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Short Communication

Characterisation of a novel plasmid containing a florfenicol resistance gene in *Haemophilus parasuis*

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ABSTRACT

Thirty clinical isolates of *H. parasuis* from pig farms in eastern China were screened for antimicrobial susceptibility. A novel plasmid, designated pHPSGC, was extracted from one isolate with evidence of resistance (elevated minimum inhibitory concentration) to florfenicol. DNA sequencing demonstrated that pHPSGC (5297 base pairs) contains three open reading frames (ORFs), corresponding to the genes *rep*, *floR* and *lysR*. The *rep* gene of pHPSGC shared 99% sequence identity with the *rep* gene of pHPS1019. In addition, the region containing *floR* and *lysR* in pHPSGC shared 99% similarity with the corresponding region of pCCK381. pHPSGC may be derived from a recombination event between pHPS1019 and pCCK381. A florfenicol resistance gene in *H. parasuis* may have been transferred via recombination between different plasmids.

Haemophilus parasuis is a nicotinamide adenine dinucleotide (NAD)-dependent Gram negative bacterium that causes Glässer's disease in pigs, which is characterised by fibrinous polyserositis, meningitis and arthritis (Macedo et al., 2015). Although antibiotics have been widely used to prevent and control outbreaks of Glässer's disease (Xu et al., 2011), antibiotic resistance in *H. parasuis* has increased and multi-drug resistance is widespread (de la Fuente et al., 2007). Antimicrobial resistance is often conveyed by plasmids carrying antibiotic resistance genes.

Several *H. parasuis* plasmids conferring antibiotic resistance genes have been reported, for example, plasmid pFS39 isolated from strain FS39 (Yang et al., 2013). Florfenicol, which has been licensed in China since 1999 for the control of respiratory tract diseases and enteric infections in food-producing animals, has been used widely in China after chloramphenicol was banned (Zhao et al., 2016). Thus far, five florfenicol resistance genes (*floR*, *fexA*, *fexB*, *cfr* and *optrA*) have been reported in bacteria of animal origin (Zhao et al., 2016). In Gram negative bacteria, the *floR* gene is the most widely reported contributor to florfenicol resistance (He et al., 2015; Wang et al., 2015). The first plasmid, pHPSF1, carrying a *floR* gene in *H. parasuis* was identified in 2015 in China (Li et al., 2015). In the present study, we screened 30 clinical isolates of *H. parasuis* for antimicrobial susceptibility and identified one strain with an elevated minimum inhibitor concentration (MIC) to florfenicol.

Thirty clinical isolates of *H. parasuis* were obtained from pig farms in eastern China from 2005 to 2016. Isolates were grown on trypticase soy agar plates (TSA, Oxoid) containing 5% bovine serum (Gibco) and 10 μg/mL NADH (β-NAD, reduced form, disodium salt, Biosharp; TSA-S-NAD) and were classified taxonomically by PCR (Oliveira et al., 2001). Since there is no standard method for testing *H. parasuis* for susceptibility to antibiotics, broth microdilution tests were performed as described by Pruller et al. (2017). Colonies from a 24 h growth on chocolate agar were suspended in sterile normal saline that had been adjusted to 0.5 McFarland standard and the suspension was diluted 1:200 in Mueller-Hinton broth (MHB, Oxoid) containing 0.0025% NADH and 1% heat-inactivated sterile filtered chicken serum (Gibco). Cell suspension (50 μL) was added to each well of a microtitre plate already loaded with 50 μL two-fold concentration of antibiotic (Table 1). One strain, designated HPSGC, exhibited a high MIC value of 16 μg/mL for florfenicol, while the remaining 29 strains exhibited low MIC values ranging from 0.25 to 4 μg/mL.

HPSGC was cultured to a high density (optical density of 1.0 at 600 nm) in 50 mL trypticase soy broth (TSB, Oxoid) containing 5% bovine serum, 10 μg/mL NADH (TSB-S-NAD) and 8 μg/mL florfenicol. Bacteria were harvested by centrifugation and plasmids

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Table 1
Minimum inhibitory concentrations (MICs) of 30 *Haemophilus parasuis* isolates.

	Number of isolates with each MIC (μg/mL)																
Agent	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
PEN							1	2	7	3	4	7	6*				
AMP					2	4	4	6	3	3	2	6					
XNL						28	1		1								
GEN				2	3	4	13	3	5								
NEO		2	1	1		1	3	5	10	6	1						
TCY			1	1	3	6	7	7	3	2							
TYL									26	2			2				
FFC					3	7	8	5	6		1						
TIA					1	1	4	12	9	3							

The tested range of MICs for each antimicrobial agent is represented by the white area. The value with an asterisk (*) in the grey shaded area denotes the number of isolates with MIC values greater than or equal to the tested concentration. PEN, penicillin (Biosharp); AMP, ampicillin (Selleck); XNL, ceftiofur (Selleck); GEN, gentamicin (Biosharp); NEO, neomycin (Aladdin); TCY, tetracycline (Aladdin); TYL, tilmicosin (Sigma); FFC, florfenicol (Aladdin); TIA, tiamulin (Aladdin).

were extracted using the Qiagen Plasmid Midi Kit. The presence of *floR* in the plasmid DNA was confirmed by PCR using the primers *floR*F (5′-GCGATATTCATTACTTTGGC-3′) and *floR*R 5′-TAGGATGAAGGTGAGGAATG-3′) (Li et al., 2015); the plasmid was designated pHPSGC.

To confirm that elevated MIC for florfenicol is conferred by pHPSGC, we transferred the plasmid into *E. coli* strain Top10 by the $CaCl_2$ method (Mandel and Higa, 1970). The transformant exhibited an elevated MIC for florfenicol (125 μg/mL) in comparison with the recipient strain (8 μg/mL). Three florfenicol-resistance colonies were selected and passaged continuously in Luria Bertani (LB) medium containing 21 μg/mL florfenicol, then the cultures were processed for plasmid extraction (Fig. 1). After 19 passages, the bacteria were unable to grow in the presence of florfenicol and plasmids were not detected (data not shown). Although the plasmid was not maintained stably in the *E. coli* Top10 strain, these results suggest that pHPSGC carries the *floR* gene and is responsible for florfenicol resistance in HPSGC.

pHPSGC was sequenced completely (GenBank KX966395); the plasmid is 5297 base pairs (bp) and contains three complete ORFs, encoding Rep, FloR and LysR, as well as a partial copy of the *mobB* mobility gene (Fig. 2). Two large regions (positions 1–1574 and 3972–5279) share 99.9% and 100% similarity, respectively, with corresponding regions in plasmid pHPS1019 of *H. parasuis* (HQ622101) (Fig. 2). Additionally, the Rep protein, consisting of 325 amino acids, is identical to the corresponding protein encoded by pHPS1019. pHPSGC encodes a complete FloR protein of 404 amino acids and has an open reading frame for a LysR-like transcriptional regulator of 99 amino acids. The region containing *floR* and *lysR* (positions 1668–3972) in pHPSGC shares 99.5% homology with the matching region in plasmid pCCK381 (AJ871969) (Kehrenberg and Schwarz, 2005). In contrast, the homology between pHPSGC and pHPSF1 is only 90.2% (Li et al., 2015). pHPSF1 appears to be a composite from different sources (He et al., 2015) and it seems likely that pHPSGC consists of elements originating from pHPS1019 and pCCK381 that have been brought together by recombination events to generate a new resistance plasmid.

In summary, a *H. parasuis* isolate (designated HPSGC) with an elevated MIC to florfenicol was identified in eastern China. A plasmid (HPSGC) containing the *floR* gene was recovered from this isolate. pHPSGC differs from the previously reported florfenicol

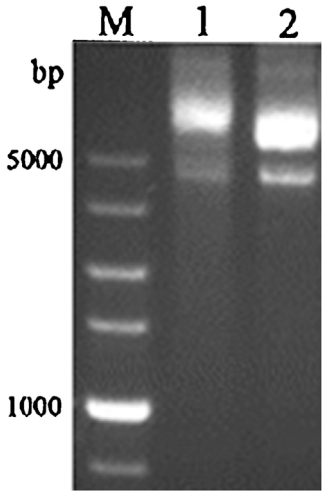

Fig. 1. Comparison of pHPSGC derived from HPSGC and Top10 transformants. Line 1, pHPSGC extracted from Top10. Line 2, pHPSGC isolated from HPSGC.

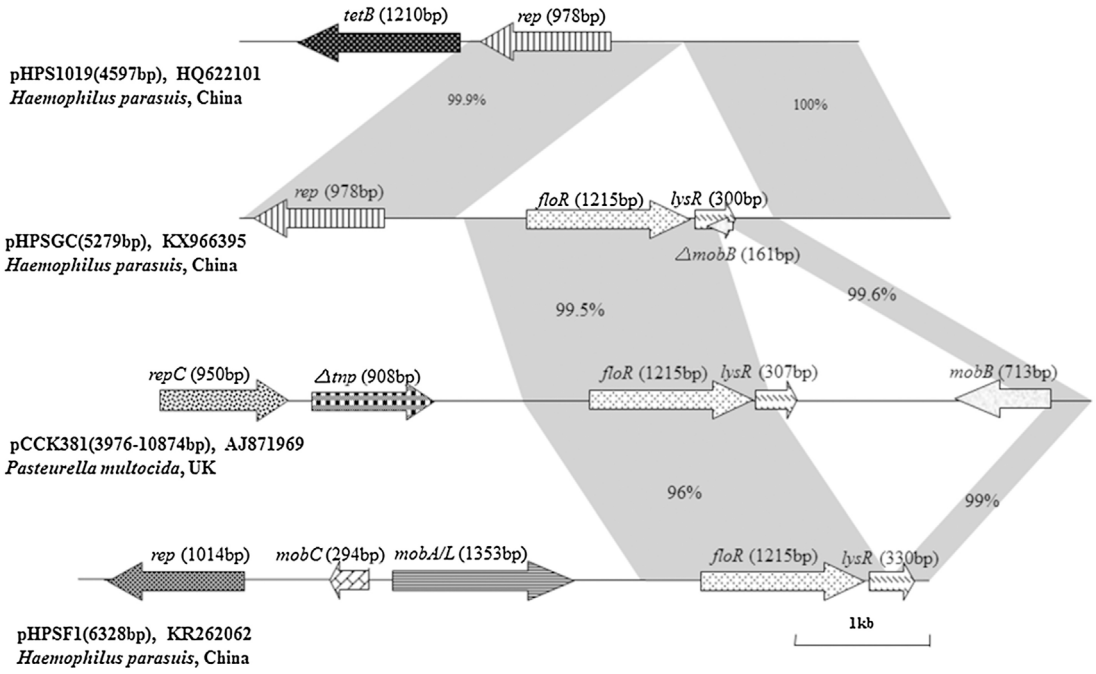

Fig. 2. Comparison of plasmids pHPS1019, pHPSGC, pHPSGF1 and pCCK381. Arrows indicate gene positions and transcriptional orientation. Regions with extensive similarity are marked by grey shading, in which sequence identity is shown as a percentage.

resistance plasmid pHPSF1 in several ways: (1) pHPSGC consists of *rep*, *lysR*, *floR* and a partial copy of the *mobB* gene, while pHPSF1 consists of *rep*, *floR*, *mobC* and *mob(A/L)*; (2) the plasmids have different lengths (5279 bp vs. 6328 bp); (3) the *rep* genes of pHPSGC and pHPSF1 have diverged significantly (63% identity); and (4) Δ*mobB* (161 bp) was not found in pHPSF1.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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