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Characterization of multidrug-resistant, *qnrB2*-positive and extended-spectrum-β-lactamase-producing *Salmonella* Concord and *Salmonella* Senftenberg isolates

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Objectives: To characterize plasmids and resistance genes of multidrug-resistant (MDR) *Salmonella* Senftenberg and *Salmonella* Concord isolated from patients in the Netherlands.

Methods: The resistance genes of four MDR *Salmonella* isolates (three *Salmonella* Concord and one *Salmonella* Senftenberg) were identified by miniaturized microarray, PCR and sequencing. Plasmids were characterized by S1 nuclease-PFGE and PCR-based replicon typing (PBRT). Linkage between plasmids and genes was determined by conjugation experiments and microarray analysis. The genetic relationship between the three *Salmonella* Concord isolates was determined by XbaI-PFGE.

Results: A large variety of resistance genes was detected, including *qnrB2* and the β-lactamase genes bla_{TEM-1} and bla_{SHV-12} in all isolates; moreover all *Salmonella* Concord isolates also harboured $bla_{CTX-M-15}$. *Salmonella* Senftenberg harboured a large IncHI2 plasmid. The three *Salmonella* Concord isolates harboured two large plasmids typed as IncHI2 and IncA/C.

Conclusions: We detected the first plasmid-mediated MDR *Salmonella* isolates in the Netherlands harbouring both *qnr* and extended-spectrum β-lactamase (ESBL) genes. In *Salmonella* Senftenberg one large plasmid (IncHI2) and in *Salmonella* Concord two large plasmids (IncHI2 and IncA/C) were responsible for the multidrug resistance.

Keywords: ESBLs, fluoroquinolones, microarray, plasmids

Introduction

Worldwide, *Salmonella* is one of the major causes of foodborne infections in humans. In the majority of the cases these infections are self-limiting. However, for patients at risk and for invasive or prolonged infections antibiotic treatment is indicated. Fluoroquinolones and third-generation cephalosporins are drugs of choice for these cases.[1] Infections caused by multidrug-resistant (MDR) *Salmonella* will affect the available treatment options. This may result in treatment failure and an increase in complications.

Although extended-spectrum β-lactamase (ESBL)-producing *Salmonella* Concord isolates from adopted Ethiopian children have been reported previously from different European countries including the Netherlands,[2–4] there is still scarce information about the genetic background of *Salmonella* Concord isolates carrying both *qnr* and ESBL genes. In addition, no information on the characterization of MDR *Salmonella* Senftenberg isolates is available to date.

The aim of the study was to characterize genes and plasmids of the first *qnr*-positive, ESBL-producing MDR *Salmonella* isolated from patients in the Netherlands.

Materials and methods

Susceptibility tests and detection of resistance genes

In 2007, four *Salmonella* isolates expressing a remarkable type of multidrug resistance were identified. The isolates were selected for further study, since all four strains showed resistance to third-generation cephalosporins and exhibited an unusual quinolone resistance phenotype; being low-level resistant to ciprofloxacin, but still susceptible to nalidixic acid. In addition, all isolates were resistant to most classes of antibiotics tested. Three *Salmonella* Concord isolates (199.69, 206.54 and 210.52) originated from adopted Ethiopian children and a *Salmonella* Senftenberg

isolate (200.27) was obtained from a male adult patient who had recently travelled to Egypt. Susceptibility to antimicrobials was tested by broth microdilution according to ISO standards (ISO 20776-1: 2006) in microtitre trays with a custom-made dehydrated panel of antibiotics (Sensititre©, Trek Diagnostic Systems, UK). The results were interpreted using epidemiological cut-off values as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org). The panel included the following antibiotics: ampicillin, cefotaxime, ceftazidime, tetracycline, sulfamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin and colistin.

To detect antimicrobial resistance genes a miniaturized microarray (AMR04, Identibac, Veterinary Laboratories Agency, UK)[5] was used followed by PCR for confirmation of the detection of plasmid-mediated quinolone resistance genes[6–10] and of the β-lactamase genes bla_{TEM},[11] bla_{CTX-M}[12] and bla_{SHV} (www.medvetnet.org/pdf/Reports/Appendix_2_Workpackage_9.doc). PCR products were purified by the QIAquick PCR Product Purification Kit (Qiagen GmbH, Germany). Sequences were determined by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a 3100-Avant Genetic Analyzer (Applied Biosystems). Sequence data were analysed with the Sequencher 4.6 program. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for gene sequences homologous to the nucleotide sequences found.

Plasmid characterization and location of resistance genes

Transfer of resistance was tested by standard broth mating experiments using a rifampicin-resistant, indole-negative *Escherichia coli* K12 as recipient. Transconjugants were selected on MacConkey agar with 75 mg/L rifampicin and 1 mg/L cefotaxime. The plasmids in the donor strains and the transconjugants were analysed by PCR-based replicon typing (PBRT).[13] IncHI2-positive plasmids were further typed using 10 different PCRs, based on the sequences of the IncHI2 plasmids R478 and pAPEC-01-R.[14] The sizes of the plasmids were determined using PFGE of S1 nuclease digests of total DNA.[15] Linkage between plasmids and resistance genes was determined by miniaturized microarray analysis of the transconjugants. The location of $bla_{CTX-M-15}$ genes in the *Salmonella* Concord isolates was determined by PFGE of I-CeuI and XbaI digests followed by Southern blot hybridization using a PCR-generated digoxigenin-labelled CTX-M-15 probe. To determine the genetic relationship between the three *Salmonella* Concord isolates, XbaI-PFGE was performed according to the PulseNet protocol (www.pulsenet.com).

Results

Susceptibility tests and detection of resistance genes

All *Salmonella* isolates were resistant to ampicillin, ceftazidime, cefotaxime, tetracycline, sulfamethoxazole, trimethoprim, chloramphenicol, gentamicin and streptomycin; the *Salmonella* Concord isolates were also resistant to florfenicol and the *Salmonella* Senftenberg isolate was resistant to kanamycin. Furthermore, all isolates were low-level resistant to ciprofloxacin (MIC: 0.12–0.5 mg/L), but still susceptible to nalidixic acid (MIC: 8–16 mg/L). The resistance genes *qnrB2*, bla_{TEM-1}, bla_{SHV-12}, *sul1*, *dfrA*19, *tet*(D), *strA* and *strB* were detected in all isolates. Some resistance genes were only detected in *Salmonella* Concord, including $bla_{CTX-M-15}$, *floR*, *sul2* and *tet*(A). The aminoglycoside resistance gene *aac(6′)-1b* was only detected in *Salmonella* Senftenberg. This classical variant of the gene was confirmed by sequencing the amplicon. In addition, the resistance genes *qnrC*, *qnrD* and *qepA* (not included in the microarray) were not detected by PCR in any of the four isolates.

Plasmid characterization and location of resistance genes

Salmonella Senftenberg 200.27 harboured one 310 kb IncHI2 plasmid, which was transferred to the *E. coli* K12 strain by conjugation. All nine resistance genes identified in the donor strain were detected in transconjugant 200.27-T1 (Table 1). The IncHI2 plasmid of the *Salmonella* Senftenberg isolate was characterized as an R478-like plasmid.

Both *Salmonella* Concord 199.69 and *Salmonella* Concord 206.54 harboured two plasmids identified by PBRT as IncHI2

Table 1. Replicon types of plasmids and resistance genes detected in donor strains and transconjugants

Isolates	IncHI2	IncA/C	intI1	TEM	SHV	CTX-M	*qnrB*	*tet*(A)	*tet*(D)	*sul1*	*sul2*	*dfrA19*	*floR*	*strA*	*strB*	*aac(6′)-1b*
200.27 (D)	+		+	+	+		+		+	+		+		+	+	+
200.27-T1	+		+	+	+		+		+	+		+		+	+	+
199.69 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
199.69-T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
199.69-T2		+	+	+		+	+	+		+	+	+	+	+	+	
206.54 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
206.54-T1		+	+	+		+	+	+		+	+	+	+	+	+	
210.52 (D)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
210.52-T1	+		+	+	+		+		+	+	+	+		+	+	
210.52-T7		+		+		+		+		+	+		+	+	+	

Replicon types of plasmids and resistance genes detected in donor strains (D) and transconjugants (-T) of the *Salmonella* Senftenberg isolate (200.27) and *Salmonella* Concord isolates (199.69, 206.54 and 210.52).

(200 kb) and IncA/C (230 kb). Transconjugant 199.69-T1 harboured both plasmids including all 12 resistance genes identified in the donor strain. Transconjugants harbouring IncA/C lacked two resistance genes identified in the donor strain [bla_{SHV} and *tet*(D)]. Transconjugants with only IncHI2 were not obtained. *Salmonella* Concord 210.52 also harboured IncHI2 and IncA/C plasmids, but of different sizes: 170 kb (IncA/C); and 290 kb (IncHI2). Conjugation experiments with *Salmonella* Concord 210.52 resulted in transconjugants with either IncHI2 or IncA/C plasmids. Transconjugant 210.52-T1 harbouring an IncHI2 plasmid lacked $bla_{CTX\text{-}M\text{-}15}$, *tet*(A) and *floR*, whereas transconjugant 210.52-T7 harbouring an IncA/C plasmid lacked $bla_{SHV\text{-}12}$, *qnrB2*, *tet*(D) and *dfrA19* (Table 1). All IncHI2 plasmids in the three *Salmonella* Concord isolates were characterized as R478-like plasmids. However, all plasmids lacked three genes present in R478 (*arsB*, *smr136* and *tnsD*) and harboured the 01R_160 locus as in pAPEC-O1-R.

Southern blot hybridization experiments demonstrated that the $bla_{CTX\text{-}M\text{-}15}$ gene was only located on an IncA/C plasmid in all three *Salmonella* Concord isolates (results not shown). Finally, XbaI-PFGE revealed a unique digestion pattern for all three *Salmonella* Concord isolates indicative of the genetic variation of MDR *Salmonella* Concord strains originating from Ethiopia (results not shown).

Discussion

Salmonella Senftenberg is a common serotype in the Netherlands; in the last decade, a total of 581 isolates (3%), originating from different sources, were tested for antibiotic susceptibility. Until 2007, all *Salmonella* Senftenberg isolates were susceptible to third-generation cephalosporins. On the contrary, *Salmonella* Concord is a very rare serotype; in the last decade, only nine isolates of human origin (<0.01%) were tested. Moreover, eight of these nine isolates were resistant to third-generation cephalosporins. However, until 2007, all *Salmonella* Concord isolates were susceptible to ciprofloxacin. These figures show the rarity of *Salmonella* isolates that are resistant to both third-generation cephalosporins and fluoroquinolones in the Netherlands. Although a simultaneous increase in *qnr*-positive *Salmonella*[16] and ESBL-producing *Salmonella* has been reported in our national surveillance programme since 2003 (www.cvi.wur.nl/NL/publicaties/rapporten/maranrapportage/), this is the first report of both *qnr*- and ESBL-positive *Salmonella* isolated from human patients in the Netherlands.

The dissemination of *qnr* genes in Enterobacteriaceae including *Salmonella* of human origin is reported with increasing frequency. Recently, an IncHI2 plasmid associated with *qnrB2* and $bla_{SHV\text{-}12}$ was identified in a human *Salmonella* Bredeney isolate.[17] In our study *qnrB2* was detected on two different types of conjugative plasmids: IncHI2; and IncA/C. To our knowledge, this is the first description of a *qnrB2* gene on an IncA/C plasmid in *Salmonella enterica*.

Fabre *et al.*[2] detected $bla_{CTX\text{-}M\text{-}15}$ genes in *Salmonella* Concord isolates on chromosomal DNA, but also on an InHI2 plasmid and on a fusion plasmid of IncY and IncA/C2. However, in our study we detected $bla_{CTX\text{-}M\text{-}15}$ genes only on InA/C plasmids (negative for IncY) and not on chromosomal DNA. In addition, we identified $bla_{SHV\text{-}12}$ genes only on IncHI2 plasmids. A study by Hendriksen *et al.*[4] included four Dutch *Salmonella* Concord isolated from 2001 to 2006, which showed resistance to third-generation cephalosporins, but were completely susceptible to ciprofloxacin. In this Danish study the co-existence of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$ genes was reported on a single plasmid. Nonetheless, our study revealed the co-existence of these resistance genes on two different plasmids in all *Salmonella* Concord isolates; $bla_{CTX\text{-}M\text{-}15}$ on IncA/C plasmids and $bla_{SHV\text{-}12}$ on IncHI2 plasmids. The microarray revealed that the smaller IncA/C plasmid of transconjugant 210.52-T7 lacked a class 1 integron (intI1) and two resistance genes (*dfrA19* and *qnrB2*) compared with the plasmids of transconjugants 199.69-T2 and 206.54-T1. This indicates that a fragment harbouring a complex integron is lacking on the IncA/C plasmid of *Salmonella* Concord 210.52. Finally the IncHI2 plasmids of all *Salmonella* Concord isolates were identically characterized as R478-like plasmids, all lacking the *arsB* gene. To our knowledge, this is the first description of such an R478-like plasmid.

The findings of this study provide additional information on the genetic background of ESBL-producing, *qnr*-positive *Salmonella* Concord and *Salmonella* Senftenberg isolates. The potential human health impact of infections with such MDR *Salmonella* emphasizes the need to monitor these resistance patterns in *Salmonella* carefully.

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Transparency declarations

None to declare.

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