum

Serum dilution	Antigen dilution					
	1:50	1:100	1:200	1:400	1:800	Results
1:40	0.821	0.943	0.915	0.875	0.867	Positive
	0.214	0.207	0.215	0.206	0.203	Negative
	3.836	4.556	4.256	4.248	4.271	P/N
1:80	0.913	0.935	0.924	0.926	0.890	Positive
	0.217	0.215	0.212	0.222	0.185	Negative
	4.207	4.349	4.358	4.171	4.811	P/N
1:160	1.038	1.219	1.123	1.084	1.070	Positive
	0.172	0.170	0.182	0.179	0.168	Negative
	6.035	7.170	6.170	6.056	6.369	P/N
1:320	1.010	0.992	0.956	0.897	0.828	Positive
	0.165	0.171	0.169	0.163	0.161	Negative
	6.121	5.801	5.657	5.503	5.143	P/N
1:640	0.895	0.979	0.948	0.89	0.801	Positive
	0.163	0.173	0.172	0.167	0.168	Negative
	5.491	5.659	5.512	5.329	4.768	P/N

dilution ratio of the rabbit anti-pig IgG were shown in Table 4. It could be seen that when the dilution ratio of the secondary antibody was 1:5000 the P/N value reached the maxium.

Determination of optimal coating time: With indirect ELISA the D_{490mm} values were measured at 37°C for 1 h and then at 4°C overnight, at 37°C for 2 h and then at 4°C overnight, at 37°C for 2 h, at 4°C overnight, respectively. Since, the D_{490mm} value 0.945 was the nearest to 1 and the maximum of the P/N values was 6.217, the optimal coating time was the condition, at 37°C for 2 h and then at 4°C overnight.

Determination of optimal blocking condition: The detection results indicated, when PBST containing 1 mL L^{-1} BSA was used to block for 1 h, the value of the positive serum was the minimum and therefore the optimal blocking time was 1 h.

Determination of optimal antigen-antibody action time: Because when the antigen-antibody action time was 60 min, the D_{490mm} value was 0.821 which is nearest to 1 and the P/N value was the maximum, 5.906, 60 min was determined to be the optimal antigen-antibody action time.

Determination of secondary enzyme-labeled antibody action time: When the secondary enzyme-labeled antibody action time was 60 min, the D_{490mm} value was 0.847 which is nearest to 1 and the P/N value was the maximum, 6.274 and therefore 60 min was determined to be the optimal secondary enzyme-labeled antibody action action time.

Determination of optimal substrate color-development time: When at 37°C in the dark the reaction lasted 15 min, the D_{490mm} value was 0.892 which is nearest to 1 and the P/N value was the maximum, 6.110 and therefore 15 min was determined to be the optimal substrate color-development time.

Determination of negative and positive reaction standards: After 20 serum samples detected to be negative with Clinical Indirect Coagulation test were detected with indirect ELISA, the measured D_{490mm} values were divided by the S/N values (Table 5) of the standard negative serum samples. By calculating according to the equation:

$$SD = \sqrt{\frac{\Sigma\left(X_n - \overline{X}\right)^2}{n-1}}$$

$\overline{X}$ of 20 serum samples was 1.73, the SD was 0.171 and hence $\overline{X}$+2SD to determine the threshold between the negative reaction and the positive one was 2.07. Therefore when S/N≥2.07 for the serum to be detected, the reaction was judged to be positive.

Sensitivity test: When the coating antigen was diluted in a ratio of 1:100 and the serum was diluted in ratios of 1:10. 1:100, 1:1000, 1:10000 and 1:100000, the P/N values were determined. The results were shown in Table 6. From Table 6 it could be seen that this method had high sensitivity.

Specificity test: Taking the negative serum as the control, the positive serum samples from Swine pasteurellosis, *Haemophilus parasuis* Serovar 4, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* Serovar 5 were detected with the established indirect ELISA. The results were shown in Table 7.

From Table 7 except for the D_{490mm} values of the serum samples of *Haemophilus parasuis* Serovar 4 and 5 were greater than the threshold and the P/N value was the biggest, those of other bacteria serum samples were all smaller than the threshold and these serum samples may be judged to be negative. This revealed that this indirect ELISA Method had high specificity.

Table 5: Result of Negative Serum test by the indirect ELISA

Serum No.	S/N	Serum No.	S/N
1	1.99	11	1.65
2	2.21	12	1.53
3	1.63	13	1.57
4	1.71	14	1.82
5	1.73	15	1.66
6	1.87	16	1.57
7	1.89	17	1.61
8	1.77	18	1.61
9	1.59	19	1.87
10	1.55	20	1.75

Table 6: The result of Sensitivity test for indirect-ELISA

Antigen	Serum				
1:100	1:10	1:100	1:1000	1:10000	1:100000
Positive	1.200	1.100	0.800	0.750	0.500
Negative	0.173	0.182	0.193	0.179	0.168
P/N	6.936	6.064	4.145	4.190	2.976

Table 7: The result of Pecificity test for indirect-ELISA

Serotypes	Pasteurella	*E. coli*	HPS4	APP	HPS5	Negative
$D_{490\,mm}$	0.243	0.217	1.216	0.293	1.291	0.165
P/N	1.473	1.315	7.370	1.776	7.824	1.000
Result	-	-	+	-	+	-

Table 4: Determination of the optimal dilution of peroxidase

HRP secondary antibody the optimal dilution	1:2000	1:3000	1:4000	1:5000	1:6000
Positive serum	0.992	1.088	1.139	1.185	1.129
Negative serum	0.214	0.203	0.186	0.172	0.169
P/N	4.636	5.360	6.124	6.890	6.680

Table 8: The reproducibility of ELISA in detection of serum samples

Detect the serial number	Sample No.									
	1	2	3	4	5	6	7	8	9	10
First	0.913	0.909	0.886	0.929	0.893	0.898	0.907	0.931	0.921	0.917
Second	0.892	0.915	0.897	0.868	0.925	0.848	0.874	0.929	0.917	0.911
Third	0.835	0.879	0.883	0.919	0.851	0.852	0.929	0.905	0.902	0.903
Result	+	+	+	+	+	+	+	+	+	+

Repeatability test: The D_{490mm} values (Table 8) of 10 positive serum samples at different time had small change range and this indicated that this method had good repeatability.

Results of clinical test: Two batches of serum samples to be detected in total quantity of 120 were detected with the indirect ELISA Method established in this study. The results showed that 17 serum samples were positive and the detection rate was 22.5%.

Haemophilus parasuis is a bacterium which is most hard to culture and usually is very difficult to isolate the pure culture of those samples of animals with the disease and especially after those pigs with the disease are treated with antibiotics isolation of this bacterium becomes even more complicated. The requirements for growth environment of *Haemophilus parasuis* are most strict and its growth depends on exogenous NAD (factor V) (Kielstein *et al.*, 2001). Ideal samples of *Haemophilus parasuis* for isolation should be collected from those pigs with acute infection which manifest the characteristic symptoms of this disease and are not treated with antibiotics. Therefore, isolation and identification of this pathogen has big difficulty while is critical in prevention and treatment of the disease caused by it because only if the pathogen is isolated further detection work such as antibiotic susceptibility test or serovar classification which has great benefit for control of the disease can be carried out. According to the results of clone morphology, biochemical characteristics and satellite phenomenon, the isolated strain in this study can be judged preliminarily as *Haemophilus parasuis*; with PCR amplification, the target DNA fragment in the same length as expected has been amplified successfully and this proves that the isolates strain is just HPS. However, the results of Antibiotic Susceptibility test have indicate that this isolates strain is tolerable to gentamycin, enrofloxacin, compound sulfamethoxazole and penicilin and highly susceptible to cephalosporin IV, cefotaxime, sulbactam, furaxone, chloromycetin which has certain difference from the related reports home and abroad. This may be associated with difference in the susceptibility of the HPS strains of different serovars from different regions to the same antibiotic. Meanwhile, the same antibiotic has different susceptibility to different strains. And hence, clinical administration of antibiotics should be performed under the instruction of the results of related Antibiotic Susceptibility tests. It should be avoided that improper antibiotic administration leads to development of the antibiotic-resistant strain. For infection of *Haemophilus parasuis* in different pig farms, a set of immunization procedure with strong purpose should be formulated and the susceptible drug should be selected to be used for treatment. In Serovar Identification test, the isolated strain has reaction with the HPS Serovar 5 standard serum which produce white laminar coagulate but had no reaction with other serum samples and normal saline which reveals that the this strain belongs to Serovar 5. Some related serum epidemic investigations carried out in other countries have reported the Serovar 4, 5 and 13 were most epidemical in German, the USA, Canada, Japan and Spain and >20% of the isolated strains can not be determined (Cai *et al.*, 2005). From this researchers can see, epidemical law of the serovars of *Haemophilus parasuis* in Sichuan is basically similar to that in other countries but has certain difference.

Among Serological Detection Methods, the indirect ELISA Method is very sensitive, specific and more accurate, more sensitive and more objective than the indirect coagulation test (Oliveira, 2003; Yin *et al.*, 2004). Referring to the method reported by Xu *et al.* (2010) and Morozumi and Nicolet (1986), the bacteria solution with concentration of inactivated *Haemophilus parasuis*, 1×10^9 cfu mL$^-$ was adopted as the immunogen and enhancing immunization after the third immunization had stronger effect. Selection of suitable coating antigen is the critical step for establishment of indirect ELISA Method. Shi *et al.* (2007) and Wang *et al.* (2006), selected capsule polysaccharide and complete bacterium as the coating antigen, respectively to establish the indirect ELISA Methods which had ideal test results and could be used for diagnosis and epidemic investigation. Feng *et al.* (2009) selected the supernatant of the ultraosonicated bacteria solution as the immunogen which contains a great amount of soluble protein and polysaccharide antigen of the bacteria which often are used as the antigen for Serological Diagnosis Methods.

CONCLUSION

This study, based on the supernatant of the ultrosonicated bacteria solution, the coating antigen was prepared by filtering with filtering unit, a specific and sensitive indirect ELISA Method was established successfully which provided scientific evidence and a reliable antibody detection way for prevention and treatment of the *Haemophilus parasuis* disease in this region. Due to less impurity in the filtered supernatant, soluble protein and polysaccharide antigen are purer and interference for antigen coating reduces much and hence the coating antigen has characters of easy preparation, simple quality control, broad spectrum and high sensitivity. In this study researchers used the established Indirect ElISA Method to detect 120 serum samples to be detected and the positive detection rate was 22.5% which is almost consistent with that reported by Zhou *et al.* (2009) which suggests that epidemic situation of HPS in Sichuan should be concerned more.

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