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Short communication

# Plasmid-mediated florfenicol resistance in *Mannheimia haemolytica* isolated from cattle

Ken Katsuda [a,*], Mariko Kohmoto [a], Osamu Mikami [a], Yukino Tamamura [b], Ikuo Uchida [b]

[a] Tohoku Research Station, Viral Disease and Epidemiology Research Division, National Institute of Animal Health, 31 Uminai, Shichinohe, Kamikita, Aomori 039-2586, Japan
[b] Hokkaido Research Station, Dairy Hygiene Research Division, National Institute of Animal Health, 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan

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ABSTRACT

The aim of this study was to analyse a florfenicol-resistant *Mannheimia haemolytica* isolated from a calf to determine the genetic basis of its florfenicol-resistance. The antimicrobial susceptibility and plasmid content of the isolate were determined. A florfenicol resistant plasmid carrying the *floR* gene was identified by PCR and transformed into *Escherichia coli* JM109 and HB101 strains. The plasmid was then mapped and sequenced completely.

The isolate was resistant to chloramphenicol, florfenicol, oxytetracycline, kanamycin, dihydrostreptomycin, nalidixic acid, ampicillin, and amoxicillin; it carried a *floR* plasmid of 7.7 kb, designated pMH1405.

The mobilisation and replication genes of pMH1405 showed extensive similarity to the 5.1-kb pDN1 plasmid from *Dichelobacter nodosus* and the 10.8-kb pCCK381 plasmid from *Pasteurella multocida*. An adjacent 2.4-kb segment was highly homologous to the Tn*floR* region of the *E. coli* BN10660 plasmid. A plasmid-mediated *floR* gene was responsible for florfenicol resistance in the bovine respiratory tract pathogen *M. haemolytica*. The pMH1405 plasmid is the smallest *floR*-carrying plasmid reported to date. To the best of our knowledge, this is the first report of a florfenicol-resistant gene in *M. haemolytica*.



## 1. Introduction

*Mannheimia haemolytica* is consistently detected as a complicating agent in bovine respiratory disease (BRD) and is recognised as an important pathogen in feedlots (Rice et al., 2007). Commercial vaccines currently used to prevent *M. haemolytica* do not provide complete protection and only appear to be efficacious in approximately 50% of field studies (Confer et al., 2006). Therefore, antimicrobials, including florfenicol, remain effective tools for the control of *M. haemolytica* infections. Although antimicrobials have been effectively utilized to treat bacterial BRD infections, many *M. haemolytica* isolates are now resistant to penicillin, ampicillin, tetracycline, sulfonamides, and tilmicosin (Apley, 1997; Welsh et al., 2004). Previously, we reported that approximately, 50% of *M. haemolytica* isolates were resistant to at least one antimicrobial agent. Resistance rates for the antimicrobial agents were as follows: dihydrostreptomycin, 31.4%; oxytetracycline, 20.5%; ampicillin, 19.2%; amoxicillin, 16.6%; kanamycin, 11.4%; chloramphenicol, 10.5%; nalidixic acid, 17.0%; enrofloxacin, 4.8%; and danofloxacin, 4.8%, respectively (Katsuda et al., 2009). Infections caused by antimicrobial-resistant bacteria represent a severe and costly animal health problem as they prolong illness. Furthermore, if not treated in a timely manner with more costly, alternative antimicrobial agents, they can lead to increased morbidity and mortality.

* Corresponding author at: Tohoku Research Station, Environmental/Enzootic Diseases Research Team, National Institute of Animal Health, 31 Uminai, Shichinohe, Kamikita, Aomori 039-2586, Japan.
Tel.: +81 176 62 5115; fax: +81 176 62 5117.
*E-mail address:* katsuda@affrc.go.jp (K. Katsuda).

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Florfenicol is a fluorinated structural analogue of thiamphenicol and chloramphenicol that has been approved worldwide for use in veterinary medicine for the control of respiratory pathogens in cattle and pigs. In Japan, florfenicol was approved for veterinary use by the government in 1995, and it is currently used to control of bacterial infection in BRD. Florfenicol resistance in various Gram-negative enteric bacteria has been reported (Bolton et al., 1999; White et al., 2000). In contrast, resistant bacteria were not found in respiratory pathogens such as *Pasteurella multocida*, *M. haemolytica*, *Histophilus somni*, and *Actinobacillus pleuropneumoniae*; the $MIC_{90}$ values ($\leq$2.0 mg/L) for these pathogens have remained stable (Kehrenberg et al., 2004; Klima et al., 2010). Recently, Kehrenberg et al. reported that florfenicol resistance in *Pasteurella* species is plasmid-mediated, and that the plasmid can replicate in different Gram-negative hosts (Kehrenberg et al., 2006, 2008; Kehrenberg and Schwarz, 2005). Here, we investigated a florfenicol-resistant *M. haemolytica* bovine isolate to determine the genetic basis of its florfenicol resistance.

## 2. Materials and methods

### 2.1. Bacterial strain and MIC measurement

The *M. haemolytica* isolates 1405 was obtained from the lung of a 6-month-old calf affected by pneumonic pasteurellosis in the Tohoku district of Japan in 2009. Clinically, the animal presented with pneumonia for 4-days prior to death. The calf was treated with ampicillin 3 days prior to death and florfenicol 1 day prior to death. The isolate was identified biochemically and through 16S rRNA sequence analysis (Angen et al., 2002). To determine the susceptibility of the *M. haemolytica* isolate antimicrobial agents, the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) subcommittee on Veterinary Antimicrobial Susceptibility Testing was used (CLSI, 2005). The following quality control strains were also tested: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. Additionally, *M. haemolytica* NCTC 9380 was included in the majority of the assays.

### 2.2. DNA manipulation

PCR analysis for resistance gene for florfenicol (*floR*), chloramphenicol (*cat3A*), tetracycline (*tetB*, *tetG*, *tetH*, *tetL*, *tetM*), kanamycin (*aphA1*), dihydrostreptomycin (*strA*) and penicillins ($bla_{ROB-1}$, $bla_{TEM-1}$, $bla_{PSE-1}$) was conducted as described previously (Kehrenberg and Schwarz, 2001; Mak et al., 2009; White et al., 2000). Plasmid DNA was prepared using a modified alkaline lysis procedure described previously (Kehrenberg and Schwarz, 2001). Plasmids were then transformed into *E. coli* JM109 and HB101 by the $CaCl_2$ method, and transformants were selected on LB agar supplemented with chloramphenicol (32 mg/L) and florfenicol (32 mg/L). Plasmid DNA from *M. haemolytica* transformants was subjected to restriction mapping; restriction fragments were cloned into either pBluescript II SK+ (Stratagene, Tokyo, Japan) or pCR-Blunt II-TOPO (Invitrogen, Tokyo, Japan) vectors and transformed into the *E. coli* recipient strains JM109 or TOP10, respectively. Sequence analyses were performed using the ABI PRISM 310 Genetic Analyzer Automatic Sequencer (Applied Biosystems, USA). For this analysis, the commercially available standard M13 reverse and forward primers were utilized. Nucleotide sequence comparisons were performed with Genetyx version 9.0 (Genetyx Corporation, Tokyo, Japan). The nucleotide sequence of the plasmid reported in this paper will appear in the GenBank/EMBL/DDBJ nucleotide sequence databases under accession number: AB621552.

### 2.3. Plasmid elimination experiments

The florfenicol-resistant *M. haemolytica* isolate was subjected to evaluated temperature-mediated plasmid elimination by sequential passages in brain heart infusion broth at 42 °C twice daily for a period of 3 weeks. After 3 weeks, cultures were plated on Mueller–Hinton plates to obtain single colonies (Leavitt et al., 2010). Colonies were plated onto control Mueller–Hinton plates and Mueller–Hinton plates that contained different concentrations of florfenicol (8.0–32.0 mg/L). Colonies that failed to grow in the presence of florfenicol were suspected to be cured from the plasmid and were further analysed for *floR* loss by PCR, antimicrobial testing, and plasmid profile.

## 3. Results and discussion

Biochemical, serological and 16S rRNA sequence analyses confirmed that the *M. haemolytica* isolate belonged to the A1 serotype. The isolate was resistant to chloramphenicol (MIC, 64 mg/L), florfenicol (32 mg/L), oxytetracycline (64 mg/L), ampicillin (>512 mg/L), amoxicillin (512 mg/L), kanamycin (>512 mg/L), dihydrostreptomycin (>512 mg/L) and nalidixic acid (256 mg/L). The isolate was susceptible to cefazolin (1.0 mg/L), ceftiofur ($\leq$0.125 mg/L), cefquinome ($\leq$0.125 mg/L), enrofloxacin (0.5 mg/L), and danofloxacin (0.5 mg/L). PCR analysis of antimicrobial resistance genes in the isolate identified the *floR*, *cat3A*, *tetH*, $bla_{ROB-1}$, *strA*, and *aphA1* genes. The isolate carried 3 plasmids, including a 7.7 kb plasmid, designated pMH1405, that mediated resistance to florfenicol and chloramphenicol when transferred into recipient *E. coli* strains. PCR analysis of the plasmid transformants indicated the presence of the chloramphenicol–florfenicol resistance gene *floR*. A map of pMH1405 is shown in Fig. 1. Complete sequencing of this plasmid revealed a total plasmid size of 7654 bp. pMH1405 is the smallest *floR*-carrying plasmid reported to date.

The initial 5.1 kb of the pMH1405 plasmid corresponded closely to the pCCK381 plasmid from *P. multocida* (position 15–5175) and the 5.1 kb pDN1 plasmid from *Dichelobacter nodosus*. In pMH1405, the gene products of this initial section contained 6 open reading frames for plasmid replication proteins (repA-C) and plasmid mobilisation proteins (mobA-C). The order and orientation of these open reading frames in pMH1405 were the same as those in pCCK381 and pDN1. Furthermore, these 6 open