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CHARACTERISATION OF A MULTIRESISTANT *PASTEURELLA MULTOCIDA* STRAIN ISOLATED FROM CATTLE

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The emergence of simultaneous resistance to multiple classes of antibiotics presents an increasing threat. Plasmid-borne multiresistance and integrative conjugative elements have been reported in *Pasteurella multocida*. We report an alternative strategy for the development of multiresistance observed in a *P. multocida* strain (Pm238) isolated from calf pneumonia. We identified genes integrated into the chromosomal DNA without known integrative and conjugative elements. These genes conferred resistance to streptomycin (*strA*), tetracycline (*tetB*), chloramphenicol (*catAIII*), and sulphonamides (*sulII*). We also detected mutation in the quinolone-resistance-determining regions of *parC*. No plasmids could be isolated from strain Pm238. These results suggest that *P. multocida* can accumulate multiple resistance determinants on the chromosome as single genes.

Key words: *Pasteurella multocida*, cattle, antimicrobial resistance, multilocus sequence typing

Pasteurella multocida is a widespread Gram-negative opportunistic pathogen. In the presence of predisposing factors, it may cause respiratory tract infections in a wide range of avian and mammalian species, including humans. It is the primary causative agent of fowl cholera, atrophic rhinitis in pigs, and haemorrhagic septicaemia in buffalo and cattle (Rhoades and Rimler, 1989; De Alwis, 1992; Magyar and Lax, 2002), and a secondary invader in pneumonia of swine and ruminants, and in various respiratory tract diseases of rodents (Boyce et al., 2010).

Pasteurella multocida strains can be classified into five capsular serogroups (A, B, D, E, and F) and 16 somatic serotypes (1–16) based on their capsular structure and lipopolysaccharide antigens (Carter, 1955; Heddleston et al., 1972; Rimler and Rhoades, 1987). Thirteen biovars can also be differentiated according to their fermentation of different carbohydrates (Fegan et al., 1995; Blackall et al., 1997).

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Antimicrobial resistance has been reported in *P. multocida* with increasing frequency, including the detection of isolates with resistance to most classes of antimicrobial agents commonly used in veterinary practice. San Millan et al. (2009) reported *P. multocida* strains that contained multiple plasmids carrying resistance genes, while other studies have described multiresistant *P. multocida* strains harbouring resistance genes integrated into the chromosomal DNA by integrative and conjugative elements (ICE*Pmu1*, ICE*Pmu2*) (Michael et al., 2012; Moustafa et al., 2015). In this study, we detected a multiresistant *P. multocida* strain isolated from calf pneumonia with a genetic background different from that of previously reported strains.

Materials and methods

Isolation and characterisation of P. multocida

Pasteurella multocida strain Pm238 was obtained from a case of bovine respiratory tract infection on a cattle farm in 2016. A 3-month-old calf showed respiratory signs including nasal discharge and laboured breathing. Despite treatment with enrofloxacin (5 mg/kg, sc., once a day) for 4 days, the animal died suddenly. Necropsy revealed severe pneumonia accompanied by multiple abscess formation.

A sample from the lung was cultured on Columbia agar (Lab M Ltd., Bury, UK) plates supplemented with 5% sheep blood under aerobic conditions at 37 °C for 24 h. The identity of the *P. multocida* isolate was confirmed by species-specific polymerase chain reaction (PCR) assay (Townsend et al., 1998). Combinations of oligonucleotide primers were used to amplify fragments from the *kmt1* (species identification), *toxA* (*P. multocida* toxin), and *hyaC-hyaD* (capsular serogroup A) genes in a single, multiplex reaction (Gautam et al., 2004; Register and DeJong, 2006). The somatic serotype was established using the gel diffusion precipitin test (Heddleston et al., 1972). The biovar was determined as described previously (Sellyei et al., 2008).

Multilocus sequence typing (MLST) was performed according to the scheme described by Subaaharan et al. (2010). PCR products were sequenced by Macrogen Europe (Amsterdam, The Netherlands). Nucleotide sequences were aligned and compared using BioEdit software (version 7.2.3) (Hall, 2011). MLST alleles were assigned to the RIRDC MLST database (http://pubmlst.org/pmultocida_rirdc/).

Susceptibility testing

Antibiotic resistance was tested using minimal inhibitory concentration (MIC) test strips (Liofilchem, Roseto, Italy). Susceptibility to 18 antimicrobial agents (penicillin, ampicillin, cefalotin, streptomycin, gentamicin, spectinomycin,

tetracycline, doxycycline, erythromycin, clindamycin, florfenicol, chloramphenicol, sulphamethoxazole, trimethoprim-sulphamethoxazole, enrofloxacin, ciprofloxacin, nalidixic acid, and colistin) was tested. *Escherichia coli* ATCC 25922 served as positive control. The strains were cultured on Mueller–Hinton agar plates supplemented with 5% sheep blood at 37 °C for 24 h. Bacterial suspensions in phosphate-buffered saline, adjusted to a density of 0.5 McFarland, were spread onto Mueller–Hinton agar using a sterile swab. An MIC test strip was placed on each plate after approximately 10 min. The plates were incubated at 37 °C for 24 h, after which MIC values were read according to the manufacturer's instructions. We interpreted the breakpoints according to the recommendations of the Clinical and Laboratory Standards Institute.

Antibiotic resistance genes were detected by PCR. PCR primers were chosen from antibiotic resistance genes including chloramphenicol (*catAIII*), sulphonamide (*sulII*), streptomycin (*strA*), quinolones (*parC*), tetracycline (*tetB*), and macrolides *[erm(42), msr(E), mph(E)]* (Table 1). PCR products were sequenced by Macrogen Europe.

Table 1

Antibiotic resistance gene-specific PCRs used in this study

Target gene	Forward (F) and reverse (R) primer sequences (5'–3')	Product length (bp)	Annealing temperature (°C)	Reference
catAIII	F: ACCATGTGGTTTTAGCTTAACA R: GCAATAACAGTCTATCCCCTTC	470	64	Kehrenberg and Schwarz, 2001
sulII	F: ACAGTTTCTCCGATGGAGGCC R: CTCGTGTGTGCGGATGAAGTC	700	64	Kehrenberg and Schwarz, 2001
strA	F: TGACTGGTTGCCTGTCAGAGG R: CCAGTTGTCTTCGGCGTTAGCA	650	64	Kehrenberg and Schwarz, 2001
parC	F: GATGGCTTGAAACCGGTGCA R: GCCATTCCCACCGCAATCC	425	55	Katsuda et al., 2009
tetB	F: TACGTGAATTTATTGCTTCGG R: ATACAGCATCCAAAGCGCAC	206	55	Aminov et al., 2002
erm(42)	F: TGCACCATCTTACAAGGAGT R: CATGCCTGTCTTCAAGGTTT	173	68	Rose et al., 2012
msr(E)	F: ATGCCCAGCATATAAATCGC R: ATATGGACAAAGATAGCCCG	395	68	Rose et al., 2012
mph(E)	F: TATAGCGACTTTAGCGCCAA R: GCCGTAGAATATGAGCTGAT	271	68	Rose et al., 2012

All the five PCR assays targeting integrative conjugation element (ICE)-associated genes from ICE*Pmu1* were performed as described by Klima et al. (2014). Plasmid purification was done using a Qiagen Plasmid Mini Kit (Hilden, Germany) according to the manufacturer's instructions. The isolated DNA was checked by electrophoresis in 0.7% agarose gel. The positive control of plasmid isolation was a plasmid harbouring *Riemerella anatipestifer* strain (1119).

Results and discussion

The identity of *P. multocida* Pm238 was confirmed by species-specific PCR, and capsular and somatic typing classified the strain as A:3. It was assigned to biovar 9 based on its ability to ferment trehalose, xylose, and sorbitol; it showed no ornithine decarboxylase activity, and did not produce acid from arabinose, maltose, lactose, or dulcitol. The *toxA* gene was not detected.

MLST analysis of concatenated sequences demonstrated sequence type 79 (ST79), with the allelic profile *adk* 26, *est* 11, *pmi* 9, *zwf* 10, *mdh* 4, *gdh* 7, and *pgi* 8. ST79 has been associated with bovine cases of pneumonia (Hotchkiss et al., 2011) and belongs to the clonal complex 13, which contains sequence types typical of *P. multocida* strains isolated from pneumonias in cattle and pigs.

Based on MIC values, strain Pm238 exhibited resistance to streptomycin (48 μg/ml), tetracycline (16 μg/ml), doxycycline (24 μg/ml), erythromycin (> 256 μg/ml), clindamycin (> 256 μg/ml), chloramphenicol (96 μg/ml), sulphamethoxazole (> 1024 μg/ml), enrofloxacin (3 μg/ml), and nalidixic acid (> 256 μg/ml). The strain was susceptible to penicillin (0.016 μg/ml), ampicillin (0.032 μg/ml), cefalotin (0.094 μg/ml), gentamicin (0.094 μg/ml), spectinomycin (2 μg/ml), florfenicol (0.125 μg/ml), trimethoprim-sulphamethoxazole (0.125/2 μg/ml), ciprofloxacin (0.019 μg/ml), and colistin (2 μg/ml).

Strain Pm238 contained no detectable plasmids. On the other hand, in accordance with the above phenotype, PCR-based analysis of Pm238 revealed the presence of chloramphenicol (*catAIII*), sulphonamide *(sulII*), streptomycin (*strA*), and tetracycline (*tetB*) resistance genes. No macrolide- or lincosamide-resistance determinants, and no chromosome-borne mobile genetic elements were detected. A resistance-mediating mutation was also detected in *parC*. As previously described, quinolone resistance is generally caused by mutations in the genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*), and amino acid changes in the quinolone-resistance-determining regions (QRDRs) play a role in the evolution of a high level of resistance to quinolones (Cárdenas et al., 2001). Sequence analysis of the QRDRs of *parC* identified a mutation in codon 84, resulting in an amino acid alteration (Glu → Lys). Fluoroquinolone resistance in *P. multocida* isolates has rarely been observed. Moreover, *in vitro* resistance to ciprofloxacin is generally considered to be associated with resistance to en-

rofloxacin as well. Interestingly, Pm238 was susceptible to ciprofloxacin, whereas it exhibited resistance to enrofloxacin and nalidixic acid. To date, only a limited number of studies has focused on these three antibiotics or has analysed the QRDRs of *P. multocida*. Kong et al. (2014) found no difference in MIC values to ciprofloxacin and enrofloxacin with nucleotide substitutions in the QRDR sequences. In another study, Cárdenas et al. (2001) determined the MICs of *P. multocida* to ciprofloxacin and nalidixic acid, but enrofloxacin has not been tested. Analysis of the gene *gyrA* revealed amino acid changes in the QRDR (Ser 83 → Ile, Asp 87 → Gly), and strains with these mutations exhibited increased nalidixic acid MIC values and decreased susceptibilities to fluoroquinolones. Recently, Vanni et al. (2014) have described *Escherichia coli* strains that, in the same way as Pm238, showed resistance to enrofloxacin and susceptibility to ciprofloxacin with QRDR mutations in the *gyrA* and *parC* sequences. Our results indicate that previously unknown fluoroquinolone resistance phenotypes of *P. multocida* could exist, and the amino acid substitutions of DNA gyrase and topoisomerase IV are responsible for the development of this kind of resistance.

Phenicol resistance in *P. multocida* is encoded by several resistance genes, therefore chloramphenicol-resistant but florfenicol-susceptible isolates may emerge. Chloramphenicol resistance is mainly mediated by the enzymatic inactivation of the drug via chloramphenicol acetyltransferases, and in *P. multocida*, the most commonly identified resistance gene responsible for chloramphenicol resistance is *catAIII* (Schwarz et al., 2004), which was detected also in Pm238. Kehrenberg and Schwarz (2005) identified florfenicol resistance gene (*floR*) carrying plasmids in *P. multocida*. *FloR* codes for a membrane-associated exporter protein that promotes the efflux of florfenicol and chloramphenicol from the bacterial cell. To date, this resistance gene has only been found in plasmids of various bacteria: *E. coli* (Cloeckaert et al., 2000), *Mannheimia haemolytica* (Katsuda et al., 2012), and *Actinobacillus pleuropneumoniae* (Bossé et al., 2015). Therefore, the presence of *catAIII* and the lack of plasmids might explain the phenicol resistance phenotype of Pm238.

Multiresistance typically results from the accumulation of mutations or resistance genes (Michael et al., 2012). San Millan et al. (2009) found multiresistance in *P. multocida* related to the coexistence of multiple, small plasmids encoding determinants that conferred resistance. Other studies described multiresistant but plasmid-free *P. multocida* isolates, establishing that resistance genes were linked to the integrative and conjugative elements, ICE*Pmu1* or ICE*Pmu2* (Michael et al., 2012; Moustafa et al., 2015). These elements consist of resistance gene cassettes flanked by sequences of transposases or insertion sequences, indicating that the resistance genes were inserted by an integration or recombination process mediated by an insertion sequence. In Pm238, PCR assays targeting chromosome-borne mobile genetic elements failed to identify any accessory genes related to such elements (Klima et al., 2014), strongly suggest-

ing that Pm238 accumulated resistance genes by several consecutive gene transfer steps, rather than by conjugal transfer of a plasmid or a transferable element carrying multidrug-resistance genes. Further studies, including more extensive sequencing of Pm238, are needed to answer the question if additional alternative strategies for gene capture exist in *P. multocida*.

The spreading of resistance among *P. multocida* isolates makes regular monitoring of its antimicrobial susceptibility important. The emergence of multiresistant strains such as Pm238 highlights the possibility that a single clone can acquire repeated gain-of-resistance genes. *Pasteurella multocida* therefore appears to be able to acquire multiresistance via various strategies, thus increasingly endangering the therapeutic efficacy of antimicrobials and supporting the spread of this microorganism. A better understanding of the mechanisms of multiresistance in *P. multocida* and limiting its spread among bacterial pathogens are currently among the most vital challenges in human and veterinary medicine.

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