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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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, adaA14, aphA1, adaB and adaA25 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 concentration were scraped off and stock cultures were prepared.
A homology analysis of nucleotide sequences was performed using nucleotide-nucleotide BLAST (BLASTN).

Expression of resistance genes should be explored in the future. Among the known mechanisms of resistance to aminoglycosides, were expressed at different levels in the isolates with different resistances; however, the relationship between MICs and the AMK and NEO, and strains harboring strB generally were resistant to SM, whereas strains harboring strB that strains harboring strA showed no effect on the MICs of other aminoglycosides.

The results of a previous study suggested there were none of the other examined resistance genes in any 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 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 were previously described [11].

### Table 1. Primers used in the study

| Primer    | Sequence (5’–3’)                          | Reference            | Annealing temp (°C) | Product (bp) |
|-----------|-------------------------------------------|----------------------|---------------------|--------------|
| strA Pm-fw | ATCCGACATAGAAGGCAAGGC | This study           | 55                  | 574          |
| strA Pm-rv | AAATGCCGAGGAAGACAGAAGGC | This study           | 54                  | 723          |
| strB Pm-fw | GCGTGTGCTCATCCTTCTCCCAT | This study           | 55                  | 418          |
| strB Pm-rv | ACCCTTCACGGCTGCTTGTG     | This study           | 55                  | 724          |
| aadB Pm-fw | CAACCCAGGTCACATTGATAAC   | This study           | 55                  | 642          |
| aadB Pm-rv | ACTGGTGTGATCTCATCAGGCA    | This study           | 55                  | 642          |
| aadA25 Pm-fw | ACTTACAGAAGTGCTAAGGCGATC | This study           | 55                  | 482          |
| aadA25 Pm-rv | CACGAAGTGACAGTGACAAATCCT | This study           | 59                  | 482          |
| aphA1 Pm-fw | AAAGCCGTTCCTGTAATGAAAGG | This study           | 55                  | 482          |
| aphA1 Pm-rv | GCACAACGGTGCCGACAAATCT    | This study           | 55                  | 482          |
| aacA4 Pm-fw | CTCGAAATGGCTGCGGTGTGTTT | This study           | 59                  | 482          |
| aacA4 Pm-rv | TTAGCGATGCCTCATGAGTGGCTA | This study           | 59                  | 482          |
| aadA14 Pm-fw | TCACCTTGGTTTGGCTCCGCAGT | [4]                  | 60                  | 642          |
| aadA14 Pm-rv | TCTTTCCGATAAAGCTGCCAGA | [4]                  | 60                  | 642          |
| 16S RNA Pm-fw | GAGATAGTAGATACACCTGCCGTCG | [5]              | 60                  | 1,740        |
| 16S RNA PmB-fw | TGATAGAGCGTGGCCTCC   | [6]                  | 60                  | 1,740        |
| 16S RNA PmC-fw | GCCTTCGAGTCAATTCAG    | [5]                  | 60                  | 2,179        |
| 16S RNA PmD-fw | TCAGCAGTGGAGAAACAGTACCA | [5]                  | 60                  | 2,055        |
| 16S RNA PmE-fw | TGTTGTCATGATGATGACCTG  | [5]                  | 60                  | 2,055        |
| 16S RNA PmF-fw | CATATGTTAGGTGTCATTGCTCT | [5]                | 60                  | 2,055        |
| 16S RNA Pm-rv | AGGAGGTTGACCAACCAGCAG  | [5]                  | 60                  | 2,055        |
| rpsE PmS5-fw | TGCAATGGCGGAAGACCAAG   | [5]                  | 55                  | 862          |
| rpsE PmS5-rw | AAGTGATTGGCAACCCGAAGG | [5]                  | 55                  | 862          |
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.

| Table 2. Aminoglycosides susceptibility* and MIC (µg/ml) of the *P. multocida* isolates |
|-----------------------------------|---|---|---|---|---|---|
| Antimicrobial agents (µg/ml) | GEN | SM | SPT | NEO | AMK | KAN |
| Pm-1 | (8) | R | S | (4) | R | R | R |
| Pm-2 | (8) | R | (32) | S | (16) | R | R |
| Pm-3 | (8) | R | S | (16) | R | (128) | R |
| Pm-4 | (8) | I | (16) | S | (8) | R | R |
| Pm-5 | S | (4) | I | (16) | S | (4) | R |
| Pm-6 | (8) | I | (16) | S | (8) | R | R |
| Pm-7 | (8) | S | (1) | S | (4) | R | R |
| Pm-8 | S | (1) | R | (32) | S | (4) | R |
| Pm-9 | (8) | I | (16) | S | (0.5) | I | R |
| Pm-10 | (8) | R | S | (4) | R | R | R |
| Pm-11 | (8) | R | S | (4) | R | R | R |
| Pm-12 | (8) | R | S | (4) | R | R | R |
| Pm-13 | (8) | R | S | (4) | R | R | R |
| Pm-14 | S | (1) | R | (32) | S | (4) | R |
| Pm-15 | (8) | I | (16) | S | (0.5) | I | R |
| Pm-16 | S | (1) | R | (32) | S | (4) | R |
| Pm-17 | (8) | R | S | (32) | S | (4) | R |
| Pm-18 | (8) | I | (16) | S | (8) | R | R |
| Pm-19 | (8) | I | (16) | S | (8) | R | R |
| Pm-20 | (8) | I | (16) | S | (8) | R | R |
| Pm-21 | (8) | I | (16) | S | (8) | R | R |
| Pm-22 | (8) | S | (1) | S | (4) | R | R |
| Pm-23 | (8) | S | (1) | S | (4) | R | R |

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

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