Tropical Biomedicine 36(2): 482–487 (2019)

Molecular detection of *Haemophilus parasuis* serotypes 4, 5 or 12 and 13 in Peninsular Malaysia

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Received 5 August 2018; received in revised form 30 January 2019; accepted 23 February 2019

Abstract. Although the economic importance of *Haemophilus parasuis* infection causing Glasser's disease is prevalent throughout pig farms in Peninsular Malaysia, there is a dearth of knowledge on its actual nature. In this study, a multiplex PCR was performed to screen for three major predominant virulent strains of *H. parasuis*, which are serotypes 4, 5 or 12 and 13. A total of 175 tissues or bodily fluid samples of various parts were collected from diseased animals from October, 2016 to February, 2018; with total of 62.9% positive detection of *H. parasuis*. The highest detection was found to be in the pericardial sac fibrin (90.9%) followed by pleural fibrin, lung, pleural fluid, tonsil, pericardial sac, peritoneal fluid, abdominal fibrin, joint fluid, brain and pericardium. Serotype 13 was the highest (40/110) followed by serotype 4(37/110), serotype 5(31/110) and 12 samples were non-typable (12/110). The presence of untypable serotype also drives to further identification of other serotypes in Malaysia.

INTRODUCTION

The fastidious gram-negative *Haemophilus parasuis* is an economically significant pathogen that causes Glasser's disease inflicting high mortality in weaners as documented in the United States (Holtkamp, Rotto, & Garcia, 2007). It colonizes the upper respiratory tract of healthy piglets as early during neonatal stage leading to fibrinous polyserositis, bronchopneumonia, arthritis and/or meningitis (Moller & Killian, 1990). Technically, successful isolation by culture is being hampered by the usage of antibiotics prior to sampling or the presence of other contaminants that tampers the growth of *H. parasuis*.

Albeit unclear reasons behind virulence, those of the serotypes 1, 5, 10, 12, 13, and 14 are very virulent while the serotypes 2, 4, 8, and 15 causes milder signs and occasional deaths in pigs (Kielstein & Rapp-Gabrielson, 1992). Vaccination of *H. parasuis* often produces variable results across farms, providing the idea that vaccine strains are different than field strain, leading to insufficient protection. Although cross-protection between some serotypes of *H. parasuis* has been proven, it did not trigger adequate immune response against the heterologous serotypes (Miniats, Smart & Rosendal, 1991; Nielsen, 1993; Rapp-Gabrielson *et al*., 1997; Bak & Riising, 2002). Therefore, the knowledge of the serotype distribution is indeed important to select the best vaccination to control this disease.

Prevalence profiling of various countries reports have indicated that serotype 4, 5 and 13 are the most prevalent serotypes around the world (Pereira *et al*., 2017). This is a preliminary study on detecting major serotypes of *Haemophilus parasuis* in this country. For this purpose,

screening PCR followed by a multiplex PCR that targets major serotypes 4, 5 and 13 was performed.

MATERIALS AND METHODS

This study was approved by the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC No. UPM/IACUC/AUP-R015/2017). A total of 175 samples from clinical cases of *H. parasuis* infection were collected from pig farms between October, 2016 to February, 2018. Samples were collected from pigs suffering from respiratory distress and/or arthritis and dead pigs that exhibited fibrinous polyserositis, arthritis, meningitis and/or bronchopneumonia. Samples included brain, lung, pericardial sac, fibrin found in the pleural, pericardial and peritoneal cavity, pericardial fluid, pleural fluid, peritoneal fluid and joint fluid. A ratio of 1:5 tissue: phosphate buffer saline (PBS) was used to homogenize the tissues.

The DNA was extracted from tissues or fluid samples using Dneasy® Blood and Tissue Kit (Qiagen®, Germany). A screening polymerase chain reaction (PCR) assay using *H. parasuis* specific primers was performed on the extracted DNA using primers as designed by Howell *et al.* (2015). Forward primer 5'-ACAACCTGCAAGTAC TTATCGGGAT-3' and reverse primer 5'-TAGCCTCCTGTCTGATATTCCCACG-3' with PCR cycling condition of 95°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 30 sec, and 72°C for 1 min followed by 72°C for 5 mins. Positive samples were then serotyped using multiplex PCR (mPCR) for three serotypes; 4, 5 or 12 and 13 using primers (Howell *et al.*, 2015) as shown in Table 1. The PCR was performed under cycling conditions of 95°C for 5 mins, 35 cycles of 94°C for 1 min, 56°C for 30 sec, and 72°C for 1 min followed by 72°C for 5 mins. The PCR reaction mixtures consisted 2x HotStarTaq Plus Master Mix, (Qiagen®, Germany) containing 1 unit HotStarTaq Plus DNA Polymerase, 200M of each dNTP, 1.5mM $MgCl_2$ and 0.2M of each primer. PCR gel was analyzed using pre-stained 2% agarose gel electrophoresed in Tris-acetate-EDTA buffer and viewed using GelDoc XR UV transilluminator (Bio-rad, USA). Then, PCR product was purified and sequenced using Sanger's method by Bioneer (South Korea). The assembled sequences were subjected to basic local alignment tool (BLAST) searches to further confirm the amplicon as *H. parasuis*.

RESULTS

A total of 110 out of 175(62.9%) samples were positive for *H. parasuis* via conventional PCR (Figure 1). Among these, *H. parasuis* was detected in all types of samples and their respective positive rates were shown in Table 2. Positive samples of each sample type from different farms were selected and serotyped using mPCR. Preliminary, *H. parasuis* serotype 4, 5 or 12 and 13 were indeed detected in Peninsular Malaysia (Figure 2).

Most of the time only one serotype prevailed in one type of sample of one animal. Among all samples, only nine samples were positive for two serotypes and only one sample was detected to be positive for all three serotypes. Besides, there were samples that were negative for all three serotypes that were screened. Overall, serotype 13 was detected most followed by serotype 4 and 5 with minimal untypable serotypes (Table 3).

Table 1. Primer sets for multiplex PCR of serotypes 4, 5 or 12 and 13 (Howell *et al.*, 2015)

Target	Serotype	Forward primer (5' to 3')	Reverse primer (5' to 3')
wciP	4	GGTTAAGAGGTAGAGCTAAGAATAGAGG	CTTTCCACAACAGCTCTAGAAACC
wcwK	5 or 12	CCACTGGATAGAGAGTGGCAGG	CCATACATCTGAATTCCTAAGC
gltP	13	GCTGGAGGAGTTGAAAGAGTTGTTAC	CAATCAAATGAAACAACAGGAAGC

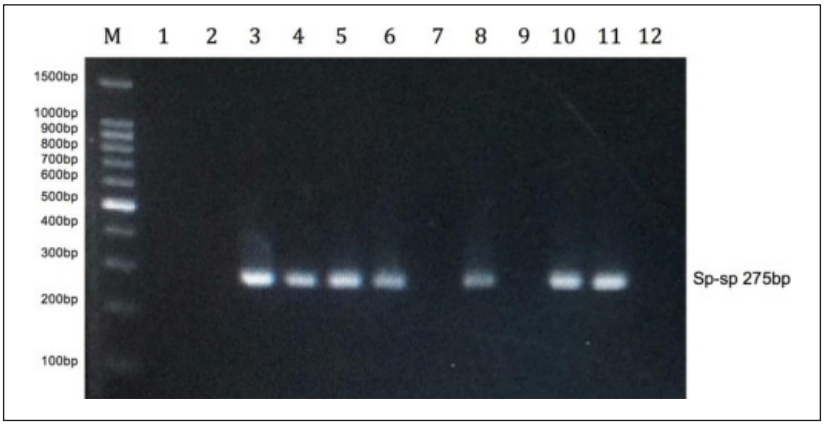

Figure 1. Detection of *H. parasuis* using conventional screen PCR. Lane M, 100bp marker; lane 1, non-template control; lane 2, negative control; lane 3, positive control; lane 4, 5, 6, 8, 10, and 11, positive samples; and lane 7, 9 and 12, negative samples; Sp-sp, species specific marker.

Table 2. Positive detection rates of *H. parasuis* via conventional PCR

Samples	No. of samples	No. of positives	Percentage
Fibrin from pericardial sac	11	10	90.9%
Fibrin of pleura	8	7	87.5%
Lung	37	27	73.0%
Pleural fluid	10	7	70.0%
Tonsil	13	9	69.2%
Pericardial sac	9	6	66.7%
Peritoneal fluid	19	12	63.2%
Peritoneal fibrin	13	8	61.5%
Joint fluid	27	13	48.1%
Brain	12	5	41.7%
Pericardial fluid	16	6	37.5%
Total	175	110	62.9%

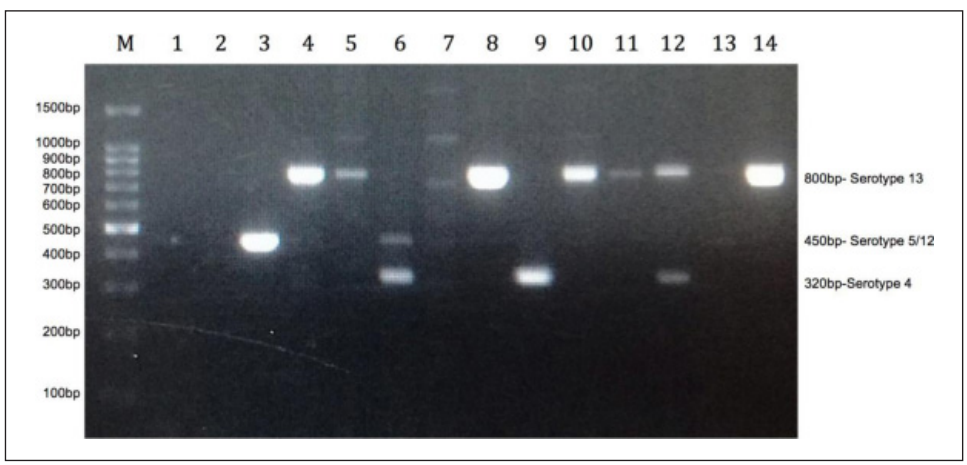

Figure 2. Multiplex PCR for serotyping. Lane M, 100bp marker; lane 1, Non-template control; lane 2, negative control; lane 3 and 6, positive for serotype 5/12; lane 4, 5, 8, 10, 11, 12, and 14, positive for serotype 13; lane 6, 9 and 12, positive for serotype 4; lane 7 and 13, negative for serotype 4, 5/12 and 13 samples.

Table 3. The detection of serotypes 4, 5 or 12 and 13 in various samples types

Samples	No. of positive samples	Serotype 4	Serotype 5 or 12	Serotype 13	Unknown
Pericardial Fibrin*	10	5	2	5	0
Pleural fibrin*	7	3	1	4	0
Pleural fluid	7	3	3	0	1
Lung*	27	6	11	8	6
Tonsil	9	3	3	2	1
Pericardial sac*	6	4	1	2	0
Peritoneal fluid	12	3	3	5	1
Abdominal fibrin*	8	3	3	2	1
Joint fluid	13	3	2	6	2
Brain*	5	0	2	4	0
Pericardial fluid*	6	4	0	3	0
Total		37	31	40	12

* More than one serotype can be found in one sample.

DISCUSSION

In general, the high detection rate (62.9%) in most samples was due to it being from diseased animals. However, this also explains why it is slightly higher than those reported elsewhere of between 50.0%–53.5% (Turni & Blackall, 2007; Oliveira, 2007). The higher detection rates (70.0%–90.9%) in the lungs indicated that *H. parasuis* may had localized or colonized the organ after death (Olvera, Segalés & Aragón, 2007).

Henceforth, lung can be a good sample for detection and serotyping but may not the best sample to identify the true serotype that causes the disease because of possible invasion of the normal flora after death (Moller & Kilian, 1990). Similarly, as *H. parasuis* is a commensal of the tonsils, serotyping the pathogen isolated in this region may not be representative of true serotype that causes the disease (Oliveira, Galina, & Pijoan, 2001). In this study, peritoneal fluid and pleural fluid in cases of fibrinous polyserositis yielded high detection and this supports that the ideal samples for bacterial isolation are bodily fluids when pigs show fibrinous polyserositis (Vahle *et al.*, 1995; Solano *et al.*, 1997).

This preliminary study revealed that *H. parasuis* serotype 4, 5/12 and 13 are present among pigs in Peninsular Malaysia. These serotypes are also the three most prevalent serotypes available in most countries such as Germany, Australia, Spain, Denmark, United States and Canada (Rapp-Gabrielson & Gabrielson, 1992; Blackall *et al.*, 1996; Rúbies *et al.*, 1999; Angen, Svensmark & Mittal, 2004; Tadjine *et al.*, 2004). These similarities could possibly resulted from animal trade or transport of animals across countries.

In addition, since serotype such as 4, 5 and 13 are described as moderately to highly virulent (Kielstein & Rapp-Gabrielson, 1992), thus is expected to be sampled from clinically ill and dead animals that exhibited variable Glasser's disease lesions (Angen, Svensmark, & Mittal, 2004). As seen in this and earlier studies, it was common to detect several serotypes within the same animal from the same farm (Blackall, Rapp-Gabrielson, & Hampson, 1996; Olvera, Cerdà-Cuéllar, & Aragon, 2006). Even so, it is not common to detect all three virulent serotypes together as very likely that one serotype is more dominant than the other.

The result of non-specific bands as well as negative band for positive *H. parasuis* sample may indicate serotype/s that was not screened, as only three serotypes were detected so far. Hence, performing PCR using primers targeting the rest of the serotypes would be useful in identifying

more *H. parasuis* serotypes in Malaysia and to further discover of new potential serotypes. Identification of *H. parasuis* serotype is important in vaccination selection. Although some serotypes are proved to provide cross protection between serotypes, it does not confer full protection to all serotypes (Nielsen, 1993; Rapp-Gabrielson *et al*., 1997; Takahashi *et al*., 2001; Bak & Riising, 2002). As of now, only commercial vaccine that targets serotype 4 and 5 is available in Malaysia market. A strategic vaccination can be implemented with this finding, especially when demonstration of very virulent strains had been done. Vaccination will remain an important tool to prevent and control this disease; hence, reducing clinical signs and mortality. This subsequently reduces the need of antibiotics, which then reduces the risks of antibiotic resistance.

CONCLUSION

This study is the preliminary efforts to create a disease data on *H. parasuis*. It was found that serotype 4, 5 or 12 and 13 are present in Malaysia and potential more serotypes are likely to be found. Therefore, further investigation on serotypes of *H. parasuis* in Malaysia needs to be done.

Acknowledgments. The authors thank Universiti Putra Malaysia for providing funding through the Putra Grant - Putra Graduate Initiative (Grant no. GP-IPS/2018/9616800). The authors would also like to thank field veterinarians and participating farms for allowing sample collection as well as laboratory staffs of Universiti Putra Malaysia for guidance and support.

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