The Journal of Veterinary Medical Science

NOTE

Bacteriology

Aminoglycoside susceptibility of *Pasteurella multocida* isolates from bovine respiratory infections in China and mutations in ribosomal protein S5 associated with high-level induced spectinomycin resistance

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J. Vet. Med. Sci.
79(10): 1678–1681, 2017
doi: 10.1292/jvms.17-0219

Received: 19 April 2017
Accepted: 3 August 2017
Published online in J-STAGE: 2 September 2017

ABSTRACT. Twenty-three isolates of *Pasteurella multocida* were tested for susceptibility to six aminoglycoside agents and screened by polymerase chain reaction for the presence of aminoglycoside resistance genes. In addition, mutations in the resistance-determining region of strains showing a high level of induced resistance to spectinomycin strains were examined. Susceptibility testing showed that all of the isolates were resistant to at least two types of aminoglycosides, and that the most effective antimicrobial was spectinomycin. The resistance genes *aphA1*, *strB* and *aacA4* were present in all 23 isolates. In the three induced spectinomycin-resistant strains, a 9-bp deletion in *rpsE* that encodes ribosomal protein S5 was detected.

KEY WORDS: aminoglycoside resistance, mutations, *Pasteurella multocida*

Pasteurella multocida is one of the most prevalent pathogens responsible for bovine respiratory disease. It is an opportunistic pathogen that generally inhabits the upper respiratory tract [6, 18]. Aminoglycosides have been used clinically against gram-negative bacteria for many years [14]. Despite increasing resistance, aminoglycoside use to control bovine respiratory disease resulting from *P. multocida* infections continues. The mechanisms underlying aminoglycoside resistance have been reported for many bacteria [1, 7, 8]. However, the aminoglycoside resistance status of *P. multocida* isolates in China has rarely been reported [19]. Aminoglycoside resistance genes such as *strA, strB, aadA14, aphA1*, *aadB* and *aadA25* have been reported in *P. multocida* or other members of the family *Pasteurellaceae*, and these genes are believed to play an important role in aminoglycoside resistance [5, 12, 15]. In addition to aminoglycoside-modifying enzymes, high-level resistance to spectinomycin often results from mutations in the 16S ribosomal ribonucleic acid (rRNA) and/or *rpsE*, which encodes ribosomal protein S5 [3, 11, 21]. In the current study, we investigated the aminoglycoside resistance status and prevalence of aminoglycoside resistance genes in 23 *P. multocida* isolates from eight provinces in China. Furthermore, we identified a new mutation in *rpsE* in strains with a high level of induced spectinomycin resistance.

Twenty-three *P. multocida* field isolates (designated Pm1 to Pm23) were obtained from nasal swabs or lungs from cattle on 23 farms located in eight provinces of China (Jilin, Heilongjiang, Neimenggu, Liaoning, Shandong, Hebei, Henan and Jiangsu) from 2011 to 2014. The isolates were identified as described previously [13]. These *P. multocida* strains were routinely cultured in brain heart infusion (BHI, Oxoid, Cambridge, U.K.) broth or on BHI agar at 37°C. Isolates were tested for susceptibility to six aminoglycosides (gentamicin [GEN], kanamycin [KAN], spectinomycin [SPT], streptomycin [SM], amikacin ([AMK], neomycin ([NEO]) using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines in VET01-A4 [2]. The reference strain *Escherichia coli* ATCC 25922 served as quality control in the antimicrobial susceptibility tests, and each experiment was repeated three times. Three strains (Pm-1, Pm-3 and Pm-5) were randomly selected for *in vitro* induction of highly SPT-resistant mutants. SPT-resistant mutants were obtained by plating the bacteria on a medium supplemented with increasing concentrations of SPT, as described previously [20]. Briefly, *P. multocida* strains were grown in BHI broth to the log phase, and then the strains were plated on BHI agar plates with an SPT concentration gradient ranging from a subinhibitory level to a concentration two times the minimal inhibitory concentration (MIC). Bacterial cells from a plate with a lower antibiotic

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Table 1. Primers used in the study

Primer	Sequence (5′–3′)	Reference	Annealing temp (°C)	Product (bp)
strA Pm-fw	ATCGCAGATAGAAGGCAAGGC	This study	55	574
strA Pm-rv	AACTGGCAGGAGGAACAGGA	This study		
strB Pm-fw	GCGTTGCTCCTCTTCTCCAT	This study	54	723
strB Pm-rv	ACCTTTTCCAGCCTCGTTTG	This study		
aadB Pm-fw	CAACGCAGGTCACATTGATACAC	This study	55	418
aadB Pm-rv	ACTGGTGGTACTTCATCGGCATA	This study		
aadA25 Pm-fw	ACTATCAGAGGTGCTAAGCGTCAT	This study	55	724
aadA25 Pm-rv	CACGTAGTGAACAAATTCTTCCAAC	This study		
aphA1 Pm-fw	AAAGCCGTTTCTGTAATGAAGGAG	This study	55	642
aphA1 Pm-rv	GGCAATCAGGTGCGACAATCT	This study		
aacA4 Pm-fw	CTCGAATGCCTGGCGTGTTT	This study	59	482
aacA4 Pm-rv	TTGCGATGCTCTATGAGTGGCTA	This study		
aadA14 Pm-fw	TCACTTGTTTGGTTCCGCAGT	[4]	60	642
aadA14 Pm-rv	TCTTTCGGATAAGCTGCCAGA	[4]		
16S RNA PmA-fw	GAGATAGTAGATACACCTCGCGTCG	[5]	60	1,740
16S RNA PmB-fw	TGGATAGAGCGTTGGCCTCC	[5]	62	1,976
16S RNA PmC-fw	CGCCTTGGCAGTCAATTCAG	[5]	58	2,179
16S RNA PmD-fw	TCACAGGTGGAGAAACAGATACCA	[5]	60	2,055
16S RNA PmE-fw	TGTGGTCAATTGAGTAATGCCTG	[5]	62	2,091
16S RNA PmF-fw	CTATGTATTAGAGTCCATTGCGGATCT	[5]	60	1,935
16S RNA Pm-rv	AGGAGGTGATCCAACCGCAG	[5]		
rpsE PmS5-fw	TGCATATGGCGAAGACCAAG	[5]	55	862
rpsE PmS5-rw	AAGTGATTGCACCGAACGG	[5]		

concentration were used to seed a plate containing a higher concentration of SPT. The procedure was repeated until a final concentration was 512 μg/m*l* was reached. Chromosomal deoxyribonucleic acid (DNA) was extracted from freshly cultured *P. multocida*. Briefly, the bacterial cells were transferred to a microcentrifuge tube containing 500 μl of Tris-EDTA (TE) buffer, followed by centrifugation at 12,000 rpm for 2 min. After the supernatant was discarded, the pellet was resuspended in 200 μl of TE buffer, heated in a 100°C water bath for 10 min to release the DNA, and then centrifuged. All of the primers used to amplify the gene fragments are listed in Table 1. The primers used to amplify aminoglycoside-resistant genes were based on the *P. multocida* whole genome sequence in GenBank (accession no. CP003022), and the primers used for the six 16S rRNA and *rpsE* were previously described [11]. Polymerase chain reaction (PCR) was performed in a 25 μl reaction volume containing 2.5 μl 10× PCR buffer, 0.5 mmol L^{-1} dNTP, 0.5 μmol L^{-1} each primer, 15.6 μl PCR water, and 1 U ExTaq polymerase (Takara, Otsu, Japan). A homology analysis of nucleotide sequences was performed using nucleotide-nucleotide BLAST (BLASTN).

The MICs of the six aminoglycosides against the *P. multocida* isolates are presented in Table 2. All 23 *P. multocida* isolates were resistant to AMK and showed 100% non-susceptibility to NEO and KAN. Twelve of the 23 isolates (52.2%) were resistant to SM, and only 13.0% (3/23) were susceptible to this antibiotic. All of the isolates were susceptible to SPT and all of the isolates except four strains showed intermediate susceptibility to GEN. Further analysis of the MICs revealed that six strains were resistant to two aminoglycosides, and 17 isolates (74.0%) were resistant to at least three aminoglycosides. The three induced SPT-resistant strains showed no effect on the MICs of other aminoglycosides.

This study is the first report on aminoglycoside resistance of *P. multocida* in bovines in China. However, the number of strains in this study was small, and the rate of resistance was higher than that in other reports. Aminoglycoside resistance is emerging worldwide. Jamali *et al.* showed that, from 2012 to 2013, 169 *P. multocida* strains from cattle were frequently resistant to SM (22%) [9]. Katsuda *et al.* showed that, of 378 *P. multocida* isolates from clinically healthy and diseased calves, 9% were resistant to KAN [10]. The animal health division of Germany reported that, of 231 *P. multocida* isolates from cattle, 3% showed resistance to SPT between 2002 and 2006 [4]. In this study, SPT was the most effective antimicrobial agent against clinical *P. multocida* strains. Whether SPT can be used for the treatment of *P. multocida* infection in China is an important question. However, because the number of isolates in this study was small, and, therefore, these samples were not representative, a large-scale survey should be conducted to answer this question.

According to the National Center for Biotechnology Information database (accession nos. NG_047288.1, AP014637.1 and CP021856.1), only three aminoglycoside-resistant genes, *strB* (aminoglycoside 6'-phosphotransferase), *aphA1* (aminoglycoside 3′-phosphotransferase III), and *aacA4* (aminoglycoside adenylyltransferase), were detected in all 23 *P. multocida* isolates. However, there were none of the other examined resistance genes in any *P. multocida* isolates. The results of a previous study suggested that strains harboring *strB* generally were resistant to SM, whereas strains harboring *aphA1* generally showed resistance to KAN, AMK and NEO, and strains harboring *aacA4* generally were resistant to GEN [14]. We doubt that the three resistance genes were expressed at different levels in the isolates with different resistances; however, the relationship between MICs and the expression of resistance genes should be explored in the future. Among the known mechanisms of resistance to aminoglycosides,

Table 2. Aminoglycosides susceptibility[a)] and MIC (μg/m*l*) of the *P. multocida* isolates

Antimicrobial agents[b)]	GEN	SM	SPT	NEO	AMK	KAN
Pm-1	I (8)	R (32)	S (4)	R (128)	R (128)	R (64)
Pm-2	I (8)	R (32)	S (16)	R (128)	R (256)	R (32)
Pm-3	I (8)	R (32)	S (16)	R (128)	R (256)	I (16)
Pm-4	I (8)	I (16)	S (8)	R (128)	R (64)	I (16)
Pm-5	S (4)	I (16)	S (4)	R (64)	R (128)	R (64)
Pm-6	I (8)	I (16)	S (8)	R (64)	R (128)	R (64)
Pm-7	I (8)	S (1)	S (4)	R (128)	R (64)	R (64)
Pm-8	S (1)	R (32)	S (4)	R (128)	R (128)	I (16)
Pm-9	I (8)	I (16)	S (0.5)	I (32)	R (256)	R (32)
Pm-10	I (8)	R (32)	S (4)	R (128)	R (128)	R (64)
Pm-11	I (8)	R (32)	S (4)	R (128)	R (64)	R (32)
Pm-12	I (8)	R (32)	S (4)	R (128)	R (128)	R (64)
Pm-13	I (8)	R (32)	S (4)	R (128)	R (128)	R (64)
Pm-14	S (1)	R (32)	S (4)	R (128)	R (128)	I (16)
Pm-15	I (8)	I (16)	S (0.5)	I (32)	R (256)	R (32)
Pm-16	S (1)	R (32)	S (4)	R (128)	R (128)	I (16)
Pm-17	I (8)	R (32)	S (4)	R (128)	R (64)	R (32)
Pm-18	I (8)	I (16)	S (8)	R (128)	R (64)	I (16)
Pm-19	I (8)	I (16)	S (8)	R (128)	R (64)	I (16)
Pm-20	I (8)	I (16)	S (0.5)	I (32)	R (256)	R (32)
Pm-21	I (8)	R (32)	S (4)	R (128)	R (128)	R (64)
Pm-22	I (8)	S (1)	S (4)	R (128)	R (64)	R (64)
Pm-23	I (8)	S (1)	S (4)	R (128)	R (64)	R (64)

a) GEN=gentamicin; SM=streptomycin; SPT=spectinomycin; NEO=neomycin; AMK=amikacin; KAN=kanamycin; R=resistant; I=intermediate; S=susceptible. b) Cut-off values used were those described in CLSI document VET01-S2 and elsewhere [14].

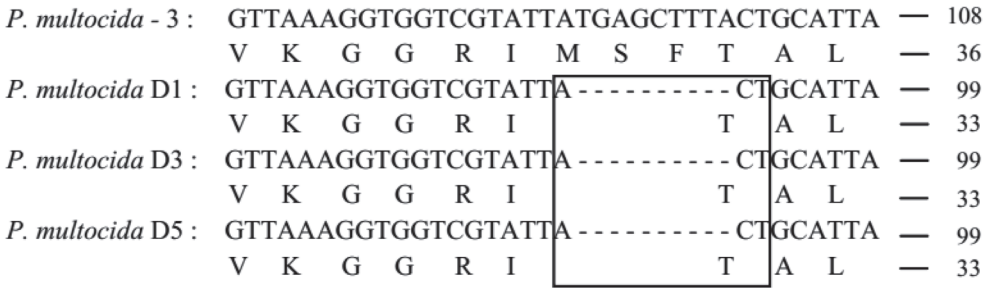

Fig. 1. Alignment of nucleotide and amino acid sequences at the 5′ end of *rpsE* and the corresponding sequences of ribosomal protein S5 in three -induced spectinomycin-resistant strains (D1, D3, D5) and Pm-3. The transparent boxes indicate the altered regions.

the most prevalent is enzyme modification, and many enzymes have been detected in clinical strains [16, 17]. However, few aminoglycoside-modified enzymes in *P. multocida* have been reported. Michael *et al.* analyzed an 82-kb integrative element of a multi-drug resistant *P. multocida* 36950 in 2011. It harbored five aminoglycoside resistance genes, *aadA25*, *strA*, *strB*, *aadB* and *aphA1* [15]. In 2015, a similar element that contained *strA*, *strB* and *aphA1* genes was identified in *Mannheimia haemolytica* [5]. In addition, this element has been shown to be easily transmitted to *P. multocida*, leading to the acquisition of resistance. However, in our examination of aminoglycoside resistance genes, we did not discover SPT resistance genes in any of the *P. multocida* isolates.

In the three strains with high-level induced SPT resistance, no mutations in the six rRNA operons were detected. However, a 9-bp deletion in *rpsE*, which resulted in the loss of the amino acids Met, Ser and Phe at positions 31 to 33, was present (Fig. 1), but the SPT susceptible isolates did not exhibit mutations in the spectinomycin resistance-determining region (SRDR) of any rRNA operons or *rpsE*. Thus, the loss of Met, Ser and Phe detected in this study is believed to affect the binding of the mutated S5 protein to the 16S rRNA. Previous research has shown that amino acids 19 to 33 of ribosomal protein S5 form a loop structure that is involved in binding of SPT to the ribosome, causing SPT resistance [3]. Kehrenberg *et al.* found a 3-bp deletion in *rpsE* that caused the loss of Lys at position 23 and resulted in a high level of spectinomycin resistance of bovine *P. multocida* [11]. In this study, we verified another mutation in ribosomal protein S5 in the induced SPT resistance strains. Thus, the loss of the highly conserved amino acids may affect the interactions between the S5 protein and the 16S rRNA.

In conclusion, we found that 23 clinical isolates of bovine *P. multocida* from eight provinces of China were resistant to several aminoglycosides, and the aminoglycoside resistance genes *aphA1*, *strB* and *aacA4* were universally present in the clinical strains. In the high-level induced SPT resistance strains, we found a new mutation in *rpsE*, which encodes ribosomal protein S5 in *P. multocida.* These results provide more evidence that mutations in highly conserved positions in ribosomal protein S5 can result in spectinomycin resistance.

ACKNOWLEDGMENTS. This research was funded by National Key Research and Development Plan (Grant number: 2016YFD0501301), National Natural Science Foundation of China (Grant number: 31702293), the Science and Technology Development Plan of Jilin Province (Grant number 20170520074JH).

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