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Identification of dfrA14 in two distinct plasmids conferring trimethoprim resistance in Actinobacillus pleuropneumoniae

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Objectives: The objective of this study was to determine the distribution and genetic basis of trimethoprim resistance in Actinobacillus pleuropneumoniae isolates from pigs in England.

Methods: Clinical isolates collected between 1998 and 2011 were tested for resistance to trimethoprim and sulphonamide. The genetic basis of trimethoprim resistance was determined by shotgun WGS analysis and the subsequent isolation and sequencing of plasmids.

Results: A total of 16 (out of 106) A. pleuropneumoniae isolates were resistant to both trimethoprim (MIC ≥ 32 mg/L) and sulfisoxazole (MIC ≥ 256 mg/L), and a further 32 were resistant only to sulfisoxazole (MIC ≥ 256 mg/L). Genome sequence data for the trimethoprim-resistant isolates revealed the presence of the dfrA14 dihydrofolate reductase gene. The distribution of plasmid sequences in multiple contigs suggested the presence of two distinct dfrA14-containing plasmids in different isolates, which was confirmed by plasmid isolation and sequencing. Both plasmids encoded mobilization genes, the sulphonamide resistance gene sul2, as well as dfrA14 inserted into strA, a streptomycin-resistance-associated gene, although the gene order differed between the two plasmids. One of the plasmids further encoded the strB streptomycin-resistance-associated gene.

Conclusions: This is the first description of mobilizable plasmids conferring trimethoprim resistance in A. pleuropneumoniae and, to our knowledge, the first report of dfrA14 in any member of the Pasteurellaceae. The identification of dfrA14 conferring trimethoprim resistance in A. pleuropneumoniae isolates will facilitate PCR screens for resistance to this important antimicrobial.

Keywords: animal infections, antibiotic resistance, respiratory tract

Introduction

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, an economically important endemic disease that can be difficult to control.1 Good husbandry practices and vaccination can help to reduce the incidence of acute disease, and the early use of effective antimicrobials is essential to limit its spread and severity. A knowledge of the antimicrobial susceptibility patterns of A. pleuropneumoniae is important so that informed treatment decisions can be made.
In the UK, the most commonly used antimicrobials for the treatment of food animals (86% of which are used for pigs and poultry) are tetracyclines, β-lactams and trimethoprim/sulphonamides. Sulphonamides have been widely used since the 1930s for the treatment of both human and veterinary diseases. Trimethoprim, introduced in the 1960s, is often coadministered with sulphonamides.

Resistance to both trimethoprim and sulphonamides can be mediated either by mutations in the chromosomally encoded target enzymes (dihydropteroate synthase and dihydrofolate reductase, respectively) or by the acquisition of transferable genes encoding alternative drug-insensitive enzymes. There are three known genes encoding alternative dihydropteroate synthases (sul1, sul2 and sul3) and >30 dfr genes encoding trimethoprim-insensitive dihydrofolate reductases.

Sulphonamide resistance conferred by sul2, carried on small plasmids, has been reported for A. pleuropneumoniae and other Pasteurellaceae. However, little is known regarding the genetic basis of trimethoprim resistance in the Pasteurellaceae. Single bovine and porcine isolates of Pasteurella multocida and Pasteurella aerogenes have harboured plasmids carrying dfrA20 and dfrA1, respectively, whereas trimethoprim-resistant Haemophilus influenzae has been shown to have mutations in the chromosomally encoded dihydrofolate reductase.

In this study, we have identified the genetic basis of trimethoprim resistance in A. pleuropneumoniae using WGS followed by plasmid isolation and confirmatory sequencing. Two distinct plasmids carrying dfrA14 were found, the first known description of this gene in the Pasteurellaceae.

**Materials and methods**

**Bacterial strains and antimicrobial resistance testing**

A total of 106 clinical isolates of A. pleuropneumoniae, cultured from the pneumatic lungs of pigs submitted for diagnostic investigation to the then Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England between 1998 and 2011, were selected for study. The majority of isolates were from 2005–10 (20, 26, 11, 12, 14 and 8 isolates, respectively), with none from 2000–01 and only 1–4 from each of the other years. Serovars 2 (11%), 2005–10 (20, 26, 11, 12, 14 and 8 isolates, respectively), with none from 2011, were selected for study. The majority of isolates were from Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England between 1998 and 2011, were selected for study. The majority of isolates were from 2005–10 (20, 26, 11, 12, 14 and 8 isolates, respectively), with none from 2000–01 and only 1–4 from each of the other years. Serovars 2 (11%), 2005–10 (20, 26, 11, 12, 14 and 8 isolates, respectively), with none from 2011, were selected for study. The majority of isolates were from Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England between 1998 and 2011, were selected for study. The majority of isolates were from Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England between 1998 and 2011, were selected for study. The majority of isolates were from Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England between 1998 and 2011, were selected for study.
separate small contigs (768–963 bp) in all other isolates. BLASTx analysis of the dfra14-containing-contigs of MIDG2664 and MIDG3349 revealed partial strA sequences flanking the dfra14 gene in both cases. Furthermore, alignments of the dfra14-containing-contigs showed that the strA\(^\text{5}^-\)-dfra14-strA\(^\text{3}^-\) sequences were identical in all 16 isolates, although the shorter contigs in MIDG2664 and MIDG3349 were missing the first 205/529 bp of the strA\(^\text{5}^-\) sequence, which was not detected by ResFinder. Alignments of the dfra14-containing-contigs also suggested two different trimethoprim resistance plasmids: contigs from MIDG2664 and MIDG3349 were identical over the 3429 bp common to both, and contigs from the remaining isolates showed 100% identity where alignment was possible, given the different lengths of the contigs.

The distribution of sequences among the small contigs suggested the possibility of multiple plasmids sharing common sequences. No known plasmids were detected in the draft genomes using PlasmidFinder (www.genomicepidemiology.org), but an analysis of the endogenous plasmid profiles for MIDG3224 and MIDG3389 suggested multiple plasmids, at least in the latter (Figure 1a). Conjugal transfer from MIDG3224 and MIDG3389 into a plasmid-free recipient strain (MIDG3331\(\Delta\text{ureC::}\text{nadV}\)) was used to isolate trimethoprim resistance plasmids pM3224T and pM3389T prior to complete nucleotide sequencing (Figure 1a). Trimethoprim-resistant transconjugants were positive for dfra14 and nadV by PCR, indicating the successful mobilization of plasmids from MIDG3224 and MIDG3389 into MIDG3331\(\Delta\text{ureC::}\text{nadV}\) (Figure 1b). Furthermore, the amplification of sul2 sequences from transconjugants suggested that both pM3224T and pM3389T also encode sulphonamide resistance (Figure 1b), and the MICs of trimethoprim and sulfoxazole were the same for the transconjugants as for the donor strains. A primer walking strategy was used to determine the complete nucleotide sequences of pM3224T and pM3389T as representatives of the two different trimethoprim resistance plasmids indicated above.

Plasmid pM3224T (6050 bp) was found to share the greatest similarity (99% identity with 81% coverage) with pB1003 (accession no. EU360945) isolated from P. multocida from pigs in Spain\(^\text{19}\) (Figure 1c). These two plasmids have identical mobilization genes (306 bp mobC, 972 bp mobA and 261 bp mobB located in the 3’ end of mobA) that belong to the HEN family of relaxases common in mobilizable plasmids in the Pasteurellaceae.\(^\text{19,20}\) In pB1003, a complete (804 bp) strA and partial (294 bp) strB gene are found downstream of sul2, and a similar gene linkage has been reported in other Pasteurellaceae plasmids.\(^\text{21}\) In pM3224T, however, there is a 711 bp strB gene, and the strA gene is disrupted by the insertion of a 568 bp element carrying dfra14, a gene arrangement that has previously been reported in plasmids pCERC1 (accession no. NC_019070; Figure 1c) and pST0JO (accession no. NG_035503) from Enterobacteriaceae isolated from humans,\(^\text{22,23}\) pYR1521 (accession no. NG_041026) from Yersinia ruckeri isolated from fish\(^\text{14}\) and pRSB206 (accession no. NC_025062) from an uncultured bacterium from wastewater.\(^\text{25}\) All of these dfra14-containing plasmids share an almost identical 3 kb region from sul2 to strB, although the strB gene is truncated (711/837 bp) in pM3224T, suggesting a common origin of this region, with recombination into the different plasmids. The insertion of dfra14 in a secondary site within strA was first noted in pUK3129 isolated from an E. coli of human origin in Scotland in 1995 (accession no. Z50805) but only a 681 bp fragment was sequenced. Since then, this sequence has been detected in 6.8 kb plasmids in Enterobacteriaceae of human and animal origin from

### Table 1. Genes identified by ResFinder in A. pleuropneumoniae isolates from the UK with resistance to trimethoprim and sulfoxazole

| MIDG number | Year | Location | Serovar | Trimethoprim (mg/L) | Sulfisoxazole (mg/L) | dfra14\(^\text{a}\) | sul2\(^\text{b}\) | strA\(^\text{c}\) | strB\(^\text{d}\) |
|-------------|------|----------|--------|---------------------|---------------------|----------------|----------------|--------------|--------------|
| 2356        | 1998 | Bury St Edmunds | 7      | >32                 | >512                | 6              | 3451           | 6            | 3451         |
| 2657        | 2005 | Winchester | 8      | >32                 | >512                | 31             | 1757           | 26           | 943          |
| 2664        | 2005 | Bury St Edmunds | 8      | >32                 | >512                | 65             | 1421           | 55           | 943          |
| 3346        | 2006 | Thirsk    | 8      | >32                 | >512                | 48             | 1757           | 57           | 943          |
| 3201        | 2006 | Bury St Edmunds | 8      | >32                 | >512                | 10             | 1765           | 28           | 951          |
| 3221        | 2006 | Bristol   | 8      | >32                 | >512                | 20             | 1761           | 54           | 947          |
| 3349        | 2006 | Thirsk    | 8      | >32                 | >512                | 47             | 1421           | 60           | 943          |
| 3224        | 2007 | Bury St Edmunds | 8      | >32                 | >512                | 49             | 3429           | 49           | 3429         |
| 3322        | 2007 | Thirsk    | 8      | >32                 | >512                | 12             | 1761           | 20           | 947          |
| 3370        | 2009 | Thirsk    | 8      | >32                 | >512                | 57             | 1759           | 106          | 945          |
| 3371        | 2009 | Thirsk    | 8      | >32                 | >512                | 50             | 1759           | 95           | 945          |
| 3372        | 2009 | Thirsk    | 8      | >32                 | >512                | 45             | 1759           | 102          | 945          |
| 3378        | 2009 | Bury St Edmunds | 8      | >32                 | >512                | 56             | 1753           | 15           | 4128         |
| 3388        | 2009 | Thirsk    | 8      | >32                 | >512                | 6              | 1777           | 22           | 963          |
| 3389        | 2009 | Thirsk    | 8      | >32                 | >512                | 30             | 1610           | 16           | 961          |
| 3385        | 2010 | Thirsk    | 8      | >32                 | >512                | 5              | 636            | 26           | 963          |

\(^{a}\)99.8% identity (483/483 bp) with dfra14 from Salmonella enterica subsp. enterica serovar Typhimurium (DQ388123).

\(^{b}\)100% identity (816/816 bp) with sul2 from Acinetobacter bereziniae (G0421466).

\(^{c}\)100% identity (529/804 bp) with strA from a Shigella flexneri plasmid (AF321551) for MIDG3224 and MIDG3324, and 99.8% identity (529/804 bp) with strA from an Erwinia amylovora plasmid (M96392) for all others with strA (NB: in MIDG3395 only 512/804 bp of the gene were detected).

\(^{d}\)99.9% identity (705/837 bp) with strB from an Erwinia amylovora plasmid (M96392).
Figure 1. Isolation and characterization of newly identified dfrA14-containing A. pleuropneumoniae plasmids. (a) Comparison of plasmid extracts from MIDG2331 ΔureC::nadV (Lane 1), conjugal donor strains (Lane 2 = MIDG3224 and Lane 4 = MIDG3389) and respective trimethoprim-resistant transconjugants, showing the transfer of plasmids (Lane 3 = pM3224T and Lane 5 = pM3389T) into MIDG2331 ΔureC::nadV. (b) PCR amplification of dfrA14 (343 bp amplicon; Lane 1 in each section), sul2 (220 bp amplicon; Lane 2 in each section) and nadV (1.5 kb amplicon; Lane 3 in each section) from MIDG2331 ΔureC::nadV, MIDG3224, MIDG2331 ΔureC::nadV+pM3224T, MIDG3389 and MIDG2331 ΔureC::nadV+pM3389T, as indicated for each section of the gel. (c) Schematic comparison of pM3224T with the most closely related Pasteurellaceae plasmid, pB1003, and pCERC1, a dfrA14-containing plasmid found in Enterobacteriaceae. (d) Schematic comparison of pM3389T with the most closely related Pasteurellaceae plasmid, pG1, and pCERC1. Reading frames are indicated by arrows, with arrowheads showing the direction of transcription; only relevant genes have been annotated (sul2: sulphamamide resistance; strA, strB: streptomycin resistance; dfrA14: trimethoprim resistance; mobA, mobB, mobC: plasmid mobilization; strB′: partial strB; strA′: partial strA). Dark grey blocks between sequences indicate ≥99% nucleotide sequence identity.
around the world, as well as 5 and 53 kb plasmids in Y. ruckeri and an uncultured bacterium, respectively, but it has not been described in plasmids from any member of the Pasteurellaceae.

The complete nucleotide sequence of PM3389T is 6101 bp and shares greatest similarity (99% identity with 87% coverage) with plG1 (accession no. U57647) from P. aerogenes and an identical plasmid found in P. multocida HN06 (Figure 1d). These previously identified 5360 bp plasmids encode the strA gene upstream of sul2, as well as the HEN mobilization genes mentioned above, although the mobA gene in these plasmids is 1131 bp in length, with a 273 bp mobB gene encoded within the 3′ end. In PM3389T, there is an insertion of 173 bp that disrupts the entire mobA and mobB, resulting in a 924 bp mobA gene with an altered 3′ end and no functional mobB gene. In addition, the strA gene is disrupted by the same 568 bp dfrA14-carrying element described above. Therefore, this is the first known description of this gene arrangement upstream of sul2, indicating the separate recombination of just the ΔstrA-dfrA14-ΔstrA cassette instead of the entire sul2-DstrA-dfrA14-ΔstrA region.

In both pM3224T and pM3389T, there is an 823 bp sequence upstream of mobC with 99% identity to the putative oriV originally identified in pLS88 (accession no. L23118) and common in numerous Pasteurellaceae plasmids. Although plasmids with similar oriV regions have been reported to replicate in E. coli, attempts to transform pM3224T and pM3389T into E. coli Stellar cells by heat shock have not been successful. It is possible that these plasmids could be transformed into E. coli by electroporation, but this was not investigated as isolation of the plasmids was achieved by conjugation into MIDG2331::ureC::nadV. A graphical analysis of the pM3224T and pM3389T sequences revealed that the region containing the oriV and mobilization genes has a GC content of 41%–42%, reflecting the average for Pasteurellaceae, whereas the regions containing the antimicrobial resistance genes have a GC content of 54%–55% and are likely of enterobacterial origin, as previously suggested for antimicrobial resistance genes in other Pasteurellaceae plasmids.

When the complete sequences of pM3224T and pM3389T were used to search the draft genomes of the remaining trimethoprim-resistant isolates using BLASTn, contigs were identified that could be assembled into plasmids with high identity (99%–100%) to either the 6050 bp plasmid (MIDG2356 and MIDG3224) or the 6101 bp plasmid (all other trimethoprim-resistant isolates). These data indicate that the 6050 and 6101 bp plasmids have been in the UK A. pleuropneumoniae population since at least 1998 and 2005, respectively. The use of trimethoprim/sulphonamide combinations to treat A. pleuropneumoniae infection and other diseases in pigs provides selective pressure for maintenance, and the coexistence of different pathogens may facilitate the transfer of these antimicrobial resistance plasmids between different species.

In conclusion, we report here for the first time, to our knowledge, dfrA14 in the Pasteurellaceae, which will facilitate the development of PCR assays for resistance to trimethoprim, a clinically important antimicrobial.

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Transparency declarations

None to declare.

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