Investigation of *Haemophilus parasuis* from healthy pigs in China

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Highlights

- Updated the epidemiologic data of *Haemophilus parasuis* from healthy pigs in China.

- Serovars distribution was different from previous reports and showed regional difference.
- Elevated MICs were observed for majority of the tested antimicrobial agents.

Abstract

Haemophilus parasuis is a common colonizer of the upper respiratory tract of swine and frequently causes disease, especially in weaner pigs. To date, limited epidemiological data was available for *H. parasuis* from healthy pigs, which might be carriers of potential pathogenic strains. In this study, from September 2016 to October 2017, we investigated the prevalence and characteristics of *H. parasuis* from healthy pigs in China. Totally, we obtained 244 isolates from 1675 nasal samples from 6 provinces. *H. parasuis* isolation was more successful in weaner pigs (22.6%, 192/849), followed by finisher pigs (9.3%, 43/463), and sows (2.5%, 9/363). The most prevalent serovars were 7 (20.1%, 49/244), followed by 3 (14.8%, 36/244), 2 (14.3%, 35/244), 11 (12.7%, 31/244), 5/12 (5.7%, 14/244) and 4 (2.5%, 6/244). Bimodal or multimodal distributions of MICs were observed for most of the tested drugs, which suggested the presence of non-wild type populations. It was noted that the MIC90 values of tilmicosin (64 μg/ml) was relatively higher than that reported in previous studies. Our results suggest that: 1) potentially pathogenic serovars of *H. parasuis* are identified in healthy pigs, and 2) elevated MICs and presence of mechanisms of

resistance not yet described for clinically important antimicrobial agents would increase the burden of disease caused by *H. parasuis*.

Keywords

Prevalence

Healthy pigs

Antimicrobial susceptibility

Serovars distribution

Introduction

Haemophilus parasuis is an important swine pathogen that causes serious diseases, such as Glässer's disease, pneumonia and septicemia (Oliveira and Pijoan, 2004). In the United States, *H. parasuis* was the leading bacterial health challenge for nursery pigs (Holtkamp et al., 2007). The Animal and Plant Health Agency (APHA) reports that the annual incidence of *H. parasuis* disease shows a steady increasing trend (except 2012 and 2014) in England, Wales, and Scotland, from nearly 8% in 2002 to 14% in 2016 (APHA, 2016). *H. parasuis* is a common resident organism of the upper respiratory tract of swine and includes strains with different degree of virulence (Galofré-Milà et al., 2017). Basically, all piglets are colonized by the bacterium, but only certain types of strains are capable of causing disease, often following a perturbation to their host (e.g. co-infections, stress, medication) (Aragon et al., 2012).

Pathogenesis of *H. parasuis* is a multifactorial process, which is associated with virulence genes, serovars, biofilm production, etc. Although it is controversial whether serovar of *H. parasuis* is an indicator of virulence, serotyping is still an important feature of Glässer's disease diagnosis, as it is necessary for veterinarians and farmers to formulate a vaccination strategy. To date, a total of 15 serovars (serovars 1 through 15) of *H. parasuis* have been defined using a gel immuno-diffusion assay as well as molecular serotyping method (the latter cannot discriminate between serovars 5 and 12) (Howell et al., 2015). Serovars 5 and 4 of *H. parasuis* are widely considered disease-causing serovars and are the most common serovars of *H. parasuis* isolated from clinically sick pigs worldwide (Oliveira and Pijoan, 2004). On the other hand, serovars 3 and 7 of

H. parasuis are generally recognized as non-virulent, although they can be isolated from pathological samples and some reports also indicate that isolates from those serovars can cause disease (Aragon et al., 2010; Zhang et al., 2012; Costa-Hurtado et al., 2013). The prevalence and distribution of serovars of *H. parasuis* isolated from clinical cases has been widely studied but not so from those isolated from healthy carrier pigs.

In China and many other countries, antimicrobial agents are still largely used and play an important role for the prevention and treatment of bacterial diseases. Tilmicosin and florfenicol are the drugs of choice for prevention and treatment of *H. parasuis* infections in China. Historically, *H. parasuis* isolates showed high susceptibility to frequently used drugs (El Garch et al., 2016; Zhou et al., 2010), but recently multiple publications indicate a decreased susceptibility of *H. parasuis* to several antimicrobial agents from a number of countries (Brogden et al., 2018; Dayao et al., 2014). In China, the last nationwide surveillance of antimicrobial susceptibility of *H. parasuis* was reported in 2010 (Zhou et al., 2010), which showed that Chinese *H. parasuis* isolates were susceptible to most of the antimicrobial agents. However, isolates from healthy piglets has been shown to carry plasmids with antimicrobial resistance genes, which may play an important role in the spreading of these resistances (Moleres et al., 2015).

In order to understand the current situation of *H. parasuis* from healthy pigs, here, we investigated the prevalence and characteristics of this organism from 6 provinces in China during 2016–2017. Our findings provide more information for better understanding of the serovars and antimicrobial resistance distribution in *H. parasuis*.

Materials and methods

Farms, animals and collection of samples

From September 2016 to October 2017, nasal swabs were collected from healthy pigs on 13 pig farms and 2 slaughterhouses located in 6 provinces (Table 1). Samples were collected from different growth stages of pigs: sows, weaned pigs and finishers. Nasal swabs were placed in tryptic soy broth (TSB) containing nicotinamide adenine dinucleotide (NAD) (10 μg/ml) and 5% (v/v) fetal calf serum (FCS), and then transported to the laboratory in a Styrofoam container with ice packs (within one day). A brief questionnaire was collected from each farm. The contents of the questionnaire included the type of pig production, herd size, breeds of pigs, age of piglets and history of antimicrobial usage.

Table 1. Source of 244 *H. parasuis* isolates from healthy pigs 2016–2017.

Province	Percentage of isolation (No. of nasal samples)			Total
	Sow	Weaner	Finisher	
Beijing	1.2% (86)	17.3% (52)	16.7% (60)	10.1% (198)
Shandong	4.5% (134)	18.3% (153)	8.8% (114)	11.0% (401)
Henan	2.7% (37)	22.7% (97)	–	17.2% (134)
Shanghai	–	31.0% (29)	8.2% (158)	11.8% (187)
Sichuan	1.5% (68)	12.8% (226)	2.2% (89)	8.4% (383)

Province	Percentage of isolation (No. of nasal samples)			Total
	Sow	Weaner	Finisher	
Chongqing	0% (38)	32.5% (292)	19.0% (42)	27.7% (372)
Total	2.5% (363)	22.6% (849)	9.3% (463)	14.6% (1675)

Bacterial isolation and identification

Nasal samples were vortexed vigorously upon arrival at the laboratory. A loopful of suspension from each sample was streaked onto tryptic soy agar (TSA) plates containing NAD (10 μg/ml) and 5% (v/v) FCS, and then incubated at 37℃ for 36 h. One or two suspect colonies from the agar plate were subjected to further identification by polymerase chain reaction (PCR) as reported previously (Oliveira et al., 2001).

DNA preparation and serotyping

The DNA templates for PCR amplification were prepared by boiling. Briefly, 1 ml of an overnight culture was spinned down at 12,000 × g for 5 min and the bacterial pellet was resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was heated at 100 °C for 10 min, followed by cooling on ice for 10 min. After centrifugation at 12,000 × g for 5 min, the supernatant was transferred to a nucleic acid-free tube and stored at 20 °C.

Serovars of the *H. parasuis* isolates were determined using a previously described one-step multiplex PCR (Howell et al., 2015).

Antimicrobial susceptibility testing

Currently, the recommended method for antimicrobial susceptibility testing of *H. parasuis* is not available in the Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentrations (MICs) of *H. parasuis* to a panel of antimicrobial agents were determined by the broth microdilution method (Clinical and Laboratory Standards Institute, 2013) using cation-adjusted Mueller-Hinton broth containing NAD (25 μg/ml) and 1% (v/v) sterile filtered heat-inactivated chicken serum as suggested by a previous report (Prüller et al., 2017). Antimicrobial agents and concentrations tested were: amoxicillin/clavulanic acid (0.06/0.03–64/32 μg/ml), ceftiofur (0.015–16 μg/ml), enrofloxacin (0.015–16 μg/ml), florfenicol (0.06–64 μg/ml), erythromycin (0.12–128 μg/ml), tilmicosin (0.12–128 μg/ml), doxycycline (0.06–64 μg/ml), and tetracycline (0.12–128 μg/ml). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains.

The MIC_{50} and MIC_{90} were defined as the MIC value at which ≥ 50% and ≥ 90% of the isolates within a test population are inhibited. If the resulting number was not an integer, the next integer following the respective value represented the MIC_{50} and MIC_{90} (Schwarz et al., 2010).

Data analysis

The comparison between categorical variables was carried out via $\chi 2$ test using GraphPad prism v7.0. P values lower than 0.05 were considered significant.

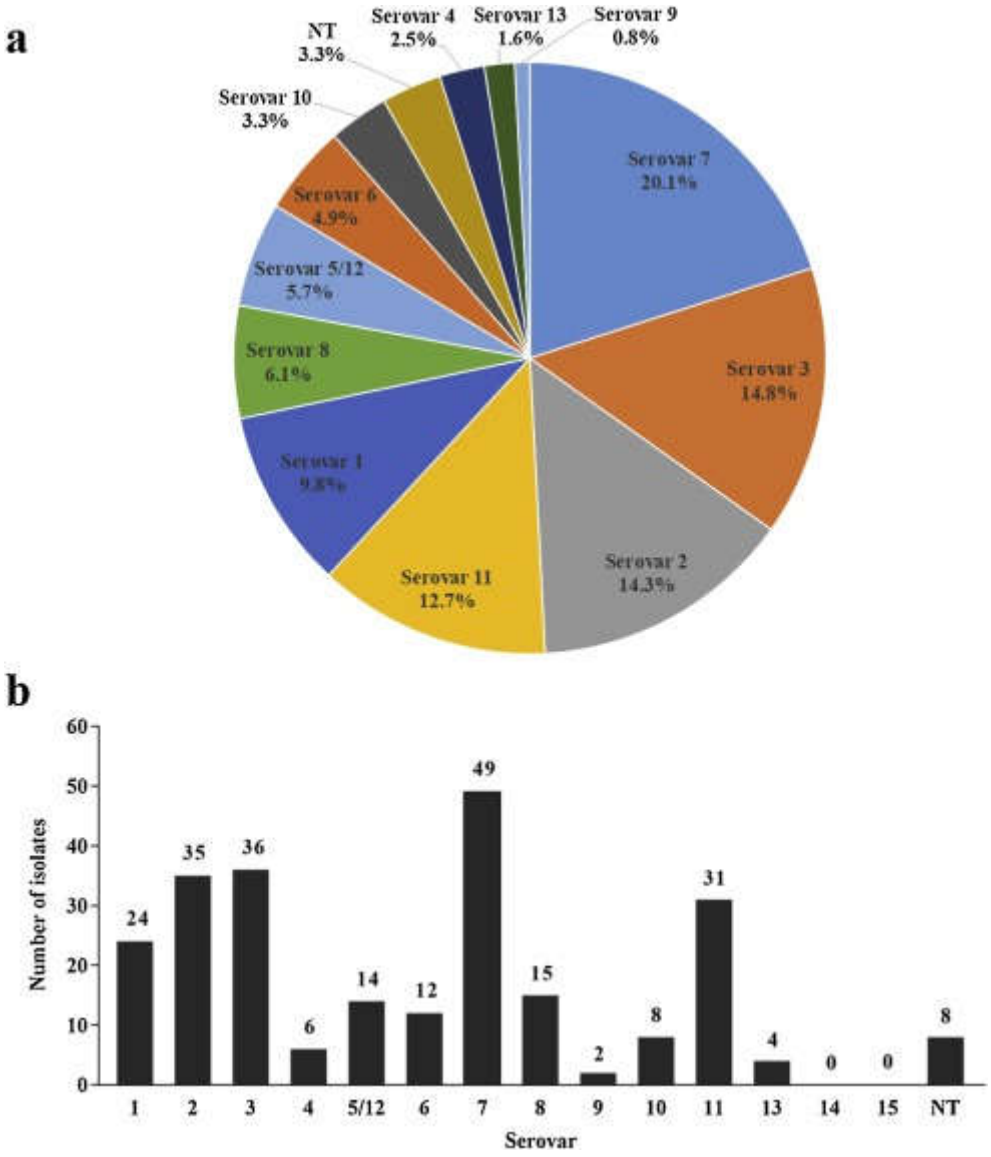
Results

Prevalence of *H. parasuis*

During the study period, a total of 244 *H. parasuis* isolates were obtained from 1675 nasal samples (Table 1): 9 isolates from sows, 192 isolates from weaners and 43 isolates from finishers. The overall isolation rate was 14.6% (244/1675). Highest prevalence was observed in weaners (22.6%, 192/849), followed by finishers (9.3%, 43/463) and sows (2.5%, 9/363).

Serovar distribution

Using the multiplex PCR method, 244 isolates were assigned to 12 serovars, and 8 isolates were non-typeable. The highest prevalence was observed for serovar 7 (20.1%, 49/244), followed by serovar 3 (14.8%, 36/244), serovar 2 (14.3%, 35/244), serovar 11 (12.7%, 31/244), serovars 5/12 (5.7%, 14/244) and serovar 4 (2.5%, 6/244) (Fig. 1). Serovars 14 and 15 were not detected.



180

181 Fig. 1. Serovar distribution of 244 *H. parasuis* isolates from healthy pigs by (a)

182 percentages and (b) number of isolates. NT means non-typeable.

183

184 As shown in Fig. 2, serovars 1 and 3 were detected from samples from all the 6

185 provinces, while serovar 11 was detected only from Chongqing and Beijing.

186 Most of the serovar 2 isolates were from Shandong, Shanghai and Beijing. For

187 serovar 3, most of the isolates were from Sichuan and Chongqing.

188

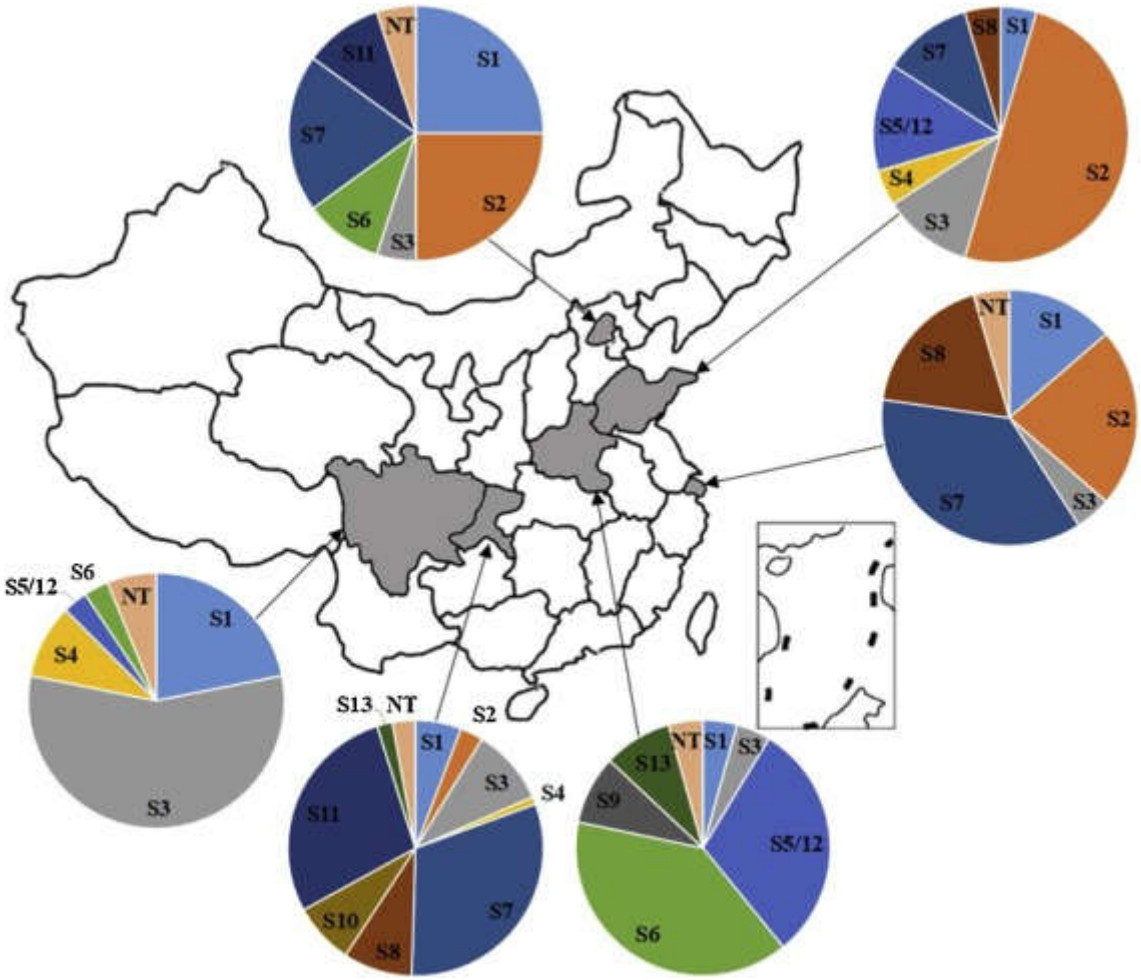


Fig. 2. Geographic distribution of serovars of 244 *H. parasuis* isolates from healthy pigs in 6 provinces of China. The pie chart showed the proportion of the serovar type in each province. NT means non-typeable.

Antimicrobial susceptibility profiles

The MIC distribution of 8 antimicrobials for the 244 *H. parasuis* isolates are summarized in Table 2. A bimodal or multimodal distribution of MICs was detected for enrofloxacin, florfenicol, erythromycin, tetracycline, tilmicosin, and doxycycline.

199 Table 2. MIC distribution of 244 *H. parasuis* isolates from healthy pigs 2016–

200 2017.

Antimicrobial agent	No. of isolates with MIC values (μg/ml) of:																
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Amoxicillin/Clavulanic acid[a]			54*	2	48	47	45	32	12	4	0	0	0	0			0.25	1
Ceftiofur	126*	2	36	39	13	12	8	4	4	0	0	0					<0.015	0.25
Enrofloxacin	40*	3	26	29	14	18	29	49	27	5	3	1					0.25	2
Florfenicol				0	5	66	140	15	1	0	0	11	6	0			0.5	1
Erythromycin				2*	0	3	11	77	77	28	18	18	6	0	2	2*	2	16
Tilmicosin				1*	1	5	23	47	21	27	17	21	33	29	18	1*	4	64
Doxycycline			5*	2	19	76	38	45	25	13	18	3	0	0			0.5	4
Tetracycline				4*	4	23	59	67	10	7	26	25	19	0	0		1	16

201 a

202 Concentrations for amoxicillin given, tested with clavulanic acid in a concentration ratio 2:1.

203 *

204 Number of isolates with MIC values equal to or higher/lower than concentrations of the test

205 range. The white areas represent the tested range of an antimicrobial agent.

206

207 The MIC_{50} and MIC_{90} values for *H. parasuis* were also calculated (Table 2). For

208 antimicrobial agents most frequently used in preventing and treating diseases

209 caused by *H. parasuis* (e.g. tilmicosin and florfenicol), elevated MICs were

210 observed compared to that from previous reports (Zhou et al., 2010). For

211 antimicrobial agents less frequently used for the treatment of *H. parasuis*

212 infections, the MIC_{90} and MIC_{50} values were relatively low. For example, MIC_{50}
213 and MIC_{90} values of ceftiofur were 0.015 μg/ml and 0.25 μg/ml, respectively.

214

215 **Discussion**

216 *H. parasuis* is one of the most important pathogens in pigs. Certain serovars of
217 this organism are clearly associated with clinical infections. In this study, we
218 obtained 244 *H. parasuis* isolates from 1675 nasal samples from healthy pigs
219 across 6 provinces. We also characterized the serovars and antimicrobial
220 susceptibility of the *H. parasuis* isolates. Our result indicated the high
221 prevalence and increased antimicrobial resistance of *H. parasuis* from healthy
222 pigs. In addition, pathogenic serovars were observed in these isolates.

223 The overall prevalence by culture (14.6%) of *H. parasuis* among healthy pigs in
224 this study was slightly lower than that found in diseased pigs (22.1%) in a
225 previous report (Cai et al., 2005), but this discordance can be explained by the
226 differences between both studies. Cai et al. (2005) included isolates collected
227 from lesions of diseased pigs, which more probably were weaned pigs, while in
228 our study, samples were obtained from the nasal cavities of healthy sows,
229 weaners and finishers. Our results confirm that the prevalence of *H. parasuis* in
230 weaned pigs was significantly higher than in finisher pigs (22.6% vs 9.3%, $P < 0.0001$)
231 and sows (22.6% vs 2.5%, $P < 0.0001$), as previously reported (Angen
232 et al., 2007; Cerdà-Cuéllar et al., 2010). Moreover, even in weaned pigs, the
233 isolation rate varied between different farms, ranging from 0% to 51.6% (data
234 not shown). The phenomenon was also observed in previous reports (MacInnes
235 et al., 2008; Turni and Blackall, 2010).

Serovars 7, 3, 2, and 11 were the most prevalent serovars (all above 10%), which are largely different from previous reports with clinical isolates, where serovars 5 and 4 were the predominant serovars (Angen et al., 2004; Cai et al., 2005; Castilla et al., 2012; Ma et al., 2016). The difference is probably due to the sampling sites: all the nasal samples in this study were collected from healthy pig herds, and in other studies the samples were lungs, fluids from joints, etc. which were obtained from sick pigs. Serovars 7, 3, and 11 were used to be considered as avirulent, and serovar 2 as moderate virulent according to challenge experiment using a small number of isolates (Aragon et al., 2010; Kielstein and Rapp-Gabrielson, 1992). In fact, serovars 3 and 11 isolates were also reported virulent or even highly virulent (Aragon et al., 2010; Dai et al., 2016). Serovars 5 and 4 isolates are widely considered disease-causing strains, and they were most commonly isolated from sick pigs with Glässer's disease (Howell et al., 2014; Tadjine et al., 2004). In the present study, serovars 5/12 and 4 were detected in a low percentage (5.7% and 2.5% for 5/12 and 4, respectively). However, this low isolation of serovars 5/12 and 4 in healthy herds can be potentially significant because these isolates may epidemically spread under certain circumstances. It was mentioned that 93.5% (29/31) of serovar 11 *H. parasuis* were isolated from Chongqing province, which showed an obvious regional difference.

MICs distribution is one of the most important parameters for reporting results of antimicrobial susceptibility testing (Schwarz et al., 2010). If the MICs distribution is bimodal or multimodal, it usually indicates the presence of resistance mechanism/s (Morrissey et al., 2014). The MICs of most of the antibiotics in our study showed bimodal or multimodal distributions (enrofloxacin, florfenicol,

erythromycin, tetracycline, tilmicosin, and doxycycline). For example, florfenicol MICs showed a bimodal distribution, and isolates with MICs > 4 μg/ml (distributed around the second peak) were proved to harbor the phenicol resistance gene floR (data not published). It was noteworthy that known macrolide resistance mechanisms cannot completely account for the multimodal distribution of tilmicosin MICs (data not published), which might imply a new tilmicosin resistance mechanism. This was of great concern, as nasal isolates were considered as an important reservoir for antimicrobial resistance. Bimodal or multimodal distributions of tilmicosin MICs were also reported in Germany and Australia recently (Brogden et al., 2018; Dayao et al., 2014, 2016).

For most of the tested antimicrobials, elevated MICs were observed compared to previous reports. For example, tilmicosin MIC_{90} values were 64 μg/ml in our study, which are 16-fold higher than that in Germany (Brogden et al., 2018) and 32-fold higher than previously described in China (Zhou et al., 2010). Florfenicol MIC_{90} values (1 μg/ml) were similar to those in Germany (0.5 μg/ml) and China in 2010 (1 μg/ml). However, no isolates had MICs > 1 μg/ml in the two previously reported studies, while in our study, 7% (17/244) of the isolates had MICs > 8 μg/ml.

Conclusions

In conclusion, serovar distribution was different from previous reports with clinical isolates and showed regional differences. Elevated MICs of *H. parasuis* for most of the antimicrobial agents were possibly due to the spread of multiple resistance mechanisms by the selection of antibiotics, especially for those

frequently used for preventing and treating diseases caused by this genus, which highlighted the importance of using antimicrobials more prudently. All these findings provide us an overview of current epidemiological status of *H. parasuis* and can be helpful for the prevention and understanding of the disease caused by this bacterium.

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