Boss, JT; Li, Y; Walker, S; Atherton, T; Fernandez Crespo, R; Williamson, SM; Rogers, J; Chaudhuri, RR; Weinert, LA; Oshota, O; Holden, MT; Maskell, DJ; Tucker, AW; Wren, BW; Rycroft, AN; Langford, PR; BRaDP1T Consortium, (2015) Identification of dfrA14 in two distinct plasmids conferring trimethoprim resistance in Actinobacillus pleuropneumoniae. The Journal of antimicrobial chemotherapy. ISSN 0305-7453 DOI: https://doi.org/10.1093/jac/dkv121

Downloaded from: http://researchonline.lshtm.ac.uk/2167390/

DOI: 10.1093/jac/dkv121

Usage Guidelines

Please refer to usage guidelines at http://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/
Identification of dfrA14 in two distinct plasmids conferring trimethoprim resistance in Actinobacillus pleuropneumoniae

Janine T. Bosse†, Yanwen Li†, Stephanie Walker, Tom Atherton, Roberto Fernandez Crespo, Susanna M. Williamson, Jon Rogers, Roy R. Chaudhuri, Lucy A. Weinert, Olusegun Oshota, Matt T. G. Holden, Duncan J. Maskell, Alexander W. Tucker, Brendan W. Wren, Andrew N. Rycroft and Paul R. Langford on behalf of the BRaDP1T Consortium

1Section of Paediatrics, Department of Medicine, Imperial College London, St Mary's Campus, London W2 1PG, UK; 2Animal and Plant Health Agency (APHA) Bury St Edmunds, Rougham Hill, Bury St Edmunds, Suffolk IP33 2RX, UK; 3Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK; 4The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK; 5 Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK; 6Department of Pathology and Pathogen Biology, The Royal Veterinary College, Hawkshead Campus, Hatfield, Hertfordshire AL9 7TA, UK

*Corresponding author. Tel: +44-(0)20-759-41803; Fax: +44-(0)20-759-43984; E-mail: j.bosse@imperial.ac.uk
†These authors contributed equally to this work.
‡Present address: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK.
§Members are listed in the Acknowledgements section.

Received 4 December 2014; returned 6 February 2015; revised 20 March 2015; accepted 5 April 2015

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. 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 (suI, suI2 and suI3) and >30 dfr genes encoding trimethoprim-insensitive dihydrofolate reductases.

Sulphonamide resistance conferred by suI2, 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 harbourd 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%), 7 (12%) and 8 (72.0%) and 12 (2.0%) were represented, reflecting the serovar distribution in the UK. A. pleuropneumoniae MIDG2331 is a plasmid-free serovar 8 clinical isolate that was cultured from pneumonic pig lungs in 1995. MIDG2331 was made NAD-independent by the chromosomal insertion (replacing part of ureC) of the Haemophilus ducreyi nadV gene, yielding MIDG2331 ΔureC::nadV. All the strains were grown at 37°C with 5% CO2 on blood heart infusion (BHI; Difco) agar supplemented with 0.01% NAD and, when required, with trimethoprim (10 mg/L).

For all isolates, MICs were determined for trimethoprim and sulfoxazole by agar dilution susceptibility testing, according to the CLSI M31-A3 guidance.

Genomic sequencing and analysis

Genomic DNA was extracted from the 16 trimethoprim-resistant (MIC ≥32 mg/L) A. pleuropneumoniae isolates (Table 1) using the FastDNA Spin Kit (CagenMedicus), according to the manufacturer’s protocol for bacterial cells, and 0.5 μg was used for library preparation and sequencing as previously described. ResFinder (www.genomicepidemiology.org) was used to identify acquired antimicrobial resistance genes (using a threshold of 98% identity) in the draft genomes. Contigs identified by ResFinder (Table 1) have been submitted to GenBank (accession numbers: contig006_MIDG2356 = KP196974; contig026_MIDG2657 = KP196975; contig031_MIDG2657 = KP196976; contig055_MIDG2664 = KP196977; contig065_MIDG2664 = KP196978; contig010_MIDG3201 = KP196979; contig028_MIDG3201 = KP196980; contig020_MIDG3221 = KP196981; contig054_MIDG3221 = KP196982; contig049_MIDG3224 = KP196983; contig012_MIDG3232 = KP196984; contig020_MIDG3232 = KP196985; contig048_MIDG3346 = KP196986; contig057_MIDG3346 = KP196987; contig047_MIDG3349 = KP196988; contig060_MIDG3349 = KP196989; contig106_MIDG3370 = KP196990; contig050_MIDG3371 = KP196991; contig095_MIDG3371 = KP196992; contig045_MIDG3372 = KP196993; contig102_MIDG3372 = KP196994; contig015_MIDG3378 = KP196995; contig056_MIDG3378 = KP196996; contig006_MIDG3388 = KP196997; contig022_MIDG3388 = KP196998; contig016_MIDG3389 = KP196999; contig030_MIDG3389 = KP197000; contig005_MIDG3395 = KP197001; contig026_MIDG3395 = KP197002; and contig074_MIDG3395 = KP197003).

Isolation and characterization of plasmids

Plasmids were extracted from A. pleuropneumoniae isolates MIDG3224 and MIDG3389, selected as representing two different patterns of resistance genes identified by ResFinder (Table 1), using the QiAprep Spin Miniprep kit (Qiagen). Attempts were made to transform plasmids into Escherichia coli Stellar cells (Clontech) by heat shock, with selection on LB agar containing trimethoprim (10 or 20 mg/L). The conjugal transfer of plasmids from MIDG3224 and MIDG3389 into MIDG2331 ΔureC::nadV was carried out as previously described, with transconjugants selected on BHI agar (without NAD) supplemented with 10 mg/L trimethoprim.

The MICs of trimethoprim and sulfoxazole were determined for selected trimethoprim and sulfoxazole-resistant transconjugants, as described above, and the presence of dfrA14, sul2 and nadV was determined by QiagenFast PCR (Qiagen) using primer pairs dfrA14f (CTTGATAGCTGGAAAGCGGACCGCCGCAATATTTC) and dfrA14r (GATCGTGGAATGGGCTGAGGACCC), sul2f (CTAACATAACCTGGGACAGTTTC) and sul2r (GGGAATGCCATCGCCTTAGAAC), and nadVf (CTAGAATCGGAGGCGCCTATTAG) and nadVr (GGCGCGCGGCTAGTGGTAAAG). The complete sequences of plasmids pM3389T and pM3224T, isolated from transconjugants, were determined using a primer walking strategy (GenBank accession numbers pM3224T = KP197004 and pM3389T = KP197005). These sequences were subsequently used to search the draft genomes of the remaining trimethoprim-resistant isolates using BLASTn.

Results and discussion

Trimethoprim resistance (MIC >32 mg/L) was detected in 16 out of 106 A. pleuropneumoniae isolates, and all 16 were resistant to sulfoxazole (MIC >32 mg/L) (Table 1). A further 32 isolates were resistant to sulfoxazole only (data not shown), which is not surprising given that trimethoprim, often coadministered with sulphonamides, was introduced for use 30 years after sulphonamides. Co-resistance to trimethoprim and sulfoxazole was found in serovar 7 and 8 isolates obtained from four different geographical locations in England as early as 1998 (1 out of 3 isolates), with the largest proportion identified in 2009 (6 out of 14 isolates). ResFinder analysis (Table 1) of the draft genomes identified the trimethoprim resistance gene dfrA14 on contigs ranging from 636 to 3451 bp in all trimethoprim-resistant isolates. In all but two isolates (MIDG2664 and MIDG3349) a partial strA gene was identified on the same contig as dfrA14, and in two isolates (MIDG2356 and MIDG3224) sul2 and strB were also found on the same contig as dfrA14. The sul2 gene was identified on
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 strA5′-dfra14-strA3′ sequences were identical in all 16 isolates, although the shorter contigs in MIDG2664 and MIDG3349 were missing the first 205/529 bp of the strA5′ 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 missing the first 205/529 bp of the strA gene. The gene is disrupted by the insertion of a 568 bp element carrying sul2 from a Shigella flexneri plasmid (AF321551) for MIDG2356 and MIDG3224, 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).

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

<table>
<thead>
<tr>
<th>MIDG number</th>
<th>Year</th>
<th>Location</th>
<th>Serovar</th>
<th>Trimethoprim (mg/L)</th>
<th>Sulfisoxazole (mg/L)</th>
<th>dfra14</th>
<th>sul2</th>
<th>strA</th>
<th>strB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>contig length</td>
<td>contig length</td>
<td>contig length</td>
<td>contig length</td>
</tr>
<tr>
<td>2356</td>
<td>1998</td>
<td>Bury St Edmunds</td>
<td>7</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>6</td>
<td>3451</td>
<td>6</td>
<td>3451</td>
</tr>
<tr>
<td>2657</td>
<td>2005</td>
<td>Winchester</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>31</td>
<td>1757</td>
<td>26</td>
<td>943</td>
</tr>
<tr>
<td>2664</td>
<td>2005</td>
<td>Bury St Edmunds</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>65</td>
<td>1421</td>
<td>55</td>
<td>943</td>
</tr>
<tr>
<td>3346</td>
<td>2005</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>48</td>
<td>1757</td>
<td>57</td>
<td>943</td>
</tr>
<tr>
<td>3201</td>
<td>2006</td>
<td>Bury St Edmunds</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>10</td>
<td>1765</td>
<td>28</td>
<td>951</td>
</tr>
<tr>
<td>3221</td>
<td>2006</td>
<td>Bristol</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>20</td>
<td>1761</td>
<td>54</td>
<td>947</td>
</tr>
<tr>
<td>3349</td>
<td>2006</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>47</td>
<td>1421</td>
<td>60</td>
<td>943</td>
</tr>
<tr>
<td>3224</td>
<td>2007</td>
<td>Bury St Edmunds</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>49</td>
<td>3429</td>
<td>49</td>
<td>3429</td>
</tr>
<tr>
<td>3327</td>
<td>2007</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>12</td>
<td>1761</td>
<td>20</td>
<td>947</td>
</tr>
<tr>
<td>3370</td>
<td>2009</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>57</td>
<td>1759</td>
<td>106</td>
<td>945</td>
</tr>
<tr>
<td>3371</td>
<td>2009</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>50</td>
<td>1759</td>
<td>95</td>
<td>945</td>
</tr>
<tr>
<td>3372</td>
<td>2009</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>45</td>
<td>1759</td>
<td>102</td>
<td>945</td>
</tr>
<tr>
<td>3378</td>
<td>2009</td>
<td>Bury St Edmunds</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>56</td>
<td>1753</td>
<td>15</td>
<td>4128</td>
</tr>
<tr>
<td>3388</td>
<td>2009</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>6</td>
<td>1777</td>
<td>22</td>
<td>963</td>
</tr>
<tr>
<td>3389</td>
<td>2009</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>30</td>
<td>1610</td>
<td>16</td>
<td>961</td>
</tr>
<tr>
<td>3395</td>
<td>2010</td>
<td>Thirsk</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;512</td>
<td>5</td>
<td>636</td>
<td>26</td>
<td>963</td>
</tr>
</tbody>
</table>

#99.8% identity (483/483 bp) with dfra14 from Salmonella enterica subsp. enterica serovar Typhimurium (DQ88123).

#100% identity (816/816 bp) with sul2 from Acinetobacter baeriniae (GQ421466).

#100% identity (529/804 bp) with strA from a Shigella flexneri plasmid (AF321551) for MIDG2356 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).

#99.9% identity (705/837 bp) with strB from an Erwinia amylovora plasmid (M96392).

---

**dfra14 plasmids in A. pleuropneumoniae**
Figure 1. Isolation and characterization of newly identified dfrA14-containing A. pleuropneumoniae plasmids. (a) Comparison of plasmid extracts from MIDG2331ΔureC::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 MIDG2331ΔureC::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 MIDG2331ΔureC::nadV, MIDG3224, MIDG2331ΔureC::nadV+pM3224T, MIDG3389 and MIDG2331ΔureC::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: sulphonamide 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,\textsuperscript{22} as well as 5 and 53 kb plasmids in \textit{Y. ruckeri}\textsuperscript{24} and an uncultured bacterium,\textsuperscript{25} 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 \textit{P. aerogenes}\textsuperscript{10} and an identical plasmid found in \textit{P. multocida} HN06\textsuperscript{26} (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 end of both 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. However, 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-ΔstrA-dfrA14-ΔstrA-strB 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 PL588 (accession no. L23118)\textsuperscript{27} and common in numerous Pasteurellaceae plasmids.\textsuperscript{21} Although plasmids with similar oriV regions have been reported to replicate in \textit{E. coli}, attempts to transform pM3224T and pM3389T into \textit{E. coli} Stellar cells by heat shock have not been successful. It is possible that these plasmids could be transformed into \textit{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.\textsuperscript{28}

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 \textit{A. pleuropneumoniae} population since at least 1998 and 2005, respectively. The use of trimethoprim/sulfonamide combinations to treat \textit{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, \textit{dfrA14} in the Pasteurellaceae, which will facilitate the development of PCR assays for resistance to trimethoprim, a clinically important antimicrobial.

### Acknowledgements

We wish to thank Chris Teale from the APHA for his advice and input.

### Members of the BRaDP1T Consortium

Duncan J. Maskell, Alexander W. (Dan) Tucker, Sarah E. Peters, Lucy A. Weinert, Jinhong (Tracy) Wang, Shi-Lu Luan and Roy R. Chaudhuri (University of Cambridge; present address for R. Chaudhuri: Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK); Andrew N. Rycroft, Gareth A. Maglennon and Dominic Matthews (Royal Veterinary College); Brendan W. Wren, Jon Cuculli and Vanessa S. Terra (London School of Hygiene and Tropical Medicine); and Paul R. Langford, Janine T. Bossé and Yanwen Li (Imperial College London).

### Funding

This work was supported by a Longer and Larger (LoLa) grant from the Biotechnology and Biological Sciences Research Council (grant numbers BB/G020744/1, BB/G019177/1, BB/G019274/1 and BB/G018553/1), the UK Department for Environment, Food and Rural Affairs, and Zoetis (formerly Pfizer Animal Health) awarded to the Bacterial Respiratory Diseases of Pigs-1 Technology (BRaDP1T) Consortium. M. T. G. H. was supported by the Wellcome Trust (grant number 098051). The MIC work was funded from the former AHVLA’s Research and Development Internal Investment Fund (grant number RD0030C). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

### Transparency declarations

None to declare.

### References


