Macrolide resistance conferred by rRNA mutations in field isolates of Mannheimia haemolytica and Pasteurella multocida

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Objective: To determine how resistance to macrolides is conferred in field isolates of Pasteurella multocida and Mannheimia haemolytica that lack previously identified resistance determinants for rRNA methylation, efflux and macrolide-modifying enzymes.

Method: Isolates of P. multocida and M. haemolytica identified as being highly resistant (MICs $\geq 64$ mg/L) to the macrolides erythromycin, gamithromycin, tilmicosin, tildipirosin and tulathromycin were screened by multiplex PCR for the previously identified resistance genes \textit{erm}(42), \textit{msr}(E) and \textit{mph}(E). Strains lacking these determinants were analysed by genome sequencing and primer extension on the rRNAs.

Results: Macrolide resistance in one M. haemolytica isolate was conferred by the 23S rRNA mutation A2058G; resistance in three P. multocida isolates were caused by mutations at the neighbouring nucleotide A2059G. In each strain, all six copies of the \textit{rrn} operons encoded the respective mutations. There were no mutations in the ribosomal protein genes \textit{rplD} or \textit{rplV}, and no other macrolide resistance mechanism was evident.

Conclusions: High-level macrolide resistance can arise from 23S rRNA mutations in P. multocida and M. haemolytica despite their multiple copies of \textit{rrn}. Selective pressures from exposure to different macrolide or lincosamide drugs presumably resulted in consolidation of either the A2058G or the A2059G mutation.

Keywords: veterinary macrolides, antimicrobial resistance, Pasteurellaceae

Introduction

Respiratory disease in swine and cattle caused by infection with the bacterial pathogens Mannheimia haemolytica and Pasteurella multocida can be treated with numerous antibiotics including veterinary macrolides. While the incidence of macrolide resistance remains relatively low in M. haemolytica and P. multocida, three macrolide resistance determinants, \textit{msr}(E), \textit{mph}(E) and \textit{erm}(42), have recently been uncovered in isolates associated with bovine respiratory disease.\textsuperscript{1-3} The \textit{msr}(E), \textit{mph}(E) and \textit{erm}(42) genes confer resistance by macrolide efflux, macrolide modification and target site methylation, respectively, and are chromosomally encoded within integrative conjugative elements.\textsuperscript{1,2,4}

The macrolides most frequently used against M. haemolytica and P. multocida infections are the 15-membered ring compounds gamithromycin and tulathromycin, which are semi-synthetic derivatives of the naturally occurring drug erythromycin, and tilmicosin and tildipirosin, which are derivatives of the naturally occurring 16-membered ring compound tylosin. The activities of these drugs are affected to various degrees by the \textit{msr}(E), \textit{mph}(E) and \textit{erm}(42) resistance genes.\textsuperscript{2,5,6}

There have been recent indications that M. haemolytica and P. multocida might attain macrolide resistance by other means. We have previously noted that the phenotypes of some field isolates resistant to tilmicosin but susceptible to tildipirosin could not be explained solely by the presence (or absence) of any of the three \textit{msr}(E), \textit{mph}(E) and \textit{erm}(42) genes.\textsuperscript{6} Furthermore, these three genes were absent in North American isolates of M. haemolytica that were highly resistant to all veterinary macrolides including 15- and 16-membered compounds.\textsuperscript{7} We report here that recent screening of European cattle revealed one M. haemolytica isolate and three P. multocida isolates that lacked \textit{msr}(E), \textit{mph}(E) and \textit{erm}(42) but were nevertheless resistant to a comprehensive range of macrolides and lincosamides.

Materials and methods

Macrolide antibiotics used for MIC testing were erythromycin, tilmicosin and tylosin (Sigma), gamithromycin extracted and purified from
Zactran® (Merial), tulathromycin extracted and purified from Draxxin® (Pfizer) and tildipirosin (Zuprevo®, Merck) from the MSD Animal Health group. MIC measurements were carried out as previously described.5

M. haemolytica and P. multocida strains were cultured and their genomic DNA was extracted as previously described1 and screened by multiplex PCR (Figure S1, available as Supplementary data at JAC Online). The Pm14424 genome was sequenced by an Illumina short read paired-end approach. Primer pairs (Table S1) for amplifying the six individual rrn operons (Figure 1) consisted of a common forward primer at the 3′ end of the 16S rRNA gene and a specific reverse primer for the gene immediately downstream of the respective rrn operon. These gave rise to PCR products of 4–6 kb (Figure 1c) that were sequenced by the Sanger method. RNA was extracted from mid-log phase cells,1 and primers complementary to 23S rRNA (Table S1) were used for reverse transcriptase extension8 under conditions described in the gel legends (Figures S2 and S3).

Results and discussion

Field isolates with increased macrolide MICs

P. multocida and M. haemolytica isolates were collected from bovine hosts within Europe during the period 2010–13. A small proportion exhibited high MICs (>64 mg/L) of 14-, 15- and 16-membered macrolides including erythromycin, gamithromycin, tulathromycin, tilmicosin and tildipirosin. Of these, one M. haemolytica isolate (Mh14717) and three P. multocida isolates (Pm14421, Pm14424 and Pm14426) lacked all of the ermA(42), mph(E) and msr(E) genes (Table 1 and Figure S1).

23S rRNA mutations conferring macrolide resistance in P. multocida and M. haemolytica

We sequenced the genome of the representative P. multocida strain Pm14424 and, with a few exceptions (Figure S4), its overall gene organization was similar to that of other published Pasteurella strains. None of the previously reported macrolide resistance genes5,6,9,10 was present in P. multocida Pm14424, and the sequences of relevant r-protein genes were wild-type. However, an adenosine to guanosine mutation at nucleotide 2059 (A2059G) was evident in sequences encoding 23S rRNA. There were no mutations in other 23S rRNA regions including nucleotides around A752 and A2609, which form part of the macrolide binding site (Figure S2).

We determined how many of the six 23S rRNA genes in P. multocida Pm14424 possessed the A2059G mutation by two independent approaches. First, amplification of a comprehensive set of PCR products (Figure 1) confirmed that the strain possessed all six rrn operons. The 23S rRNA gene in each rrn was sequenced and shown to contain the A2059G mutation, and no other change. Second, direct RNA analysis by reverse transcription confirmed that Pm14424 possessed a homogeneous population of

**Figure 1.** Location and annotation of the six rrn operons on the chromosome of P. multocida and M. haemolytica. (a) The genome structure of P. multocida Pm14424, and (b) PCR products from the individual rrn operons. (c) Priming sites for the PCRs in the 16S rDNA sequence and in specific genes (X) downstream of 23S rDNA (annotation from P. multocida Pm14424 and M. haemolytica USDA-ARS-USMARC-183). (d) Genome structure of M. haemolytica, and (e) PCR products from the individual rrn operons of Mh14717.
All isolates were collected from European cattle between 2010 and 2013.

TIP, tildipirosin; TUL, tulathromycin; TIL, tilmicosin; GAM, gamithromycin; Mh13103 0.25 0.5 1 0.25 1

Figures S4 and S5).

unclear, although resistance profiles suggest that lincosamides should favour either the A2058G or the A2059G mutation remains mutant ribosomes, with a concomitant increase in macrolide schemes most commonly used for other bacteria (Figure 1 and Figures S4 and S5).

Resistance conferred by rRNA mutations in bacteria with multiple rrn operons

Macrolide resistance due to rRNA mutations is already well documented in bacteria with a single (or a few) rrn operons. Although 23S rRNA mutations conferring macrolide resistance are relatively rare in bacteria with multiple rrn operons, they have been noted in Streptococcus pneumoniae isolates and Moraxella catarrhalis, each of which has four rrn operons. Furthermore, linezolid resistance has been associated with mutations in the six rrn operons of Staphylococcus epidermidis, while spectinomycin resistance has been linked with 16S rRNA mutations in P. multocida.

Conclusions

In the case of the Mh14717 and Pm14424 strains reported here, it can be envisaged that a stepwise series of events led to the mutation of all six rrn operons. An initial mutation in one rrn operon would have conferred a slight selective advantage in the presence of a macrolide drug and was presumably followed by homologous recombination between rrn operons amplifying the proportion of mutant ribosomes, with a concomitant increase in macrolide resistance. Whether initial selection and subsequent amplification should favour either the A2058G or the A2059G mutation remains unclear, although resistance profiles suggest that lincosamides and the 14-membered macrolide erythromycin might tend towards selection of A2058G, while 16-membered macrolides tend towards A2059G.

23S rRNA with A2059G (Figure S3). Identical observations were made for the other two resistant strains Pm14421 and Pm14426 (not shown).

Macrolide resistance in M. haemolytica Mh14717 was shown by similar PCR and RNA analyses to be conferred by mutation at the neighbouring nucleotide A2058G (Figure S3). This mutation was present in all six of the Mh14717 rrn operons. Other rRNA regions were unchanged. The six rrn operons of M. haemolytica and P. multocida were annotated starting from the chromosomal origin of replication, consistent with the annotation schemes most commonly used for other bacteria (Figure 1 and Figures S4 and S5).

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