Journal of Animal and Veterinary Advances 10 (20): 2669-2674, 2011
ISSN: 1680-5593

The Research of KRG Serotyping and ERIC-PCR and PCR-RFLP Genotyping of *Haemophilus parasuis* Isolates from Anhui Province of China

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Abstract: To investigate the characteristics of serology and molecular epidemiology of *Haemophilus parasuis* in Anhui area, this research adopts KRG serotyping and ERIC-PCR and PCR-RFLP genotyping to conduct systematic analyses on 18 HPS isolates from Anhui. The result shows that 55.6% bacterial strains can be serotyped where the most prevalent serotype is type 4 (27.78%) followed by type 14 (16.67%) and type 13 (11.11%). About 44.4% bacterial strains cannot be serotyped. With ERIC-PCR analyses, 18 HPS isolates contain 4-9 bands with the size ranging from 100-2000 bp and they belong to 11 different genotypes where IV is the dominant genotype (27.78%) followed by type 2 (16.67%) and type 1 (11.11%). With PCR-RFLP analyses, 18 HPS isolates lie in 6 different genotypes where the most prevalent genotype in Anhui area is DBP (44.4%) followed by BAE (22.22%) and BBB (16.67%). The result revels that HPS prevails in pigs in Anhui area and both its serotypes and genotypes are in polymorphism distribution. The result also shows that KRG serotypes, ERIC-PCR genotypes and PCR-RFLP genotypes of HPS are not significantly associated with each other but ERIC-PCR genotype is associated with the sources of bacterial strains with the same serotype. Compared with PCR-RFLP, ERIC-PCR better uncovers the genetic relationships of bacterial strains and hence reveals the genetic distance among different bacterial strains which can be especially applied to the inter-pig and inter-farm epidemic conditions of pathogenium.

Key words: *Haemophilus parasuis*, serotyping, ERIC-PCR, PCR-RFLP, genotyping, China

INTRODUCTION

Haemophilus parasuis (HPS) is a type of commensal bacteria living in the upper respiratory tract of swine. It can attack pig bodies under specific conditions and cause systemic deseases characterized by fibrinous polyserositis, arthritis and meningitis which are all generally categorized as glasser's disease of swine. As the modern pigeries develop rapidly and intensively, HPS is gradually becoming one of the pathogenic bacteria that bothers swine industry (Nedbalcova *et al*., 2006).

So far, KRG (Kieletein-Rapp-Gabriedson) serotyping divides HPS into 15 serotypes based on the variance of bacterial surface antigen (Kielstein and Rapp-Gabrielson, 1992) but 26.2-41% isolates still cannot be serotyped (Kielstein and Rapp-Gabrielson, 1992;Turni and Blackall, 2005). Moreover, a disadvantage of serological typing is that different bacterial might have the same antigen and hence cause cross reactions. In recent years, genotyping such as ERIC-PC and PCR-RFLP has become an important approach to molecular epidemiology research on microbes under the development of molecular biology.

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence is a non-coding, conservative and repetitive sequence found in enteric bacterial genome with length of 126 bp. Both of its position in chromosome and the number of its duplicates have species specialty and hence can be applied to the research and analyses on genome (Hulton *et al*., 1991; Versalovic *et al*., 1991). ERIC-PCR is actually a random PCR and can obtain stable and highly repetitive DNA finger-print. ERIC-PCR was first used on HPS genotyping (Rafiee *et al*., 2000) in 2000 and was later applied to the inter-pig and inter-farm epidemic conditions of HPS (Oliveira *et al*., 2003). Restriction Fragment Length Polymorphism (RFLP) is based on restriction endonuclease in different individual genome where the base of enzyme restriction site has mutations or the insertion or loss of base happens between enzyme restriction sites that causes the change on the size of enzyme restriction sites. This change can be detected by specific hybridization probe and hence can be used to compare the variance of DNA in different individuals (Li *et al*., 2999). PCR-RFLP uses restriction endonuclease to digest PCR products and then reveals the specific gene polymorphism based on the changes of electrophoresis patterns. In 2003, PCR-RFLP on Ttransferrin-binding protein A (*TbpA*) gene as a specific gene was used on HPS genotyping (De la Puente Redondo *et al*., 2003). This research uses KRG serological typing and ERIC-PCR and PCR-RFLP

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genotyping to analyze 18 HPS Anhui isolates and hence, lays a foundation for HPS seroepidemiology and molecular epidemiology in Anhui province and provides scientific evidence for the prevention and treatment of Glasser's disease.

MATERIALS AND METHODS

Bacterial strains: About 18 HPS strains were isolated from infected pigs (symptom as high fever and dyspnea) at 12 regions (or farms) in Anhui by Animal Infectious Diseases Laboratory at Anhui Agricultural University from 2008-2010. The strains were biochemically identified and confirmed by 16S rRNA PCR and preserved. For relevant information of the isolates (Table 1).

Main reagents: PCR MasterMix and agarose gel DNA recollection kit purchased from Tiangen Biotech (Beijing) Co. Ltd., Ava I, Afa I, Taq I restriction endonuclease purchased from Takara Biotechnology (Dalian) Co. Ltd., Trypticase Soy-yeast Extract Agar (TSA-YE) and Trypticase Soy-yeast Extract Broth (TSB-YE) purchased from Hangzhou Microbial Reagent Co., Ltd., Nicotinamide Adenine Dinucleotide (NAD) purchased from Sangon Biotech (Shanghai) Co. Ltd., new born Calf serum purchased from Beijing Solarbio and Science Technology Co. Ltd. and 15 standard positive serum purchased from Wuhan Keqian Animal Biological Products Co. Ltd.

Main instruments: Super clean bench, incubator, thermostatic water bath, high speed centrifuge, gradient PCR, electrophoresis apparatus trophoresis and Peiqing TS-680D Gel documentation and analysis system, etc.

HPS heat-stable antigen preparation: Study bacterial strains were inoculated into TSA-YE plate (containing 5% new-born Calf serum and 0.01% NAD) and then cultured for 20 h at 37°C then colony on plate was flushed by 0.15 M NaCl to form 0.1 g mL^{-1} cell broth. The broth was then autoclaved for 2 h at 121°C and centrifuged in 12,000 r min^{-1} for 10 min. The supernatant was taken as the antigen (Morozumi and Nicolet, 1986).

HPS serological typing: About 1% Agarose plate was made in thickness of 4 mm. The plate was punched regularly (in diameter of 3 mm and distance between adjacent holes to be 4 mm) and was injected with samples after base finishing. Different antigen physiological saline was added into adjacent holes as comparisons. A model of standard positive serum was put into the middle hole and then the plate was put into wet box at 37°C. The plate was observed every 24 h for 3 consecutive days. A positive result would be marked if a clear white precipitation line appeared between antigen and antibody. Otherwise, a negative result would be labeled (Kielstein and Rapp-Gabrielson, 1992).

ERIC-PCR genotyping of HPS

DNA template preparation: HPS was inoculated into TSB-YE broth (containing 5% new-born Calf serum and 0.01% NAD) and was then shaken and cultured for 18 h at 37°C. Then 1 mL was taken and put into 1.5 mL centrifuge tube and was then centrifuged in 12,000 r min^{-1} for 5 min. The broth was precipitated by heavy suspended bacteria and was then boiled for 10 min and centrifuged in 12,000 r min^{-1} for 5 min. Supernatant was taken as the template.

Table 1: List of the KRG Serotyping, ERIC-PCR and PCR-RFLP genotypes and related information for the 18 *H. parasuis* strains

Strain no.	Strain code	Isolate source	Isolate time	Major clinical manifestations	Serotype	Genotype of ERIC-PCR	Genotype of RFLP-PCR
1	LJ3	Lujiang	2008/11	pleuritis	13	II	BBB
2	LJ2	Lujiang	2008/05	pleuritis	14	II	BBB
3	XC	Xuancheng	2008/09	pleuritis	14	V	BBE
4	FD	Feidong	2010/11	hydropericardium	NT	XI	DBP
5	LJ1	Lujiang	2008/05	pleuritis	NT	I	BAE
6	BZ	Bozhou	2009/03	pleuritis	4	VI	DBP
7	HS	Huangshan	2008/11	pleuritis	4	X	EBE
8	FX5	Feixi	2010/11	pleuritis	4	IV	DBP
9	FY	Fuyang	2008/11	hydropericardium, lung rou yang variable	13	II	BBB
10	FX3	Feixi	2009/08	pleuritis	NT	IV	DBP
11	FX4	Feixi	2009/09	pleuritis	NT	IV	DBP
12	FX2	Feixi	2008/02	pleuritis	4	IV	DBP
13	SZ	Suzhou	2008/09	pleuritis	14	VIII	BAE
14	SC	Shucheng	2008/04	Myocardial bleeding, Pulmonary hemorrhage	NT	I	BAE
15	QM	Qimen	2009/11	pleuritis	NT	VII	DBP
16	TC	Tongcheng	2008/04	pleuritis	NT	IX	BAE
17	GD	Guangde	2010/07	pleuritis	NT	III	EAI
18	FX1	Feixi	2008/01	pleuritis	4	IV	DBP

NT: Non-typeable

Primers: ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Rafiee *et al*., 2000). They were compounded by Sangon Biotech (Shanghai) Co. Ltd.

ERIC-PCR amplification system: The reaction had total volume of 50 μL which contained 25 μL PCR MasterMix, 1 μL forward primer (10 pmol L^{-1}), 1 μL reverse primer (10 pmol L^{-1}) and 5 μL DNA template and then was added into sterilization double steamed water to 50 μL. The reaction procedure was as follows; 5 min preheating at 95°C followed by 1 min at 95°C, 1 min at 50°C and 3 min at 72°C with 35 such cycles. Then the procedure was extended for 10 min at 72°C. About 10 μL PCR products were taken for electrophoresis with 1% agarose gel in 100 V for 80 min and were then analyzed by gel imaging system.

Electrophoresis pattern comparison and data analyses: The number and their relevant positions of segments of electrophoresis pattern within the same analysis range were analyzed. The dendrogram analysis was based on the selection of bands. ERIC-PCR products were collected by 0 or 1, i.e., on the same shift position (the same molecular weight segment), 1 if there was an amplification band and 0 otherwise. The result was processed by Cross-check software to form matrix of binary sequences. The data was input into NTSYS-pc 2.10 software for dendrogram analysis using Unweighted pair group method using averages algorithm (UPMGA) to draw heredity relationship pictures. Each strain was characterized as a taxonomy unit (OUT) and strains with similarities ≥90% were characterized as the same-origin strains (Oliveira *et al*., 2003).

PCR-RFLP genotyping of HPS

DNA template preparation: HPS was inoculated into TSB-YE broth (containing 5% new-born Calf serum and 0.01% NAD) and was then shaken and cultured for 18 h at 37°C. Then 1 mL was taken and put into 1.5 mL centrifuge tube and was then centrifuged in 12,000 r min^{-1} for 5 min. The broth was precipitated by heavy suspended bacteria and was then boiled for 10 min and centrifuged in 12,000 r min^{-1} for 5 min. Supernatant was taken as the template.

Primers: TbpA1 (5'-TTAGCCTTGCTCTTCTTAGCC-3') and TbpA2 (5'-GCTCTTGGAAACTTGGCACTCTAA-3') (De la Puente Redondo *et al*., 2003). They were compounded by Sangon Biotech (Shanghai) Co. Ltd. The size of amplification segment is 1905 bp.

TbpA PCR amplification and purification: The reaction had total volume of 25 μL which contained 12.5 μL PCR MasterMix, 1 μL forward primer (10 pmol L^{-1}), 1 μL reverse primer (10 pmol L^{-1}) and 5 μL DNA template and then was added into sterilization double steamed water to 50 μL. The reaction procedure was as follows; 5 min at 95°C followed by 1 min at 94°C, 1 min at 50°C and 2 min at 72°C with 35 such cycles. Then the procedure was extended for 10 min at 72°C. About 10 μL PCR products were taken for electrophoresis with 1% agarose gel in 100 voltages for 80 min and were then analyzed by gel imaging system. PCR products were recollected by agarose gel DNA recollection kit for enzyme cutting.

RFLP analyses: Three restriction Endonuclease reaction systems are; Afa I: Endonuclease 1 μL, 10× buffer 2 μL, 0.1% Bovine Serum Albumin (BSA) 2 μL, PCR products 15 μL. The total reaction volume was 20 μL. About 3-4 h at 37°C. Taq I: Endonuclease 1 μL, 10× buffer 2 μL, 0.1% BSA 2 μL, PCR products 15 μL. The total reaction volume was 20 μL. About 3-4 h at 65°C. Ava I: Endonuclease 1 μL, 10× buffer 2 μL, PCR products 17 μL. The total reaction volume was 20 μL.

About 3-4 h at 37°C. DNA segment pattern distribution of enzyme cutting products was observed with 1% agarose gel, analyzed and named in RFLP pattern based on methods built (De la Puente Redondo *et al*., 2003). The same macrorestriction map was given the same name. The macrorestriction map that could not be found in literature would be renamed.

RESULTS AND DISCUSSION

HPS serological typing: About 10 (55.6%) of 18 HPS Anhui isolates could be serotyped. Of the 10 strains, 5 are serotype 4 (27.78%) involving 3 regions (or farms), 3 are serotype 14 (16.67%) involving 3 regions (or farms) and 2 are serotype 13 (11.11%) involving 2 regions (or farms). Serotype 4 is the most prevalent one followed by type 14 and 13 in polymorphism distribution. The other 8 strains (44.4%) could not be identified (Table 1).

HPS ERIC-PCR genotyping: About 18 HPS Anhui isolates produced DNA finger-print with clear bands after ERIC-PCR analyses and amplification (Fig. 1). The repeated experiments validate the repeatability of the finger-print. The collected DNA finger-print contains

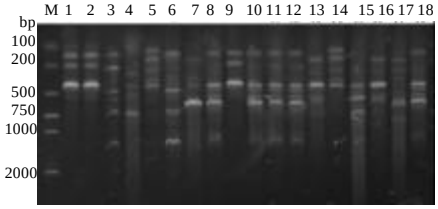

Fig. 1: ERIC-PCR fingerprints of 18 *H. parasuis* isolates M: DNA Marker DL2000; 1~18: fingerprints of 18 *H. parasuis* isolates

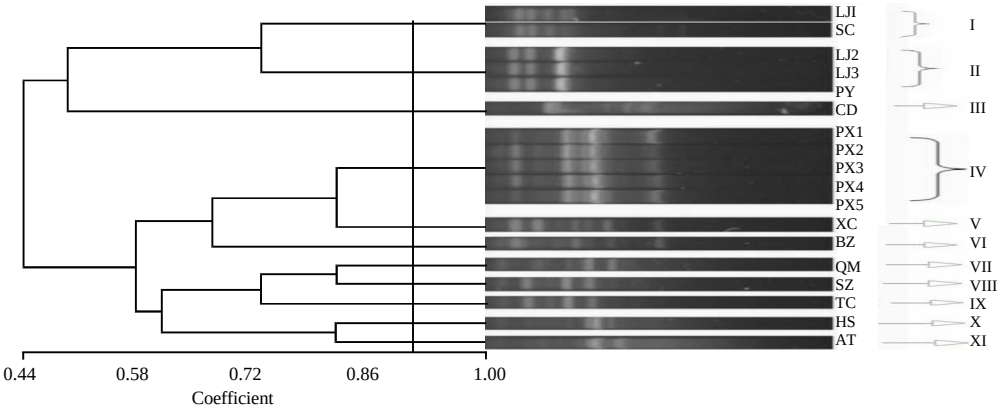

Fig. 2: ERIC-PCR dendrogram of 18 *H. parasuis* isolates

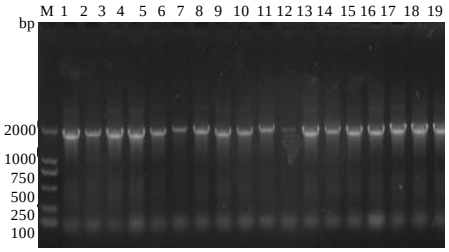

Fig. 3: TbpA amplification product of 18 *H. parasuis* isolates; M: DNA Marker DL2000; 1~18: tbpA amplification product of 18 *H. parasuis* isolates; 19: negative control

4-9 bands with the size ranging from 100-2000 bp. The further dendrogram analysis on DNA finger-print (Fig. 2) shows that 18 HPS Anhui isolates belong to 11 genotypes (Table 1 showed by I~XI). Of the 11 types, IV is the dominant genotype with the percentage of 27.78 (5/18) and is from the same region (or farm). Except for 2 isolates that could not be serotyped, other 3 are all type 4. The 2nd dominant genotype is II with the percentage of 16.67% (2/18) involving two serotypes and two regions (or farms). The 3rd is genotype I with the percentage of 11.11% (2/18) involving two regions (or farms) and neither of them could be serotyped. The other 8 isolates belong to genotype III, V, VI, VII, VIII, IX, X and XI in polymorphism distribution. The isolates that could not be serotyped are all able to be ERIC-PCR genotyped (Table 1).

HPS PCR-RFLP genotyping: About 18 HPS Anhui isolates all produced *tbpA* gene segments with approximate size of 1.9 kb after amplification which is consistent with the anticipated size of augmented segments (Fig. 3). Based on the methods built by De la Puente Redondo, PCR amplification products produced 3, 2 and 4 types of macrorestriction maps with the process of Taq I, Ava I and Afa I restriction endonuclease. The 3 macro-restriction maps produced by Taq I could be named B, D and E, respectively. The maps produced by Ava I could be named A and B, respectively. The maps produced by Afa I could be named B, E, I and P, respectively (De la Puente Redondo *et al*., 2003). After genotyping, 18 HPS isolates generated 6 different RFLP Patterns (Fig. 4) which were named genotype DBP, BAE, BBB, EBE, BBE and EAI. Total 8 isolates have genotype DBP (the most dominant genotype) with the percentage of 44.44 (8/18) involving 4 regions (or farms). Except 4 isolates that could not be serotyped, the other 4 all belong to serotype 4 and are from 2 different regions (or farms). About 4 isolates belong to genotype BAE (the 2nd dominant genotype) with the percentage of 22.22 (4/18) involving 4 regions (or farms) and only 1 strain could be serotyped as type 14. While 3 isolates belong to genotype BBB (the 2nd dominant genotype) with the percentage of 16.67 (3/18) involving 2 regions (or farms) and 2 of them are serotype 13 with the other one to be serotype 14. The remaining 3 genotypes belong to EBE, BBE and EAI, respectively and they are all from different regions (or farms) with serotypes to be 4, 14 and the unidentified, respectively. The strains that are in polymorphism distribution but could not be serotyped could all be PCR-RFLP genotyped (Table 1). So far, there are only a few reports on HPS epidemiology. It is primarily because very few laboratories are capable of serotyping HPS and serotyping itself also has many limitations which make 26.2~41% strains unidentifiable (Kielstein and Rapp-Gabrielson, 1992; Turni and Blackall, 2005). This research has 18 Anhui isolates in which 10 (55.6%) can be serotyped. The 10 isolates involve 3 serotypes, i.e., type 4, 14 and 13 which is different from what Cai *et al*. (2005) reported about the dominant HPS in China

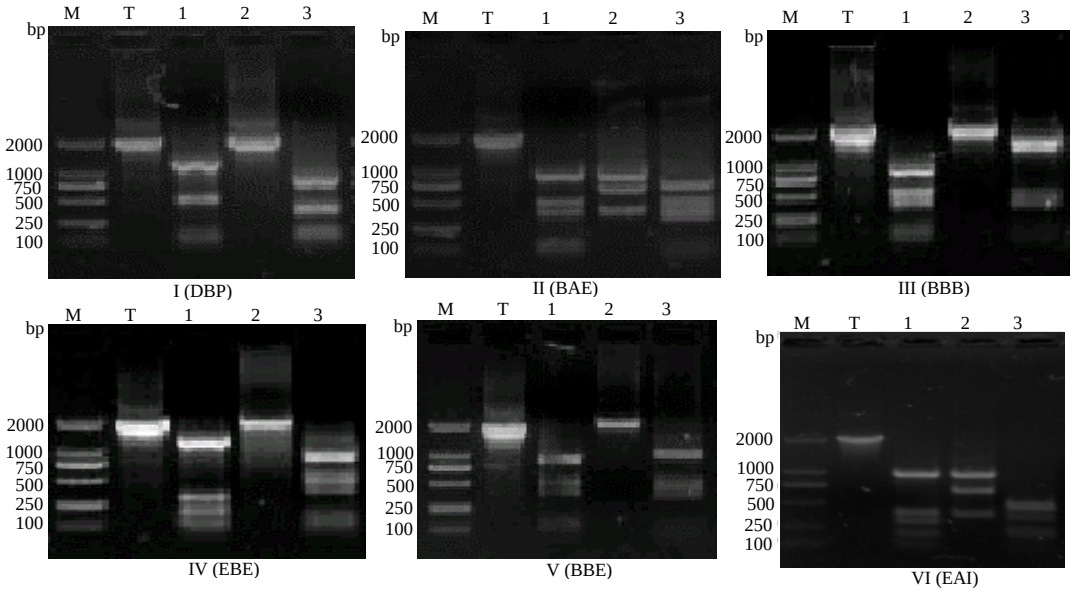

Fig. 4: TbpA amplification product from *H. parasuis* restricted with Taq I, Ava I, Afa I; M: DNA Marker DL2000; T: tbpA amplification product from *H. parasuis*; 1: tbpA amplification product with Taq 2: tbpA amplification product with Ava I 3: tbpA amplification product with Afa I

to be type 4, 5, 12 and 13. The other 8 (44.4%) strains cannot be serotyped which greatly limits people's knowledge on HPS epidemiology pattern in Anhui area and further affects the effective prevention and treatment on glasser's disease. Since, serotyping is not an effective method in practice (Blackall *et al*., 1997), genotyping of HPS and analyses and mastery of HPS molecular epidemiology has significant practical importance.

This research utilizes both ERIC-PCR and PCR-RFLP to genotype 18 HPS Anhui isolates. Both of the two methods could successfully group the 44.4% strains that could not be serotyped. This result confirms that ERIC-PCR and PCR-RFLP genotyping have stronger identification capabilities than the traditional serotyping does.

ERIC-PCR divides 18 HPS Anhui isolates into 11 genotypes in which IV is the dominant genotype including 5 strains (27.78%) from the same region (or farm). Except for 2 strains that cannot be serotyped, 3 strains are all serotype 4. This result combined with the serotypes, genotypes and sources of the other 13 strains shows that ERIC-PCR genotyping of HPS is not significantly related to KRG serotyping which is consistent with existing domestic and international reports (Oliveira *et al*., 2003; Jia *et al*., 2010). Strains with the same serotype can be categorized into different genotypes. For example, strains labeled as BZ, HS, FX5, FX2 and FX1 all belong to serotype 4 but are in three different genotypes (VI, X and IV); strains labeled as LJ2, XC and SZ all belong to serotype 14 but are in three different genotypes (II, V and IV). However, ERIC-PCR genotypes of HPS are associated with the sources of strains that have the same serotypes. For example, strains labeled as FX5, FX2 and FX1 are all from the same region (or farm) and they all belong to serotype 4 and genotype IV.

Researchers in China reported before that DBN (38%), ABN (18%) and DBP (12%) are the three most dominant genotypes in China (Li *et al*., 2009). Based on PCR-RFLP, 18 HPS Anhui isolates can be divided into 6 genotypes in which DBP is the dominant genotype but DBN and ABN were not found. This difference is probably related to region difference and the small number of inspected strains. Moreover in the 6 genotypes found in 18 HPS Anhui isolates, 3 genotypes (BAE, EBE and BBE) have not been reported yet which shows that there are many prevalent genotypes of HPS strains in Anhui area. The dominant genotype DBP contains 8 strains (44.44% of the total strains). The 8 strains are from 4 regions (or farms) and 4 of them are all serotype 4 while the other 4 are unidentifiable.

Of the 8 strains, 5 are from the same region (or farm) with the same ERIC-PCR genotype (IV); the other 3 are from different regions (or farms) with different ERIC-PCR genotypes. This result combined with the serotypes, ERIC-PCR genotypes and sources of the other 10 strains shows that PCR-RFLP genotyping of HPS is not significantly related to KRG serotyping which is

consistent with existing domestic and international reports (De la Puente Redondo *et al*., 2003; Li *et al*., 2009) and it is not significantly related to ERIC-PCR genotyping either.

Although, ERIC-PCR and PCR-RFLP have different genetic backgrounds on analyzing subjects and hence, the analysis results are not consistent both of them have strong identification capabilities. Both of their analysis results reveal that prevalent HPS strains in Anhui area have many genotypes which further shows that HPS has a large degree of genetic diversity. Compared with PCR-RFLP, ERIC-PCR genotyping better uncovers the genetic relationships of strains, reveals the genetic distance among different strains on molecular level and can be especially applied to the research on the inter-pig and inter-farm epidemic conditions of pathogenium.

CONCLUSION

The study shows that the incidence of glasser's disease and the loss caused by it has increased dramatically with no exception in Anhui area. Therefore, the sufficient and timely knowledge on HPS epidemiology is significantly important in preventing and treating Glasser's disease. This study utilizes KRG serotyping and ERIC-PCR and PCR-RFLP genotyping to systematically analyze 18 HPS Anhui isolates. It confirms that HPS prevails in pigs in Anhui area and its serotypes and genotypes are in polymorphism distribution and reveals that KRG serotype, ERIC-PCR genotype and PCR-RFLP genotype of HPS are not significantly associated with each other and ERIC-PCR genotype is related to the sources of strains with the same serotype which further provides important theoretical foundations for the effective prevention and treatment of Glasser's disease in Anhui area.

ACKNOWLEDGEMENTS

This research is financially supported by the Anhui province eleventh 5 years science and technology research projects (07010302144 and 08010302155) and Projects of Natural Science Foundation of Anhui Province (11040606M89).

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