Molecular serotyping of *Haemophilus parasuis* isolated from diseased pigs and the relationship between serovars and pathological patterns in Taiwan

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**Background:** *Haemophilus parasuis* (*H. parasuis*) is the etiological agent of Glässer’s disease, and causes severe economic losses in the swine industry. Serovar classification is intended as an indicator of virulence and pathotype and is also crucial for vaccination programs and vaccine development. According to a polysaccharide biosynthesis locus analysis, *H. parasuis* isolates could be classified by a molecular serotyping assay (except for serovars 5 and 12). The aim of this study was to identify *H. parasuis* isolates from diseased pigs in Taiwan by using a molecular serotyping assay and to analyze the relationship between serovars and pathological patterns.

**Methods:** From August 2013 to February 2017, a total of 133 isolates from 277 lesions on 155 diseased animals from 124 infected herds serotyped by multiplex PCR and analyzed with pathological data.

**Results:** The results showed that the dominant serovars of *H. parasuis* in Taiwan were serovars 5/12 (37.6%), 4 (27.8%) and 13 (15%) followed by molecular serotyping non-typable (MSNT) isolates (13.5%), which are differentiated on a genetic basis. Nevertheless, the serovar-specific amplicons were not precisely the same sizes as previously indicated in the original publication, and MSNT isolates appeared with unexpected amplicons or lacked serovar-specific amplicons. Furthermore, most *H. parasuis* isolates were isolated from nursery pigs infected with porcine reproductive and respiratory syndrome virus. The percentage of lung lesions (30.4%) showing *H. parasuis* infection was significantly higher than that of serosal lesions.

**Discussion:** Collectively, the distribution of serovars in Taiwan is similar to that found in other countries, but MSNT isolates remain due to genetic variations. Furthermore, pulmonary lesions may be optimum sites for *H. parasuis* isolation, the diagnosis of Glässer’s disease, and may also serve as points of origin for systemic *H. parasuis* infections in hosts.
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Abstract

Background: *Haemophilus parasuis* (*H. parasuis*) is the etiological agent of Glässer’s disease, and causes severe economic losses in the swine industry. Serovar classification is an indicator of virulence and pathotype and is also crucial for vaccination programs and vaccine development. *H. parasuis* isolates can be classified by a molecular serotyping assay except for serovars 5 and 12. The aim of this study was to serotype *H. parasuis* isolates from diseased pigs in Taiwan by using a molecular serotyping assay and to analyze the relationship between serovars and pathological patterns.

Methods: From August 2013 to February 2017, a total of 155 diseased animals from 124 infected herds were examined for infection with *H. parasuis*. One hundred thirty three isolates of *H. parasuis* were recovered and serotyped by multiplex PCR and correlated with pathological lesions.

Results: The dominant *H. parasuis* were serovars 5/12 (37.6%), 4 (27.8%) and 13 (15%) followed by molecular serotyping non-typable (MSNT) isolates (13.5%). The serovar-specific amplicons were not precisely the same sizes as previously indicated in the original publication, and MSNT isolates appeared with unexpected amplicons or lacked serovar-specific amplicons. Most *H. parasuis* isolates were isolated from nursery pigs infected with porcine reproductive and respiratory syndrome virus. The percentage of lung lesions (30.4%) showing *H. parasuis* infection was significantly higher than that of each serosal lesions.

Discussion: Collectively, the distribution of *H. parasuis* serovars in Taiwan is similar other countries, but MSNT isolates remain due to genetic variations. Our data suggests those prevalent serovar isolates prefer to cause both serosal and pulmonary lesions rather and only pulmonary lesions. Pulmonary lesions are optimum sites for *H. parasuis* isolation and may also serve as
points of origin for systemic *H. parasuis* infections in hosts.

Keywords: *Haemophilus parasuis*, Glässer’s disease, polyserositis, serotyping

**Introduction**

*Haemophilus parasuis (H. parasuis)*, a part of normal upper respiratory microbiota, is the etiological agent of Glässer’s disease which induces sudden death, polyserositis, polyarthritis, meningitis and poor production performance, resulting severe economic losses in the swine industry (Amano et al. 1994; Moller & Kilian 1990; Vahle et al. 1997; Zhang et al. 2014).

Vaccination is an effective strategy for preventing increased mortality and economic losses caused by virulent *H. parasuis* (Miniats et al. 1991a; Smart & Miniats 1989). However, only partial protection is observed with heterologous *H. parasuis* strain challenges due to poor cross-protection (Miniats et al. 1991b; Nielsen 1993; Takahashi et al. 2001). Thus, serotyping of *H. parasuis* is very important, not only for epidemiological research but also for choosing efficacious inactivated whole-cell bacterial vaccines.

Fifteen serovars, conventional serotyping cross-reactive (CSCR) and non-typable (CSNT) isolates of *H. parasuis* have been described and demonstrated by gel immunodiffusion assay (GID) (Kielstein & Rapp-Gabrielson 1992). Due to the persistence of cross-reactivity or non-reaction to antisera, there are still approximately 15% to 40% CSCR and CSNT isolates reported in a variety of countries by GID (Table S1) (Blackall et al. 1996; Cai et al. 2005; Castilla et al. 2012; Del Rio et al. 2003; Kielstein & Rapp-Gabrielson 1992; Luppi et al. 2013; Ma et al. 2016; Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Rubies et al. 1999; Tadjine et al. 2004). Despite using an indirect hemagglutination assay (IHA) designed to reduce the proportion of CSCR isolates, 7.5% to 18% of isolates are still untypable (Table S1) (Angen et al. 2004; Cai et al. 2005; Del Rio et al. 2003; Dijkman et al. 2012; Howell et al. 2015). This phenomenon
makes it more difficult to conduct an effective vaccination program against \textit{H. parasuis}.

Conventional serotyping is used extensively (Kielstein et al. 1991; Morozumi & Nicolet 1986; Rapp-Gabrielson & Gabrielson 1992). The Kielstein-Rapp-Gabrielson (KRG) scheme recognizes 15 serovars of \textit{H. parasuis} on the basis of a GID test with specific rabbit antisera and the authors noted a correlation between serovar and virulence (Kielstein & Rapp-Gabrielson 1992). According to serotyping results, serovar 4 tends to be found in pulmonary infections; CSNT and CSCR isolates are mainly found in systemic infections (Angen et al. 2004).

Unfortunately, others report little correlation between serovar and virulence as isolates in the same serovar often exhibit different virulence levels (Aragon et al. 2010; Olvera et al. 2007).

Previous studies established the serovar and pathotype of \textit{H. parasuis} are based on differences at the genome level (Brockmeier et al. 2014; Howell et al. 2013; Howell et al. 2017).

A multiplex PCR (mPCR) based on a polysaccharide biosynthesis locus analysis was employed to molecularly serotype \textit{H. parasuis} serovars (Howell et al. 2015). As a result, 14 of 15 serovars of \textit{H. parasuis} (serovars 5 and 12 could not be differentiated) were identified using this assay (Howell et al. 2015) Using the molecular typing assay many of the CSNT and CSCR isolates were successfully typed in a recognized serovar.

Although Glässer’s disease is common in Taiwan, serotyping of pathogenic \textit{H. parasuis} isolates from Taiwanese pigs is not clear. The principal aim of this study was to molecularly serotype \textit{H. parasuis} isolated from Taiwanese diseased pigs, and correlate serovars with pathological patterns.

\textbf{Materials & Methods}

\textbf{Bacterial isolate collection and identification}

\textit{H. parasuis} field isolates were collected from diseased pig herds between August 2013 and
February 2017 in Taiwan (Table S2). Lesions suspected of being caused by *H. parasuis* in diseased pigs were located in the meninges, pleura, pericardia, peritonea, synovial cavities of joints and lungs. Lesions were swabbed and plated on chocolate agar (at 37°C, 5% CO₂, 18 to 72 hours for growth rate variation for various isolates), blood agar (at 37°C, 16 to 24 hours) and MacConkey agar (at 37°C, 16 to 24 hours). The bacterial isolates were identified by colony morphology, Gram stain (Gram negative bacillus), nicotinamide adenine dinucleotide (NAD) dependence (only growing on chocolate agar) and virulence-associated trimeric autotransporter group 3 colony PCR (Pina et al. 2009).

**Molecular serotyping mPCR**

The molecular serotyping assay for *H. parasuis* isolates was modified from a previously published method (Howell et al. 2015). The sp-sp amplicon was used as an internal control. A loopful of bacteria from a passaged plate of pure culture was resuspended in 30 μL ultrapure H₂O, which was heated to 100°C for 30 min then centrifuged at 4,000 x g for 1 min. The supernatant was used in the mPCR reaction. Isolates from various lesions or pigs from the same herd were serotyped. If they belonged to same serovar, they were considered one isolate.

Each PCR reaction was performed in a total volume of 30 μL containing ultrapure H₂O, 1 x DreamTaq buffer, 250 μM dNTP, 0.2 μM concentrations of forward and reverse serovar-specific primers, 0.04 μM concentrations of forward and reverse species-specific primers, 1.25 U of DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μL of supernatant. The thermocycling conditions consisted of 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, and then a final extension at 72°C for 5 min. The molecular serotyping mPCR amplicons were stained with ethidium bromide and analyzed using a 20-cm-long 2% agarose gel. A 50-bp DNA ladder RTU (GeneDireX, Las Vegas, NV, USA) and Bio-1D software (Vilber Lourmat, Collégien, France) were used to estimate molecular size.
The electrophoresis conditions were an electric field 6 V/cm (300 V, 50-cm full-length electric field) and 3 hr. The results were confirmed by twice repeating tests.

**Sequencing and analysis of unexpected PCR-amplified products**

Unexpected amplicons of the molecular serotyping mPCR products were cloned using a TA cloning kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan) and sequenced using an automated DNA sequencer (ABI 3730XL, USA). Sequence data were analyzed using MEGA7 (Molecular Evolutionary Genetics Analysis Version 7.0) software and BLAST (Basic Local Alignment Search Tool) database.

**Pathological examination**

Cases of sick animals or fresh, complete carcasses were subjected to necropsy for gross morphological examinations and H&E staining. Histopathological examination focused primarily on meningeal, pleural, pericardial, peritoneal, and synovial cavities of joints, and lungs. Typical meningeal gross lesions were characterized by yellow to white exudate accumulation in the subarachnoid space, on pia mater and in the sulci (Fig. S1). Meningeal histopathological lesions were principal neutrophils and few mononuclear inflammatory cells infiltration on pia mater with fibrin and cellular debris deposit (Fig. S2). Serosal lesions were characterized by yellow to white exudate accumulation in pleural, pericardial, abdominal and joint synovial cavities and yellow to white fibrin covering the visceral and parietal serosa (Fig. S3; Fig. S4; Fig. S5). The histopathological lesions of serositis were principal neutrophils and few mononuclear inflammatory cells infiltration with fibrin deposit (Fig. S6). In typical cases, *H. parasuis* resulted in bronchopneumonia with numerous neutrophils, mononuclear inflammatory cells, erythrocytes, cellular debris and fibrinous exudate accumulation in alveoli (Fig. S7; Fig. S8). Due to disease duration, lesions varied in field. Other lesions infected *H. parasuis* were also involved to determine pathological patterns, including chronic fibrous serositis with angiogenesis and
mononuclear inflammatory cells infiltration (Fig. S9), and meningitis with principal
macrophages infiltration (Fig. S10).

**Detection of porcine reproductive and respiratory syndrome virus**

Nucleic acid extraction of pulmonary tissue was performed on a MagNA Pure LC 2.0 by
using the MagNA Pure LC total nucleic acid isolation kit (Roche Applied Science, Indianapolis,
IN, USA). Following cDNA synthesis was using PrimeScript™ RT reagent kits (Takara, Kyoto,
Japan). Porcine reproductive and respiratory syndrome virus (PRRSV) reverse transcription real-
time PCR was performed as previously described (Lin et al. 2013).

**Statistical analysis**

Fisher’s exact test was used to compare the frequency of *H. parasuis* infected lesions and
the percentage of various lesion patterns using GraphPad Prism software (GraphPad Software,
La Jolla, CA, USA). Variables were considered significant at a 0.05 level (two-sided).

**Results**

**H. parasuis isolates, origins and pathological lesion patterns**

One hundred thirty three isolates of *H. parasuis* were isolated from August 2013 to
February 2017. The isolates were taken from 277 lesions on 155 diseased animals from 124
infected herds. Isolates from a herd serotyped as a single serovar were calculated as one isolate.

Of 155 *H. parasuis* cases, 12 cases (7.7%) belonged to suckling pigs (≤ 3-week-old), 133 cases
(85.2%) belonged to nursery pigs (4- to 12-week-old), 7 cases (4.5%) belonged to growing pigs
(13- to 26-week-old) and one case belonged to a breeding boar. Age information for two cases
was unknown. Eighty-six cases (55.5%) had *H. parasuis* isolated from lung lesions with or
without serosal lesions.

One hundred eight animals were necropsied with complete pathological examination and
further correlated to pathological pattern and isolation proportion (Table S3). Of the *H. parasuis* infected animals, 54.6% had serositis and pulmonary tissue lesions, 41.7% had serosal lesions only, and 3.7% displayed only pulmonary lesions (Fig. 1).

One hundred six cases (98.1%) had bronchopneumonia, 64 cases (59.3%) displayed *H. parasuis* positive lung lesions. Seventy-eight cases (72.2%) registered as positive for PRRSV via reverse transcription real-time PCR screening. The proportion of 204 *H. parasuis* infected lesions from 108 animals with complete pathological examination were meninges (10.3%), pleura (20.1%), pericardium (16.2%), peritoneum (13.7%), joint synovial cavity (9.3%) and lung (30.4%) (Fig. 2). The percentage of lung lesions showing *H. parasuis* infection was significantly higher than the percentage of serosal lesions (*P* < 0.05).

**Serovar distribution by molecular serotyping assay**

Of the 133 isolates, 91 (68.94%) isolates were typed using molecular serotyping mPCR. The most common serovars were serovar 5/12 (38.2%) and serovar 4 (27.5%) followed by serovar 14 (2.3%), serovar 1 (0.8%), serovar 2 (0.8%) and serovar 9 (0.8%) (Fig. 3). However, the product sizes of the serovar-specific primers analyzed by Bio-1D software were varied from the original publication (Howell et al. 2015). Furthermore, there were still 41 isolates (29.8%) classified as MSNT; these were divided into four groups based on the appearance of unexpected amplicons or the lack of serovar-specific amplicons. Eighteen isolates (13%) positive for a species-specific (sp-sp) marker were categorized as MSNT group 1. Nineteen isolates (14.5%) were placed in MSNT group 2; these displayed amplicons of 300, 830, and 1000 bp. Two isolates (1.5%) which showed unexpected amplicons at 500 and 660 bp were categorized as MSNT group 3. One isolate (0.8%), showing an amplicon of 300 bp was categorized as an MSNT group 4 isolate (Fig. 3; Fig. S11; Fig. S12; Fig. S13; Fig. S14; Fig. S15).

**Identification of serovar-specific amplicons**
The amplicons generated from molecular serotyping mPCR analyzed using Bio-1D software were not precisely the same sizes as previously indicated in the original description of this assay (Howell et al. 2015). The product size of a specific amplicon found in serovar 4 was mentioned at 320 bp in the original publication but the PCR run generated an amplicon of nearly 350 bp which might be confused with serovar 6. In serovar 5, the PCR results generated an amplicon larger than 450 bp mentioned in the original publication which might be confused with serovar 7. The product size of serovar 9 serovar-specific primers, mentioned at 710 bp in the original publication, was smaller than the 700 bp ladder marker and might be confused with serovar 8. In light of these conflicting results, the isolates were serotyped again to confirm the sizes, and the amplicons were subsequently sequenced. Comparisons of the molecular serotyping original publication described, Bio-1D software analyzed, and BLAST product sizes are shown in Table 1 (Howell et al. 2015). The product sizes analyzed by Bio-1D software, BLAST and sequence are more consistent.

The unexpected PCR products of the MSNT isolates were cloned for sequencing (Table 2). The MSNT group 2 amplicon was 297 bp; this product was generated with a serovar 13 specific forward and a serovar 14 specific reverse primer pair targeting gltP gene as a marker of serovar 13 in a polysaccharide biosynthesis locus. These primers were paired because the target sequences in the respective serovars shared homologous segments. The other PCR generated an amplicon product of the MSNT group 2 isolate determined to be 836 bp, and was identified as a serovar 13 specific product. The Bio-1D software measured a 500 bp product of the MSNT group 3 isolate as 499 bp; this amplicon was identified as a serovar 7 specific product. The 300 bp product found in the MSNT group 4 isolate, (sequencing results indicated it was 297 bp) was generated by pairing a serovar 13 specific forward primer with a serovar 14 specific reverse primer. This result was the same as that generated using the same primer pair of DNA isolated
Serovar distribution based on molecular serotyping assay and sequencing

The molecular serotyping assay combined with sequencing results reduced the percentage of isolates classified as MSNT from 30.1% to 13.5% (Fig. 3). The dominant serovars were serovar 5/12 (37.6%), serovar 4 (27.8%) and serovar 13 (15%) followed by serovar 14 (2.3%), serovar 7 (1.5%), serovar 1 (0.8%), serovar 2 (0.8%) and serovar 9 (0.8%). Combining the sequencing results showed that serovar 13 is a common serovar.

Relationship between pathological lesion patterns and serovars

The distribution of *H. parasuis* serovars in lesions from necropsied animals were serovar 5 (42.6%), serovar 4 (21.3%), serovar 13 (20.4%), and MSNT group 1 (13%). These categories were further subdivided into animals displaying both serosal and pulmonary lesions, those with only pulmonary lesions, and those with lesions found only in serosa. The respective percentages of lesions vs. serovars, and the pattern of lesions in infected animals were showed in Fig. 1 and Table S4. Necropsied animals with both serosal and pulmonary lesions were the most frequent; animals with pulmonary lesions alone were the least frequent (*p* < 0.0001). Serovars 4 and 5/12 showed similar results, the MSNT group 1 both serosal and pulmonary lesions were more frequent than serosal lesions alone. Serovar 13 had more serosal lesions than the combination of serosal and pulmonary lesions.

Nine herds (7.3%) had populations infected with two *H. parasuis* serovars. One herd contained a population with lesions infected with serovars 1 and 4. Three herds were infected with serovars 4 and 5. Serovar 5, 13 and 5, 14 co-infections were seen in single herds. The infected lesions were located in animals displaying a variety of tissue lesion patterns. Other 4 herds contained individuals co-infected with two *H. parasuis* serovars. One clinical case showed pleural and pulmonary lesions coinfecteared with *H. parasuis* serovars 4 and 7, respectively. A
separate herd contained one case of pulmonary lesions with serovar 5, as well as pleural, pericardial, and peritoneal lesions infected with *H. parasuis* serovar 13. One case was co-infected with serovars 5 (pulmonary) and 13 (pleura and pericardium). A fourth case showed coinfection with serovar 4 and an MSNT group 1 isolate taken from separate pulmonary lesions.

**Discussion**

This is the first study describing serovars of *H. parasuis* defined by molecular serotyping in Taiwan. The most common serovars are serovar 5/12, 4 and 13, followed by MSNT isolates. Even though serotyping assays vary, the serovar population profile of *H. parasuis* in Taiwan is similar to profiles described in several other studies (Table S1) (Angen et al. 2004; Blackall et al. 1996; Cai et al. 2005; Castilla et al. 2012; Del Rio et al. 2003; Dijkman et al. 2012; Howell et al. 2015; Kielstein & Rapp-Gabrielson 1992; Luppi et al. 2013; Ma et al. 2016; Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Rubies et al. 1999; Tadjine et al. 2004). Most commercial *H. parasuis* vaccines are inactivated vaccines, which provide protection against the same serovar but are unable to provide protection from challenge using different serovars (Miniats et al. 1991b; Nielsen 1993; Smart & Miniats 1989; Takahashi et al. 2001). Candidate serovar composition in *H. parasuis* vaccine determines the success of a vaccine strategy against *H. parasuis* (Takahashi et al. 2001). Therefore, the distribution of serovars in herds is an important factor in outlining vaccination strategies and vaccine developments aimed at the prevention and control of Glässer’s disease.

IHA was applied to *H. parasuis* serovar differentiation to decrease the proportion of *H. parasuis* isolates classified as CSCR (Cai et al. 2005; Del Rio et al. 2003). De-encapsulation due to multiple passages results in non-reaction with antisera and cross reactivity of isolate antigens to diagnostic (immune-based) test reagents are the primary factors behind CSNT and CSCR *H. parasuis*.
parasuis isolates, respectively (Cai et al. 2005; Kielstein & Rapp-Gabrielson 1992; Oliveira et al. 2003; Rapp-Gabrielson & Gabrielson 1992; Turni & Blackall 2005). The presence of CSNT and CSCR isolates confounds epidemiological surveys used to assess H. parasuis isolate population profiles, and impairs efforts to generate effective vaccines against this pathogen. The correlation between the capsule and serovar of H. parasuis is well established; a multiplex serotyping PCR was developed with this in mind (Howell et al. 2015; Howell et al. 2013). This protocol can be employed to type isolates previously classified as CSNT and CSCR via traditional (immunological) methods. The mPCR serotyping reduced the incidence (percentage) of CSNT and CSCR H. parasuis. Molecular serotyping has not completely eliminated the issue of CSNT and CSCR isolates. One reason may be the sequence similarity of different serovar-specific primers and serovar-specific products. Another factor may be deletions and/or unknown sequences within certain antigenic markers (Ma et al. 2016). This underscores the importance of MSNT isolate whole-genome sequencing for in silico serotyping and improving the molecular serotyping assay. Emergence of MSNT isolates by the molecular serotyping assay may be due to insufficient or incomplete sequence data for H. parasuis from Asia. When this assay was developed, there were only nine Asian H. parasuis isolates in a 212-isolate database (7 from Japan, 2 from China). Investigating the sequences and gene composition of Asian H. parasuis isolate capsule loci may be key for assaying and serotyping MSNT isolates. Besides, absence of serovar-specific markers in polysaccharide biosynthesis loci in MSNT isolates may create antigenic variation impairing vaccine strategies. Therefore, it is also important to study the antigenic variation due to gene mutation and/or absence in polysaccharide biosynthesis loci in the future.

Thus far, molecular serotyping has been challenging as there are 15 serovars, making it difficult to design serovar-specific primers yielding differential results. Some primer pairs
produce amplicons from different *H. parasuis* serovars that vary by less than 20 bp—a difference that is hardly detected especially when the amplicon size is larger than 600 bp. In our study, electrophoresis using longer agarose gels was performed to enhance the ability of the procedure to discriminate DNA fragment sizes. Bio-1D software was applied to more accurately measure product size based on the intensity of the bands and decrease human operation error. In the case of molecular serotyping tests resulting in ambiguities, serovar-specific primer pairs may be used (in simplex PCR format) to confirm or classify hard-to-identify serovars. According to sequence analysis, the product sizes described in the original publication were not accurate (Howell et al. 2015). The corrected product sizes are important to avoid mis-serotyping.

According to a previous study, pigs were infected with *H. parasuis* serovars 1, 5, 10, 12, 13, and 14 showed high mortality. Pigs challenged with serovars 2, 4, 8, and 15 showed polyserositis. Pigs inoculated with serovars 3, 6, 7, 9, and 11 resulted in no clinical symptoms or lesions (Kielstein & Rapp-Gabrielson 1992). Serovars 5/12, 4, 13 and 7 are the most common serovars in most countries worldwide (Table S1). Previous study has showed serovar 4 and 13 have a higher prevalence in systemic infection than in respiratory disease only (Luppi et al. 2013). Our data also showed similar results of serovar 4, 5/12, 13 and MSNT isolates. There may be some correlation between serovar and virulence because serovars are defined by capsule which can directly interact with host cells and has been proven to be a key virulence factor relating to phagocytosis resistance (Olvera et al. 2009). Besides, it should be considered if the impact of some isolates resulting in only pulmonary lesions are underestimate in field due to absence of serositis and typical pulmonary lesions. The role and economic impact of *H. parasuis* in pulmonary infection animals related to porcine respiratory disease complex in field is also worthy of investigation in the future. In our study, serovars 7 and 9 caused serositis with or without respiratory lesions. Serovar 5 isolate was isolated from an animal with only
bronchopneumonia lesions and another with lesions in both the serosa and lung tissues in the same herd. Therefore, the results show clinical manifestations of Glässer’s disease are influenced by multiple factors, including host, stress, environment, co-infection with different serovars or other pathogens, and gene differences between infecting *H. parasuis* isolates (Boerlin et al. 2013; Howell et al. 2014; Li et al. 2009). In general, most Glässer’s disease cases in nursery pigs were co-infected with PRRSV in our data. This may be because PRRSV can cause immunosuppression by reducing non-specific bactericidal activity of pig alveolar macrophages and stimulating interleukin-10 production, which down-regulates inflammatory cytokines (Drew 2000; Flores-Mendoza et al. 2008; Suradhat & Thanawongnuwech 2003). The previous studies have showed PRRSV does not result in an increased Glässer’s disease by experimental challenge (Segales et al. 1999; Solano et al. 1997). However, significant association between *H. parasuis* and PRRSV in field was reported (Palzer et al. 2015). Recently studies also showed PRRSV can induce bronchopneumonia with *Bordetella bronchiseptica* which is a part of normal upper respiratory microbiota and predispose to colonization with *H. parasuis* (Brockmeier 2004; Brockmeier et al. 2001). Co-infection of pig alveolar macrophages with PRRSV and *H. parasuis* leads to pro-inflammatory mediated immunopathology by synergistic effect (Kavanova et al. 2015; Li et al. 2017). In the future, the synergistic effect between PRRSV and *H. parasuis* resulting in economic losses in field is worthy of further investigation. Other factors also interact with *H. parasuis* including the stress of weaning and maternal antibody reduction. However, highly virulent *H. parasuis* isolates might be considered primary pathogens (Aragon et al. 2012). In our study, some *H. parasuis* isolates caused serositis and sudden death without co-infection, even in growing pigs and breeding boars.

Previous studies showed *H. parasuis* can access the blood stream through invasion of the mucosal surface in the nasal cavity (Vahle et al. 1997). In our study, pulmonary lesions showed
higher pathogenic *H. parasuis* infection rates than serosal lesions. These results are in accordance with a previous study from the Netherlands (Dijkman et al. 2012). *H. parasuis* invasion and survival in lung tissue is likely a key feature for the onset of disease (Olvera et al. 2009; Vahle et al. 1995). Our results show *H. parasuis* infected animals with lesions found in dual anatomical locations (pulmonary and serosal) occur at a higher rate than infected animals with lesions located in only one tissue type. Previous studies also mentioned lung is one of the most successful sites for acute (serovar 12) and subacute (serovar 4) isolate recovery (Turni & Blackall 2007). Therefore, lung is an important origin for *H. parasuis* isolation and a target organ for Glässer’s disease diagnosis. Pulmonary infections may be an important step for *H. parasuis* systemic infections.

Others have reported isolation of multiple isolates from single pig farms (Cerda-Cuellar et al. 2010; Oliveira et al. 2003; Olvera et al. 2006a; Olvera et al. 2006b). Our results also show different serovars cause disease in a single herd, or even in a single animal, although the latter scenario is fairly uncommon. In most situations, Glässer’s disease is caused by one isolate (Rafiee et al. 2000), but several isolates may be present at a given farm (Turni & Blackall 2010). Therefore, it would be useful to develop a universal vaccine against multiple serovars. The possibility of cross talk between different pathogenic *H. parasuis* isolates at a given site may be worthy of investigation.

**Conclusions**

Our study shows the dominant serovars of *H. parasuis* in Taiwan are serovars 5/12, 4 and 13, followed by MSNT isolates. Proportions of isolates in those serovars resulting in both serosal and pulmonary lesions are significantly higher than pulmonary lesion. Pulmonary lesions may be
most important for *H. parasuis* isolation, and may serve as points of origin for systemic *H. parasuis* infections in hosts.

**References**

Amano H, Shibata M, Kajio N, and Morozumi T. 1994. Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method. *Journal of Veterinary Medical Science* 56:639-644. 10.1292/jvms.56.639

Angen O, Svensmark B, and Mittal KR. 2004. Serological characterization of Danish *Haemophilus parasuis* isolates. *Veterinary Microbiology* 103:255-258. 10.1016/j.vetmic.2004.07.013

Aragon V, Cerda-Cuellar M, Fraile L, Mombarg M, Nofrarias M, Olivera A, Sibila M, Solanes D, and Segales J. 2010. Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains. *Veterinary Microbiology* 142:387-393. 10.1016/j.vetmic.2009.10.025

Aragon V, Segalés J, and Oliveira S. 2012. Glässer’s Disease. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, and Stevenson GW, eds. *Diseases of Swine*. 10 ed. Chichester: Wiley-Blackwell, 760-769.

Blackall PJ, Rapp-Gabrielson VJ, and Hampson DJ. 1996. Serological characterisation of *Haemophilus parasuis* isolates from Australian pigs. *Australian Veterinary Journal* 73:93-95.

Boerlin P, Poljak Z, Gallant J, Chalmers G, Nicholson V, Soltes GA, and MacInnes JJ. 2013. Genetic diversity of *Haemophilus parasuis* from sick and healthy pigs. *Veterinary Microbiology* 167:459-467. 10.1016/j.vetmic.2013.07.028

Brockmeier SL. 2004. Prior infection with *Bordetella bronchiseptica* increases nasal colonization by *Haemophilus parasuis* in swine. *Veterinary Microbiology* 99:75-78. 10.1016/j.vetmic.2003.08.013

Brockmeier SL, Palmer MV, Bolin SR, and Rimler RB. 2001. Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. *American Journal of Veterinary Research* 62:521-525.

Brockmeier SL, Register KB, Kuehn JS, Nicholson TL, Loving CL, Bayles DO, Shore SM, and Phillips GJ. 2014. Virulence and draft genome sequence overview of multiple strains of the swine pathogen *Haemophilus parasuis*. *PloS One* 9:e103787.
Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, and Jin M. 2005. Serological characterization of *Haemophilus parasuis* isolates from China. *Veterinary Microbiology* 111:231-236. 10.1016/j.vetmic.2005.07.007

Castilla KS, de Gobbi DD, Moreno LZ, Paixao R, Coutinho TA, dos Santos JL, and Moreno AM. 2012. Characterization of *Haemophilus parasuis* isolated from Brazilian swine through serotyping, AFLP and PFGE. *Research in Veterinary Science* 92:366-371. 10.1016/j.rvsc.2011.04.006

Cerda-Cuellar M, Naranjo JF, Verge A, Nofrarias M, Cortey M, Olvera A, Segales J, and Aragon V. 2010. Sow vaccination modulates the colonization of piglets by *Haemophilus parasuis*. *Veterinary Microbiology* 145:315-320. 10.1016/j.vetmic.2010.04.002

Del Rio ML, Gutierrez CB, and Rodriguez Ferri EF. 2003. Value of indirect hemagglutination and coagglutination tests for serotyping *Haemophilus parasuis*. *Journal of Clinical Microbiology* 41:880-882. 10.1128/JCM.41.2.880-882.2003

Dijkman R, Wellenberg GJ, van der Heijden HM, Peerboom R, Olvera A, Rothkamp A, Peperkamp K, and van Esch EJ. 2012. Analyses of Dutch *Haemophilus parasuis* isolates by serotyping, genotyping by ERIC-PCR and Hsp60 sequences and the presence of the virulence associated trimeric autotransporters marker. *Research in Veterinary Science* 93:589-595. 10.1016/j.rvsc.2011.10.013

Drew TW. 2000. A review of evidence for immunosuppression due to porcine reproductive and respiratory syndrome virus. *Veterinary Research* 31:27-39. 10.1051/vetres:2000106

Flores-Mendoza L, Silva-Campa E, Resendiz M, Osorio FA, and Hernandez J. 2008. Porcine reproductive and respiratory syndrome virus infects mature porcine dendritic cells and up-regulates interleukin-10 production. *Clinical and Vaccine Immunology* 15:720-725. 10.1128/CVI.00224-07

Howell KJ, Peters SE, Wang J, Hernandez-Garcia J, Weinert LA, Luan SL, Chaudhuri RR, Angen O, Aragon V, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Maskell DJ, Tucker AW, and Consortium BRT. 2015. Development of a multiplex PCR assay for rapid molecular serotyping of *Haemophilus parasuis*. *Journal of Clinical Microbiology* 53:3812-3821. 10.1128/JCM.01991-15

Howell KJ, Weinert LA, Chaudhuri RR, Luan SL, Peters SE, Corander J, Harris D, Angen O, Aragon V, Bensaid A, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Holden MT, Tucker AW, Maskell DJ, and Consortium BT. 2014. The use of genome wide association methods to investigate pathogenicity, population structure and serovar in *Haemophilus parasuis*. *BMC Genomics* 15:1179. 10.1186/1471-2164-15-1179

Howell KJ, Weinert LA, Luan SL, Peters SE, Chaudhuri RR, Harris D, Angen O, Aragon V,
Parkhill J, Langford PR, Rycroft AN, Wren BW, Tucker AW, Maskell DJ, and
Consortium BRT. 2013. Gene content and diversity of the loci encoding biosynthesis of
capsular polysaccharides of the 15 serovar reference strains of *Haemophilus parasuis*.
*Journal of Bacteriology* 195:4264-4273. 10.1128/JB.00471-13

Howell KJ, Weinert LA, Peters SE, Wang J, Hernandez-Garcia J, Chaudhuri RR, Luan SL,
Angen O, Aragon V, Williamson SM, Langford PR, Rycroft AN, Wren BW, Maskell DJ,
and Tucker AW. 2017. "Pathotyping" multiplex PCR assay for *Haemophilus parasuis*: a
tool for prediction of virulence. *Journal of Clinical Microbiology* 55:2617-2628.
10.1128/JCM.02464-16

Kavanova L, Prodelalova J, Nedbalcova K, Matiasovic J, Volf J, Faldyna M, and Salat J. 2015.
Immune response of porcine alveolar macrophages to a concurrent infection with porcine
reproductive and respiratory syndrome virus and *Haemophilus parasuis* in vitro.
*Veterinary Microbiology* 180:28-35. 10.1016/j.vetmic.2015.08.026

Kielstein P, and Rapp-Gabrielson VJ. 1992. Designation of 15 serovars of *Haemophilus parasuis*
on the basis of immunodiffusion using heat-stable antigen extracts. *Journal of Clinical
Microbiology* 30:862-865.

Kielstein P, Rosner H, and Mueller W. 1991. Typing of heat-stable soluble *Haemophilus
parasuis* antigen by means of agar gel precipitation and the dot-blot procedure. *Journal of
Veterinary Medicine B: Infectious Diseases and Veterinary Public Health* 38.

Li J, Wang S, Li C, Wang C, Liu Y, Wang G, He X, Hu L, Liu Y, Cui M, Bi C, Shao Z, Wang
X, Xiong T, Cai X, Huang L, and Weng C. 2017. Secondary *Haemophilus parasuis*
infection enhances highly pathogenic porcine reproductive and respiratory syndrome
virus (HP-PRRSV) infection-mediated inflammatory responses. *Veterinary Microbiology*
204:35-42. 10.1016/j.vetmic.2017.03.035

Li JX, Jiang P, Wang Y, Li YF, Chen W, Wang XW, and Li P. 2009. Genotyping of
*Haemophilus parasuis* from diseased pigs in China and prevalence of two coexisting
virus pathogens. *Preventive Veterinary Medicine* 91:274-279.
10.1016/j.prevetmed.2009.06.004

Lin CN, Lin WH, Hung LN, Wang SY, and Chiou MT. 2013. Comparison of viremia of type II
 porcine reproductive and respiratory syndrome virus in naturally infected pigs by zip
nucleic acid probe-based real-time PCR. *BMC Veterinary Research* 9:181. 10.1186/1746-
6148-9-181

Luppi A, Bonilauri P, Dottori M, Iodice G, Gherpelli Y, Merialdi G, Maioli G, and Martelli P.
2013. *Haemophilus parasuis* serovars isolated from pathological samples in Northern
Italy. *Transboundary and Emerging Diseases* 60:140-142. 10.1111/j.1865-
1682.2012.01326.x
Ma L, Wang L, Chu Y, Li X, Cui Y, Chen S, Zhou J, Li C, Lu Z, Liu J, and Liu Y. 2016. Characterization of Chinese Haemophilus parasuis isolates by traditional serotyping and molecular serotyping methods. PloS One 11:e0168903. 10.1371/journal.pone.0168903

Miniats OP, Smart NL, and Ewert E. 1991a. Vaccination of gnotobiotic primary specific pathogen-free pigs against Haemophilus parasuis. Canadian Journal of Veterinary Research 55:33-36.

Miniats OP, Smart NL, and Rosendal S. 1991b. Cross protection among Haemophilus parasuis strains in immunized gnotobiotic pigs. Canadian Journal of Veterinary Research 55:37-41.

Moller K, and Kilian M. 1990. V factor-dependent members of the family Pasteurellaceae in the porcine upper respiratory tract. Journal of Clinical Microbiology 28:2711-2716.

Morozumi T, and Nicolet J. 1986. Some antigenic properties of Haemophilus parasuis and a proposal for serological classification. Journal of Clinical Microbiology 23:1022-1025.

Nielsen R. 1993. Pathogenicity and immunity studies of Haemophilus parasuis serotypes. Acta Veterinaria Scandinavica 34:193-198.

Oliveira S, Blackall PJ, and Pijoan C. 2003. Characterization of the diversity of Haemophilus parasuis field isolates by use of serotyping and genotyping. American Journal of Veterinary Research 64:435-442.

Olvera A, Ballester M, Nofrarias M, Sibila M, and Aragon V. 2009. Differences in phagocytosis susceptibility in Haemophilus parasuis strains. Veterinary Research 40:24. 10.1051/vetres/2009007

Olvera A, Calsamiglia M, and Aragon V. 2006a. Genotypic diversity of Haemophilus parasuis field strains. Applied and Environmental Microbiology 72:3984-3992. 10.1128/AEM.02834-05

Olvera A, Cerda-Cuellar M, and Aragon V. 2006b. Study of the population structure of Haemophilus parasuis by multilocus sequence typing. Microbiology 152:3683-3690. 10.1099/mic.0.29254-0

Olvera A, Segales J, and Aragon V. 2007. Update on the diagnosis of Haemophilus parasuis infection in pigs and novel genotyping methods. Veterinary Journal 174:522-529. 10.1016/j.tvjl.2006.10.017

Palzer A, Haedke K, Heinritzi K, Zoels S, Ladinig A, and Ritzmann M. 2015. Associations among Haemophilus parasuis, Mycoplasma hyorhinis, and porcine reproductive and respiratory syndrome virus infections in pigs with polyserositis. Canadian Veterinary Journal 56:285-287.

Pina S, Olvera A, Barcelo A, and Bensaid A. 2009. Trimeric autotransporters of Haemophilus parasuis: generation of an extensive passenger domain repertoire specific for pathogenic
strains. *Journal of Bacteriology* 191:576-587. 10.1128/JB.00703-08

Rafiee M, Bara M, Stephens CP, and Blackall PJ. 2000. Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis*. *Australian Veterinary Journal* 78:846-849.

Rapp-Gabrielson VJ, and Gabrielson DA. 1992. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. *American Journal of Veterinary Research* 53:659-664.

Rubies X, Kielstein P, Costa L, Riera P, Artigas C, and Espuna E. 1999. Prevalence of *Haemophilus parasuis* serovars isolated in Spain from 1993 to 1997. *Veterinary Microbiology* 66:245-248. 10.1016/S0378-1135(99)00007-3

Segales J, Domingo M, Solano GI, and Pijoan C. 1999. Porcine reproductive and respiratory syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs. *Veterinary Microbiology* 64:287-297.

Smart NL, and Miniats OP. 1989. Preliminary assessment of a *Haemophilus parasuis* bacterin for use in specific pathogen free swine. *Canadian Journal of Veterinary Research* 53:390-393.

Solano GI, Segales J, Collins JE, Molitor TW, and Pijoan C. 1997. Porcine reproductive and respiratory syndrome virus (PRRSv) interaction with Haemophilus parasuis. *Veterinary Microbiology* 55:247-257.

Suradhat S, and Thanawongnuwech R. 2003. Upregulation of interleukin-10 gene expression in the leukocytes of pigs infected with porcine reproductive and respiratory syndrome virus. *Journal of General Virology* 84:2755-2760. 10.1099/vir.0.19230-0

Tadjine M, Mittal KR, Bourdon S, and Gottschalk M. 2004. Development of a new serological test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence in North America. *Journal of Clinical Microbiology* 42:839-840.

Takahashi K, Naga S, Yagihashi T, Ikehata T, Nakano Y, Senna K, Maruyama T, and Murofushi J. 2001. A cross-protection experiment in pigs vaccinated with *Haemophilus parasuis* serovars 2 and 5 bacterins, and evaluation of a bivalent vaccine under laboratory and field conditions. *Journal of Veterinary Medical Science* 63:487-491. 10.1292/jvms.63.487

Turni C, and Blackall P. 2007. Comparison of sampling sites and detection methods for *Haemophilus parasuis*. *Australian Veterinary Journal* 85:177-184. 10.1111/j.1751-0813.2007.00136.x

Turni C, and Blackall PJ. 2005. Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis*. *Veterinary Microbiology* 106:145-151.

Turni C, and Blackall PJ. 2010. Serovar profiling of *Haemophilus parasuis* on Australian farms by sampling live pigs. *Australian Veterinary Journal* 88:255-259. 10.1111/j.1751-
Vahle JL, Haynes JS, and Andrews JJ. 1995. Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings. *Journal of Veterinary Diagnostic Investigation* 7:476-480. 10.1177/104063879500700409

Vahle JL, Haynes JS, and Andrews JJ. 1997. Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived (CDCD) swine. *Canadian Journal of Veterinary Research* 61:200-206.

Zhang B, Tang C, Liao M, and Yue H. 2014. Update on the pathogenesis of *Haemophilus parasuis* infection and virulence factors. *Veterinary Microbiology* 168:1-7. 10.1016/j.vetmic.2013.07.027

**Figure legends**

**Figure 1** Distribution of *Haemophilus parasuis* serovars according to lesion pattern.

Serositis only: animals were diagnosed with *H. parasuis* positive serosal lesions. Pulmonary lesion only: animals were diagnosed with *H. parasuis* positive pulmonary lesions. Data were analyzed by Fisher’s exact test and variables were considered significant at a 0.05 level (two-sided).

**Figure 2** *Haemophilus parasuis* isolation proportion of 204 lesions of 108 pathological diagnosed cases.

Fisher’s exact test was used to compare the frequency of *H. parasuis* isolation lesions. *P* value < 0.05 was considered a significant difference.

**Figure 3** Molecular serotyping results with or without sequence results for 133 *Haemophilus parasuis* isolates.

**Supplementary file**

**Supplementary Figure 1** Gross meningeal lesion in *H. parasuis* infected pigs.

**Supplementary Figure 2** Histopathological suppurative meningitis lesion in *H. parasuis* infected pigs.

**Supplementary Figure 3** Gross pleural and peritoneal lesions in *H. parasuis* infected pigs.
Supplementary Figure 4  Gross epicardial lesion in *H. parasuis* infected pigs.

Supplementary Figure 5  Gross joint synovial cavity lesion in *H. parasuis* infected pigs.

Supplementary Figure 6  Histopathological fibrinous serositis lesion in *H. parasuis* infected pigs.

Supplementary Figure 7  Gross lung lesion in *H. parasuis* infected pigs.

Supplementary Figure 8  Histopathological pulmonary lesion in *H. parasuis* infected pigs.

Supplementary Figure 9  Histopathological fibrous serositis lesion in *H. parasuis* infected pigs.

Supplementary Figure 10  Histopathological meningitis lesion in *H. parasuis* infected pigs.

Supplementary Figure 11  Band patterns of molecular serotyping mPCR for *Haemophilus parasuis*.
Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane G2: molecular serotyping non-typable group 2, lane S4: serovar 4, lane S9: serovar 9, lane G1: molecular serotyping non-typable group 1, lane NC: negative control.

Supplementary Figure 12  Band patterns of molecular serotyping mPCR for *Haemophilus parasuis*.
Lane M: 50 bp DNA Ladder, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G2: molecular serotyping non-typable group 2, lane S14: serovar 14, lane G1: molecular serotyping non-typable group 1, lane NC: negative control.

Supplementary Figure 13  Band patterns of molecular serotyping mPCR for *Haemophilus parasuis*.
Lane M: 50 bp DNA Ladder, lane S5: serovar 5 or 12, lane S4: serovar 4, lane G1: molecular serotyping non-typable group 1, lane G2: molecular serotyping non-typable group 2, lane NC: negative control. Histopathological bronchopneumonia lesion in *H. parasuis* infected pigs.

Supplementary Figure 14  Band patterns of molecular serotyping mPCR for *Haemophilus parasuis*.
Lane M: 50 bp DNA Ladder, lane G1: molecular serotyping non-typable group 1, lane S4: serovar 4, lane S5: serovar 5 or 12, lane S14: serovar 14, lane G4: molecular serotyping non-typable group 4, lane G3: molecular serotyping non-typable group 3, lane NC: negative control.

Supplementary Figure 15 Band patterns of molecular serotyping mPCR for *Haemophilus parasuis*.

Lane M: 50 bp DNA Ladder RTU (GeneDireX), lane G2: molecular serotyping non-typable group 2, lane S4: serovar 4, lane S5: serovar 5 or 12, lane G1: molecular serotyping non-typable group 1, lane G3: molecular serotyping the non-typable group 3, lane NC: negative control.

Ethics and consent to participate

The study did not involve any animal experiment. The Institutional Animal Care and Use Committee (IACUC) of National Pingtung University of Science and Technology did not deem it necessary for this research group to obtain formal approval to conduct this study.

Consent to publish

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

Wei-Hao Lin, Chao-Nan Lin and Ming-Tang Chiou designed this study. Wei-Hao Lin performed the laboratory experiments, analyzed data and wrote the manuscript. Hsing-Chun Shih assisted the laboratory experiments. Chuen-Fu Lin, Cheng-Yao Yang, Yung-Fu Chang, Chao-Nan Lin and Ming-Tang Chiou proofread and edited the manuscript.

Availability of data and materials

All the data supporting our findings is contained within the manuscript.

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Figure 1

Distribution of *Haemophilus parasuis* serovars according to lesion pattern.

Distribution of *Haemophilus parasuis* serovars according to lesion pattern. Serositis only: animals were diagnosed with *H. parasuis* positive serosal lesions. Pulmonary lesion only: animals were diagnosed with *H. parasuis* positive pulmonary lesions. Data were analyzed by Fisher’s exact test and variables were considered significant at a 0.05 level (two-sided).
Figure 2

*Haemophilus parasuis* isolation proportion of 204 lesions of 108 pathological diagnosed cases.

*Haemophilus parasuis* isolation proportion of 204 lesions of 108 pathological diagnosed cases. Fisher’s exact test was used to compare the frequency of *H. parasuis* isolation lesions. *P* value < 0.05 was considered a significant difference.
Figure 3

Molecular serotyping results with or without sequence results for 133 *Haemophilus parasuis* isolates.
Table 1 (on next page)

Product size by molecular serotyping assay
| Gene   | Serovar | Product size in the original publication (bp) | Product size (bp) predicted by BLAST | Aligned sequence accession number | Product size (bp) measured by Bio-1D software | Product size (bp) according to sequence |
|--------|---------|-----------------------------------------------|--------------------------------------|-----------------------------------|-----------------------------------------------|----------------------------------------|
| funB   | 1       | 180                                           | 183                                  | CL120103                          | 184                                           | 183                                    |
| wzx    | 2†      | 295                                           | 294                                  | CL120103                          | N/A                                           | N/A                                    |
| glyC   | 3†      | 610                                           | 618                                  | KC795327.1                        | N/A                                           | N/A                                    |
| wciP   | 4       | 320                                           | 349                                  | KC795356.1                        | 350                                           | 349                                    |
| wcwK   | 5 or 12 | 450                                           | 468                                  | KC795341.1                        | 469                                           | 468                                    |
| gltI   | 6†      | 360                                           | 378                                  | KC795372.1                        | N/A                                           | N/A                                    |
| funQ   | 7†      | 490                                           | 499                                  | CP009158.1                        | N/A                                           | N/A                                    |
| scdA   | 8†      | 650                                           | 634                                  | KC795411.1                        | N/A                                           | N/A                                    |
| funV   | 9       | 710                                           | 676                                  | KC795429.1                        | 675                                           | 676                                    |
| funX   | 10†     | 790                                           | 784                                  | KC795448.1                        | N/A                                           | N/A                                    |
| amtA   | 11†     | 890                                           | 883                                  | KC795474.1                        | N/A                                           | N/A                                    |
| gltP   | 13†     | 840                                           | 836                                  | KF841370.1                        | N/A                                           | N/A                                    |
| funAB  | 14      | 730                                           | 710                                  | KC795520.1                        | 708                                           | 710                                    |
| funI   | 15†     | 550                                           | 550                                  | KC795537.1                        | N/A                                           | N/A                                    |
| HPS_219690793 | All | 275                                           | 276                                  | CP020085.1                        | 276                                           | 276                                    |

†This serotype was not detected in this study.
Table 2 (on next page)

Unexpected products of serotyping multiplex PCR
## Table 2 Unexpected products of serotyping multiplex PCR

| Molecular serotyping non-typable isolate | Serovar according to sequence | Product size (bp) measured by Bio-1D software | Product size (bp) according to sequence | Amplified primer |
|-----------------------------------------|------------------------------|-----------------------------------------------|------------------------------------------|-----------------|
| Group 1                                 | Unknown†                     | None‡                                         | None                                     | None            |
|                                         |                              | 300                                           | 297                                      | S13F, S14R      |
| Group 2                                 | Serovar 13                   | 830                                           | 836                                      | S13            |
|                                         |                              | 1000                                          | N/A§                                     | N/A            |
| Group 3                                 | Serovar 7                    | 500                                           | 499                                      | S7             |
| Group 4                                 | Serovar 13                   | 660                                           | N/A                                      | N/A            |
|                                         |                              | 300                                           | 297                                      | S13F, S14R      |

1. †Serovar could not be defined without any serovar-specific product sequence result.
2. ‡There was no serovar-specific product.
3. §Cloning of serovar-specific product was failed.