Journal of Animal and Veterinary Advances 11 (8): 1106-1109, 2012
ISSN: 1680-5593

Plasmid Mediated Streptomycin and Sulfonamide Resistance in *Haemophilus parasuis*

[1, 2]Pin Chen, [3]Yingyu Liu, [3]Yang Wang, [3]Wentao Li, [1]Dingren Bi and [1, 3]Qigai He
[1]College of Veterinary Medicine,
[2]National Reference Laboratory of Veterinary Drug Residues,
[3]State Key Laboratory of Agricultural Microbiology,
Huazhong Agricultural University, 430070 Wuhan, China

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 septicaemia. 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 Pijoan, 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/sulphmethoxazole 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

Corresponding Author: Qigai He, College of Veterinary Medicine, Huazhong Agricultural University, 430070 Wuhan, China

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 GTG ATG AGG AAG GGT GGT GT 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.

Antimicrobialsusceptibilitydetermination: 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 accordance 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% CO_2).

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×l 96 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;lastacc essed 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 *Haemophlius parasuis* and molecular mechanism of resistance in China. About 110 *Haemophilus parasuis* isolates in China were susceptible to tested antimicrobial agents (ampicillin, ceftifour, erythromycin, florfenicol, penicillin, spectinomycin, tetracycline, tiamulin, tilmicosin but resistance was observed to streptomycin, kanamycin, gentamycin, sulfonamide, TMP+sulfonamide, enrofloxacin and ciprofloxacin (Zhou *et al*., 2010). *Haemophlius 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 (DHPS II). The DHPS II from the majority of the plasmids exhibited 100% identity to the gene product expressed from plasmids pMS260 of *A. pleuropneumoniae* and RSF1010 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.

Table 1: Summary of genes found on the plasmid pHPS-A67 of *H. parasuis*

Genes	Putative function	Length (aa)	Protein identity (%)	Source of homology	Nucleotide accession No.
MobA	Plasmid mobilization	339	100	*Pasteurella multocida*, pIG1	NP_054475
MobB	Plasmid mobilization	90	100	*Pasteurella multocida*, pB1005	ACN39574
MobC	Plasmid mobilization	101	100	*Pasteurella multocida*, pIG1	NP_054475
Str A	Aminoglycoside phosphotransferase	163	100	*Pasteurella multocida*, pIG1	NP_054475
sul 2	Dihydropteroate synthase	283	99	*Actinobacillus pleuropneumoniae*, pSMS35_130	YP_001969930

The streptomycin resistance gene *StrA* which specifies an aminoglycoside phosphotransferase was 100% similarity to *Escherichia coli* SMS 3-5 plasmid pSMS35_130 (Fricke *et al*., 2008), *P. multocida* plasmid pIG1 (Accession No. NP_054472), *A. pleuropneumoniae* plasmid pPSAS1522 (Accession No. CAI 46313) and *Escherichia coli* plasmid pLEW517_p54 (Accession No. YP_001096379). Indeed, the *sul 2* and *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 *P. multocida* plasmid pIG1 (Accession No. NP_054474), pJR1 (Accession No. NP_848169) and the *Mannheimia haemolytica* plasmid pMHSCS1 (Accession No. NP_073218). The MobA reading frame coded for a protein of 339 amino acids which showed 100% identity to the MobA proteins from the *P. multocida* plasmid pIG1 (Accession No. NP_054475), the *Actinobacillus Pleuropneumoniae* plasmid pMHSCS1 (Accession No. NP_073219) and the *Mannheimia haemolytica* plasmid pPSAS1522 (Accession No. YP_245434).

Within the *MobA* gene, there was a reading frame for MobB protein. The functionality of each Mob protein remains to be demonstrated. However, the presence of *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 *Pasteurella* (Wright *et al*., 1997), Haemophilus (Millan *et al*., 2007) and Actinobacillus (Ito *et al*., 2004).

The presence of the plasmid in *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 *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 in the swine pathogen *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 *E. coli* and *Actinobacillus pleuropneumoniae* from pigs and cattle. To the best of the knowledge, the present study is the first research of *StrA* and *sul 2* resistance genes on a plasmid isolated from *Haemophilus parasuis*.

ACKNOWLEDGEMENTS

This research was supported by the earmarked fund for China Agriculture Research System (CARS-36). The researchers wish to thank Xueli Zhou for his kind donation of the *Haemophilus parasuis* isolated strain which used for plasmid isolation.

REFERENCES

CLSI, Document M31-A3, 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard. 3rd Edn., Cal and Laboratory Standards Institute, Wayne, PA.

Fricke, W.F., M.S. Wright, A.H. Lindell, D.M. Harkins, C. Baker-Austin, J. Ravel and R. Stepanauskas, 2008. Insights into the environmental resistance gene pool from the genome sequence of the multidrug-resistant environmental isolate *Escherichia coli* SMS-3-5. J. Bacteriol., 190: 6779-6794.

Habi, S. and H. Daba, 2009. Plasmid incidence, antibiotic and metal resistance among enterobacteriaceae isolated from algerian streams. Pak. J. Biol. Sci., 12: 1474-1482.

Ito, H., H. Ishii and M. Akiba, 2004. Analysis of the complete nucleotide sequence of an Actinobacillus pleuropneumoniae streptomycin-sulfonamide resistance plasmid, pMS260. Plasmid, 51: 41-47.

Lancashire, J.F., T.D. Terry, P.J. Blackall and M.P. Jennings, 2005. Plasmid-encoded tet B tetracycline resistance in *Haemophilus parasuis*. Antimicrob. Agents Chemother, 49: 1927-1931.

Millan, A.S., J.A. Escudero, A. Catalan, S. Nieto and F. Farelo *et al*., 2007. β-lactam resistance in *Haemophilus parasuis* is mediated by plasmid pB1000 bearing bla_{ROB}-1. Antimicrob. Agents Chemother., 51: 2260-2264.

Nazir, K.H.M.N.H., M.B. Rahman, K.M. Nasiruddin, F. Akhtar, M.F.R. Khan and M.S. Islam, 2005. Antibiotic sensitivity of *Escherichia coli* isolated from water and its relation with plasmid profile analysis. Pak. J. Biol. Sci., 8: 1610-1613.

Nedbalcova, K., P. Satran, Z. Jaglic, R. Ondriasova and Z. Kucherova, 2006. *Haemophilus parasuis* and Glasser's disease in pigs: A review. Vet. Med., 51: 168-179.

Oliveira, S. and C. Pijoan, 2004. Haemophilus parasuis: New trends on diagnosis, epidemiology and control. Vet. Microbiol., 99: 1-12.

Oliveira, S., L. Galina and C. Pijoan, 2001. Development of a PCR test to diagnose *Haemophilus parasuis* infections. J. Vet. Diagn. Invest., 13: 495-501.

Sundin, G.W. and C.L. Bender, 1996. Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals and plants. Mol. Ecol., 50: 133-143.

Wright, C.L., R.A. Strugnell and A.L. Hodgson, 1997. Characterization of a *Pasteurella multocida* plasmid and its use to express recombinant proteins in *P. multocida*. Plasmid, 37: 65-79.

Zhou, X.L., X.J. Xu, Y.X. Zhao, P. Chen, X. Zhang, H.C. Chen and X.W. Cai, 2010. Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates. Vet. Microbiol., 141: 168-173.