Plasmid Mediated Streptomycin and Sulfonamide Resistance in *Haemophilus parasuis*

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**Abstract:** Streptomycin and sulfonamide resistance in *Haemophilus parasuis* is an emerging phenomenon that has not yet been characterized from a molecular perspective. The aim of the present research was to determine the molecular mechanism of *Haemophilus parasuis* resistant to streptomycin and sulfonamide. One high level streptomycin and sulfonamide-resistant isolate from China was submitted for MIC determination and plasmid extract, then sequencing and annotation. One plasmid extracted from the bacteria strain, designated pHPS-A67 (4.2 kb), contain fifteen open reading frames including streptomycin resistance gene *StrA*, sulfonamide resistance gene *Sul 2* and mobilization protein genes *Mob A*, *Mob B* and *Mob C*. This is believed to be the first report of native *Haemophilus parasuis* contains plasmid which mediated streptomycin and sulfonamide resistance in this microorganism.

**Key words:** *Haemophilus parasuis*, streptomycin, sulfonamide, plasmid, pig, China

**INTRODUCTION**

*Haemophilus parasuis* can cause Glasser’s disease characterized by fibrinous polyserositis, polyarthritis and meningitis or acute pneumonia without polyserositis and acute septicemia. Acute infections are occasional and the clinical disease particularly affects young animals exposed to stress (Nedbalcova et al., 2006). *Haemophilus parasuis* is commonly isolated from nasal cavities, tonsils and the upper part of trachea. Infections caused by *Haemophilus parasuis* in pigs have become worldwide at present. The consequences of these infections are economic losses due to mortality of animals in acute form of the disease and expensive antibiotic treatment.

Control of Glasser’s disease can be achieved by use of vaccination however, serovar diversity and the high number of non-typable isolates reported have affected negatively the development of effective crossprotective vaccines (Oliveira and Pjioan, 2004). For this reason, a wide variety of antibacterial agents have been used to treat this disease which is widely believed to increase the rate of antimicrobial resistance in *Haemophilus parasuis*. Resistance to antimicrobials may be encoded either by the chromosome or by a plasmid (Habi and Daba, 2009; Nazir et al., 2005). This selective pressure may still affect the resistance of the *Haemophilus parasuis* strains which reside in the tonsils without causing disease. From 2005-2008, 110 *Haemophilus parasuis* clinical isolates from diseased pigs were obtained in the laboratory at the Wuhan Keqian Animal Biological Products Co., Ltd. in the course of routine diagnosis. The antimicrobial resistance status of these *Haemophilus parasuis* isolates were determined. The result indicated that up to 44% of the clinical isolates are highly resistant to trimethoprim/sulphamethoxazole and >33.6% of the clinical isolates are highly resistant to streptomycin (Zhou et al., 2010). One high level streptomycin and sulfonamide-resistant isolate strain was then screened for the presence of resistance plasmid and sequence.

Plasmid-mediated antimicrobial resistance in *Haemophilus parasuis* has been reported in study (Lancashire et al., 2005; Millan et al., 2007) but streptomycin and sulfonamide-resistant plasmid has not been reported till date. Thus, the purpose of the present research was to determine the molecular mechanism of *Haemophilus parasuis* resistant to streptomycin and sulfonamide.

**MATERIALS AND METHODS**

**Bacteria strain and species identification:** The *H. parasuis* isolate A67 was recovered from the lung of a 2 months old pig submitted to the diagnosis laboratory...
at the Wuhan Keqian Animal Biological Products Co., Ltd. which suffering polyseritis, pneumonia and septicemia from herds located in Central China. Identification was performed using phenotypic characteristics in combination with a PCR based on species-specific amplification of the 16S rRNA gene with primers HPS-F

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and HPS-R GGC TTC GTC ACC CTC TGT essentially as described by Oliveira et al. (2001) and biochemical tests. Reference strain Histophilus somni ATCC 700025 was purchased from Beijing Zhongyuan Ltd.

**Antimicrobial susceptibility determination:** Antimicrobial Minimum Inhibitory Concentrations (MIC) of streptomycin and trimethoprim/sulfamethoxazole (China Institute of Veterinary Drug Control) were determined by the Standard Broth Doubling Dilution Method according to the CLSI, Document M31-A3 (2008). Histophilus somni ATCC 700025 was used as the control strain in the MIC determination. Bacteria were cultured on Veterinary Fastidious Medium at 35°C under microaerophilic conditions (5% CO₂).

**Species-specific PCR:** A simple PCR-based technique was developed to determine whether resistance gene was located in the plasmid. The technique is based on extraction and purification of plasmids and subsequent PCR with two primers, the sulfonamide resistance gene sul 2 probe and universal primers of the chromosomal encoded 16S rRNA.

**DNA techniques:** Plasmid Mini kit (Omega) was used for plasmid purification. Restriction, Taq polymerase for amplifying probes by PCR and DNA modification enzymes were purchased from Takara (China).

**DNA sequencing, analysis and annotation of plasmids:** DNA fragments from a mini-prep isolation of plasmid derived from strain A67 digested with Sau3AI (cloned fragment sizes, 0.7 kb) was cloned into plasmid pUC19 digested with BamHI. Following EDTA/ethanol precipitation, samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for automated sequencing using an ABI 3730×196 capillary automatic sequencer (PE Applied Biosystems) and sequenced by the Dideoxy Chain Termination Method was started with the M13 reverse and forward primers. Purified plasmid DNA from strain A67 was used as a template in subsequent sequencing reactions. After each round of sequencing, new primers were designed until a complete double-stranded sequence of the plasmid was obtained.

Sequence data were aligned and annotated using Software DNASTar and the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html; last accessed 30 March, 2011). Sequence comparisons were performed with the BLAST programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/; last accessed 30 March, 2011).

**Nucleotide sequence accession numbers:** The annotated nucleotide sequence of plasmid pHPS-A67 has been deposited in the GenBank database under Accession No. FJ670543.

**RESULTS AND DISCUSSION**

**Antimicrobial susceptibility:** The MICs for the original Haemophilus parasuis strain of streptomycin and sulfonamide were 128 and 512 μg mL⁻¹, respectively. There are only very limited number of reports on the susceptibility of Haemophilus parasuis and molecular mechanism of resistance in China. About 110 Haemophilus parasuis isolates in China were susceptible to tested antimicrobial agents (ampicillin, cefotaxime, erythromycin, florfenicol, penicillin, spectinomycin, tetracycline, tiamulin, tilmicosin but resistance was observed to streptomycin, kanamycin, gentamicin, sulfonamide, TMP-sulfonamide, enrofloxacin and ciprofloxacin (Zhou et al., 2010). Haemophilus parasuis harbored resistance gene to beta-lactam (Millan et al., 2007) and tetracycline resistance (Lancashire et al., 2005) in plasmid has been reported.

A negative signal with the 16S rRNA primers shows that no chromosomal DNA is present in the plasmid preparations whereas a positive signal with the probe primers indicates that the sul 2 gene is located in a plasmid. The data show that sul 2 of Haemophilus parasuis is encoded in an extra chromosomal plasmid and demonstrate that PCR is a valuable technique for assessing plasmid locations of genes in Haemophilus parasuis.

The plasmid is 4,248 bp in size and 15 Open Reading Frames (ORFs) were found in the plasmid. Detailed analysis of the sequence revealed that streptomycin resistance gene StrA, sulfonamide resistance gene sul 2 and plasmid mobilization genes MobA, MobB and MobC (Table 1) were contained in it.

The sulfonamide resistance gene sul 2 encode a type II Dihydropteroate Synthase (DHPIS II). The DHPIS II from the majority of the plasmids exhibited 100% identity to the gene product expressed from plasmids pMS260 of A. pleuroptuatorum and RSA1010 of E. coli (Ito et al., 2004). The sul 2 was one of the most frequent genes found in the family Pasteurellaceae and its genetic location has yet to be determined and characterized.
The streptomycin resistance gene \textit{StrA} which specifies an aminoglycoside phosphotransferase was 100\% similarity to \textit{Escherichia coli} SMS 3-5 plasmid pSMS35\textunderscore 130 (Fricke et al., 2008), \textit{P. multocida} plasmid pG1 (Accession No. NP\textunderscore 054472), \textit{A. pleuropneumoniae} plasmid pPSAS1522 (Accession No. CAI 46313) and \textit{Escherichia coli} plasmid pLEW517\textunderscore p54 (Accession No. YP\textunderscore 001096379). Indeed, the \textit{sul2} and \textit{StrA} genes were widespread among Enterobacteriaceae (Sundin and Bender, 1996) and the presence of such highly conserved resistance genes is an indication that horizontal gene transfer events have occurred recently between bacteria of these families. The result indicated that multidrug resistance plasmids are already widespread in the pig population.

Genes which may be involved in plasmid mobilization was found in the plasmids. Mobilization proteins are relaxases which cleave one of the DNA strands at a specific origin. The MobC reading frame coded for a protein of 101 amino acids which showed 100\% identity to the MobC proteins from the \textit{P. multocida} plasmid pG1 (Accession No. NP\textunderscore 054474), pR1 (Accession No. NP\textunderscore 848169) and the \textit{Mannheimia haemolytica} plasmid pMHSCSI (Accession No. NP\textunderscore 073218). The MobA reading frame coded for a protein of 339 amino acids which showed 100\% identity to the MobA proteins from the \textit{P. multocida} plasmid pG1 (Accession No. NP\textunderscore 054475), the \textit{Actinobacillus Pleuropneumoniae} plasmid pMHSCSI (Accession No. NP\textunderscore 073219) and the \textit{Mannheimia haemolytica} plasmid pPSAS1522 (Accession No. YP\textunderscore 245434).

Within the \textit{MobA} gene, there was a reading frame for MobB protein. The functionality of each Mob protein remains to be demonstrated. However, the presence of \textit{Mob} genes supports the strong potential for these antibiotic resistance plasmids to spread within the pig flora.

The frequent use of antimicrobial agents to treat or prevent respiratory and intestinal infections in pig husbandry has been selected for an antibiotic resistant flora. Members of the family Pasteurellaceae are also affected by the antimicrobial selective pressure and plasmid mediated antibiotic resistance has been reported in the genera \textit{Pasteurella} (Wright et al., 1997), \textit{Haemophilus} (Millan et al., 2007) and \textit{Actinobacillus} (Ito et al., 2004).

The presence of the plasmid in \textit{Haemophilus parasuis} and the high identity to known antibiotic resistance and mobilization genes indicate that virulence bacteria strain from pigs may accumulate antibiotic resistance determinants from other bacterial species and thereby also participate in the distribution of antibiotic resistance.

The distribution of these antibiotic resistance plasmids among different pigs and farms indicates that \textit{Haemophilus parasuis} species have a high ability to acquire antibiotic resistances and to spread them within the pig population. The location of up to two resistance genes on the same plasmid poses the evident risk of co-selecting for several resistances either by the use of sulfonamides and streptomycin. Since, streptomycin and sulfonamide are among the most used antimicrobials in veterinary medicine in China, the extensive use of such drugs may have contributed to the successful spread of these genetic determinants in zoonotic pathogens.

**CONCLUSION**

The results of this study showed that the swine pathogen \textit{Haemophilus parasuis} resistant to sulfonamide was mediated by a plasmid which also carried gene for resistance to streptomycin, composed of several segments previously found on other plasmid which has been found either in bacteria such as \textit{E. coli} and \textit{Actinobacillus pleuropneumoniae} from pigs and cattle. To the best of the knowledge, the present study is the first research of \textit{StrA} and \textit{sul2} resistance genes on a plasmid isolated from \textit{Haemophilus parasuis}.

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REFERENCES


