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Occurrence of a novel class 1 integron harboring *qnrVC4* in *Salmonella* Rissen

Sirirat Luk-in [a], Chaiwat Pulsrikarn [b], Aroon Bangtrakulnonth [b], Tanittha Chatsuwan [c,*], Wanla Kulwichit [c]

[a] Medical Microbiology Interdisciplinary Program, Graduate School, Chulalongkorn University, Bangkok, Thailand
[b] World Health Organization National Salmonella and Shigella Centre, Nonthaburi, Thailand
[c] Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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ABSTRACT

We described *qnrVC4* in *S.* Rissen 166ANSS50, a swine isolate, which was detected in the study on quinolone resistance mechanisms of nontyphoidal *Salmonella* in Thailand. The isolate was found to harbor a 17-kb non-conjugative plasmid carrying *qnrVC4* within 8.91 kb of a novel In4-like class 1 integron (In805). It contained the multi-drug resistance gene cassettes of *qnrVC4-qacH4-aacA4-cmlA7-bla*$_{OXA-10}$*-aadA1-dfrA14* and unusual 3′-CS of *mobC*-IS*6100*. This 1014-bp *qnrVC4* cassette included with promoter (P_{qnrVC4}: −35 TTGAGA and −10 TAGTCT) showed high homology with *qnrVC4* in superintegron of *V. cholerae* O1 El Tor. The *qnrVC4* recombinant plasmid resulted in 4-, 8-, and 16-fold increase in the MICs of nalidixic acid (2–8 μg/mL), ciprofloxacin (0.015–0.125 μg/mL), and norfloxacin (0.03–0.5 μg/mL), respectively. In addition, the backbone plasmid revealed a novel replicon belonging to the MOB_{Q1} group from the broad-host-range mobilisable IncQ1 plasmid RFS1010 based on relaxase sequences. This is the first known report of *qnrVC* in *Salmonella enterica*. The *qnrVC4* gene was co-transferred with other resistance genes via a novel plasmid-borne In805. This allowed the spread of this resistance gene to Enterobacteriaceae.

1. Introduction

Salmonella is one of the most common causes of foodborne diseases which occur through the consumption of contaminated food of animal origin. Fluoroquinolones, broad-spectrum antibacterial agents, are used as a key drug of choice for treating the invasively-infected patients widely used in veterinary medicine. Quinolone resistance is commonly caused by a modification of quinolone targets with DNA gyrase (*gyrA*) and/or by topoisomerase IV (*parC*) gene mutations (Ruiz, 2003). Lately, the various plasmid-mediated quinolone resistance (PMQR) mechanisms have been increasingly reported including the production of quinolone resistance protein (Qnr), the modified-acylyltransferase (AAC(6′)-Ib-cr) and the QepA efflux pump (Ruiz, 2003; Strahilevitz et al., 2009). Qnr, the pentapeptide-repeat protein that can prevent quinolones from interacting with DNA/DNA gyrase complexes during DNA replication are known as the common PMQR mechanism in Enterobacteriaceae (Strahilevitz et al., 2009). Currently, five main families with many variants of *qnr* genes have been reported; *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS* (Strahilevitz et al., 2009). Those genes are widespread among bacteria in many countries due to conjugative plasmid or various mobile genetic elements. In 1998, an additional family, *qnrVC1* was firstly discovered in *Vibrio cholera* from Brasil (Fonseca et al., 2008) and the transferability of *qnrVC*-like gene was latterly described in this organism by the present of *qnrVC* within chromosome embedded with SXT integrating conjugative element (Kim et al., 2010). Recently, *qnrVC4* has been reported within a novel complex class 1 integron in gene cassettes (*aacA4-qnrVC4-aacA4-catB3*) on a non-transferable plasmid of *Aeromonas punctate* 159 isolated from aquatic environment in China (Xia et al., 2010). To date, various *qnrVC* alleles (*qnrVC1* to *qnrVC7)* have been found in Vibrionaceae and among environmental aquatic-borne species and in different genetic contexts which were annotated in GenBank on the NCBI database (Fonseca and Vicente, 2013; Po et al., 2015). However, there are very few reports on the transfer of *qnrVC* out of Vibrionaceae. Here, we found *qnrVC4* in *S.* Rissen strain 166ANSS50 isolated from swine in Thailand. The genetic background of *qnrVC4* was characterized. This study reported the occurrence of *qnrVC* family in *Salmonella enterica* for the first time and the identification of this plasmid-mediated *qnrVC* in Enterobacteriaceae underlines a possible dissemination of these resistance determinants within Gram-negative bacteria.

2. Materials and methods

2.1. Bacterial strains

As a part of our study on quinolone resistance mechanisms, from 812 nontyphoidal *Salmonella* isolates from humans and food animals in Thailand during 2005–2007, *Salmonella* isolates that displayed

* Corresponding author. Tel: +66-2-256-4132; fax: +66-2-252-5952.
E-mail address: Tanittha.C@chula.ac.th (T. Chatsuwan).

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nonsusceptible to ciprofloxacin (MIC >0.06 μg/mL) were screened for quinolone resistance mechanisms. We found *Salmonella* Rissen strain 166ANSS50, isolated from rectal swab of a swine from a farm in Khon Kaen province in 2007, showed resistance to nalidixic acid (MIC 32 μg/mL) and intermediate resistance to ciprofloxacin (MIC 0.5 μg/mL) and norfloxacin (MIC 1 μg/mL). Mutations in *gyrA* and *parC* genes and the presence of known PMQR genes of Enterobacteriaceae were not detected. Therefore, the screening of *qnrVC* gene was performed and the *qnrVC4* gene was identified in this isolate. Identification of the isolate was performed by biochemical characteristics and confirmed by PCR using specific primers for 16S rRNA gene, 16SRNA-F (5′-GGAGGG TGCAAGCGTTAAT-3′) and 16SRNA-R (5′-GCCCCCGTCAATTCATTT-3′). The serotype of *S. enterica* was determined according to the Kauffman–White serotyping scheme (Popoff, 1992).

2.2. Antimicrobial susceptibility testing

The antimicrobials, obtained from Sigma-Aldrich (St. Louis, MO, USA), were ampicillin, ceftazidime, streptomycin, amikacin, gentamicin, kanamycin, chloramphenicol, nalidixic acid, ciprofloxacin, and norfloxacin. Minimal inhibitory concentrations (MICs) of these were determined by the agar-dilution technique and interpreted using the Clinical and Laboratory Standards Institute criteria (Clinical and Laboratory Standards Institute, 2016).

2.3. Screening for QRDR mutations and the PMQR genes

The PCR for *gyrA* and *parC* genes were amplified using primers as previously described (Griggs et al., 1996; Eaves et al., 2004). Purified PCR products were sequenced for analysis of quinolone resistance determining region (QRDR). The nucleotide sequences were compared with those of *S.* Typhimurium LT2. PMQR genes were amplified, including *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, and *qepA*, using the primers as previously described (Kehrenberg et al., 2006; Kehrenberg et al., 2007; Hee Young Kang et al., 2009). The *qnrVC* gene was identified by primers qnrVC-F (5′-GAACCTCCGCGATACACAA-3′) and qnrVC-R (5′-GCGCCAATCCATCTATTCTC-3′).

2.4. Transfer of quinolone resistance

Transfer of *qnrVC* was determined by transconjugation and transformation experiments. We performed transconjugation using filter-mating technique, as previously described (Ceccarelli et al., 2006). Azide-resistant *E. coli* UB1637 was used as a recipient strain. Transconjugant was selected on MacConkey agar plate containing 150 μg/mL of sodium azide and 0.03 μg/mL of ciprofloxacin and confirmed by PCR. Transformation was performed using electroporation techniques. Plasmid DNA was isolated by the alkaline lysis method and introduced into electrocompetent *E. coli* DH10B (Invitrogen, Cergy Pontoise, France) by Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, USA). Transformant was selected on Mueller-Hinton agar plate containing 0.03 μg/mL of ciprofloxacin and confirmed by PCR. MICs of antimicrobials for the donor, recipient, and transformant strains were compared by the agar-dilution technique. PCR-based replicon typing was used to detect major plasmid types found in Enterobacteriaceae (Carattoli et al., 2005). PCR and DNA sequencing were also performed to identify genes coding for replication protein (*repA*) and relaxase protein (*mobA*) using primers repA-F (5′-TCAGCCCTTGTATG CGATGG-3′) and repA-R (5′-ATGGCCGCCAACGATCAA-3′), mobA-F (5′-GGCGCGAAACTCGATCA-3′) and mobA-R (5′-GTGGCAATCGGGCG ATT-3′).

2.5. PFGE and Southern blot hybridisation

Total bacterial DNA was prepared in low-melt agarose plugs which were digested with S1 nuclease (Fermentas, UK) or I-CeuI nuclease (New England BioLabs, Inc., USA) and separated using a CHEF-Mapper XA pulsed-field gel electrophoresis (PFGE) system (Bio-Rad, Hercules, USA). The sizes of plasmids were estimated by S1 nuclease PFGE. A low-range PFG marker (New England BioLabs, Inc.) was used as molecular-size markers. The chromosomal DNA was estimated by I-CeuI nuclease PFGE. *E. coli* K12 was used as a reference strain for chromosomal DNA study. DNA treated with I-CeuI or S1 nuclease was blotted onto Hybond N^+ nylon membranes (Amersham, England) by using DNA capillary transfer method. The location of the *qnrVC4* gene on plasmids or in the chromosomal DNA was determined by Southern blot hybridisation by using specific probes of *qnrVC4* or 16S rRNA replicons. Probe labeling, hybridisation, and detection were performed with the DIG DNA labeling and detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocols.

2.6. Cloning of qnrVC4

The *qnrVC4* gene was amplified from *S.* Rissen 166ANSS50 by PCR using primers, qnrVC-CF (5′-ATGGATAAAACAGACCAGT-3′) and qnrVC-CR (5′-TTAGTCAGGAACTACTATTAAACC-3′) and cloned into a TA vector, pTZ57R/T (Fermentas, UK). This gene was subcloned into the pBK-CMV expression vector (Stratagene, La Jolla, CA) with EcoRI and ApaI digestion and then transformed into an *E. coli* DH10B with selection on LB agar plates containing 50 μg/mL of kanamycin. The colonies were selected by BlueWhite colony screening and confirmed by PCR and DNA sequencing.

2.7. Genetic environment of qnrVC4 gene

The presence of *qnrVC4* located in integron was determined by PCR using specific primers for *int1* and *qnrVC4* genes. Integron gene cassettes were detected by PCR using specific primers for the 5′ and 3′ conserved segment (5′-CS and 3′-CS) regions (White et al., 2000). Since this isolate did not yield an amplicon of the CS region, inverted PCR was performed to characterize the gene cassettes flanking *qnrVC4* as previously described (Lee et al., 2011). The plasmid DNA of *S.* Rissen 166ANSS50 was digested with PstI and re-ligated by T4 ligase. The re-ligated DNA fragments were used as DNA templates for PCR using specific primers INV3 (5′-GTTGTGATTTGAGCCACTCG-3′) and INV4 (5′-GCGCCAA TCCATCTATTCTC-3′) for amplification of downstream regions that flank the known sequence. The inverse PCR products were purified and sequenced. PCR mapping and DNA sequencing were used to determine complete cassettes of class 1 integron by primer-walking-strategy using the indicated primers (Supplementary Table S1). DNA sequence comparison and annotation were performed using BLASTN and BLASTP.

2.8. Nucleotide sequence accession numbers

The nucleotide sequences reported here are provided on GenBank under accession number JX173955 and KU886277.

3. Results and discussion

3.1. QnrVC4 determinant from Salmonella enterica

We identified *qnrVC4* from swine isolate of *S.* Rissen 166ANSS50. Antimicrobial susceptibility of this isolate and transformants were determined and MICs of antimicrobials were shown in Table 1. *S.* Rissen 166ANSS50 showed resistance phenotype to quinolones with resistance to nalidixic acid (MIC 32 μg/mL) and intermediate resistance to ciprofloxacin (MIC 0.5 μg/mL) and norfloxacin (MIC 1 μg/mL). This isolate had no substitutions on QRDR of *gyrA* or *parC* and none of the other PMQR genes were found. The isolate also displayed resistance phenotype to other groups of antimicrobials including chloramphenicol, aminoglycosides (streptomycin but not amikacin, gentamicin or kanamycin). It showed resistance to narrow spectrum beta-lactam