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Full Length Research Paper

Detection of mutations in the *gyrA* of clinical *Salmonella* spp.

U. Govinden[1]*, C. Mocktar[1], P. Moodley[2, 3], A. W. Sturm[2] and S. Y. Essack[1]

[1]School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.
[2]Department of Medical Microbiology, University of KwaZulu-Natal, Durban, 4000, South Africa.
[3]Department of Infection Control, University of KwaZulu-Natal, Durban, 4000, South Africa.

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The high prevalence of resistance to nalidixic acid and reduced susceptibility to ciprofloxacin of *Salmonella* spp. obtained from stool samples of neonates presenting with acute diarrhea in 2001 at the King Edward VIII hospital in Durban, South Africa, prompted this study to determine if there were any mutations in the QRDR of these isolates and to search for the *qnrA* gene. All isolates with nalidixic acid MICs > 48 µg/ml had the single mutation D87N, or D87G in the QRDR of the *gyrA* gene, and only 2 strains had an additional mutation; S83L and S83F respectively. The mutation S83T was present in only one isolate with the nalidixic acid MIC of 10 µg/ml whilst the 6 other strains with nalidixic acid MICs < 10 µg/ml had no changes in the QRDR of the *gyrA* gene. The *qnrA* gene was not found. These findings indicate that there are mutations in the *gyrA* of *Salmonella* isolates which could contribute to resistance to nalidixic acid with reduced susceptibility to ciprofloxacin and there is the co-expression of quinolone and extended-spectrum ß-lactam resistance among *Salmonella* spp.

Key words: Quinolone resistance, mutations in *gyr*A.

INTRODUCTION

In gram-negative bacteria the principal target of quinolone including fluoroquinolone activity is the type II topoisomerase, DNA *gyrA*se. DNA *gyrA*se is a tetramer composed of two *GyrA* subunits (encoded by *gyrA* gene) and two GyrB subunits (encoded by *gyrB* gene) (Kilmartin et al., 2005). DNA *gyrA*se catalyses the negative supercoiling of DNA and is therefore essential for maintenance of DNA topology. Topoisomearse IV is also a tetrameric enzyme consisting of two ParC and two ParE subunits and is involved in the segregation of replicated daughter chromosomes during DNA replication. Topoisomerase IV is a homologue of DNA *gyrA*se and the parC and parE genes have strong sequence identity to *gyrA* and gyrB. Fluoroquinolones stabilize the breaks in the DNA made by the DNA *gyrA*se or topoisomerase IV,and the resulting drug/enzyme/DNA complex inhibits DNA synthesis (Hopkins et al., 2005).

*Corresponding author. E-mail: Govindenu@ukzn.ac.za. Tel: + 2731-2608251. Fax + 2731-2607792.

Plasmid-mediated quinolone resistance (qnrA) was originally reported in a *Klebsiella pneumoniae* clinical isolate from the USA in 1998 and has since been identified in many enterobacterial species including *Salmonella* spp. Six variants of QnrA are known (QnrA1-QnrA6). The *qnrA* gene codes for a 218 amino acid protein belonging to the pentapeptide family that protects DNA from quinolone binding to *gyrA*se and topoisomerase IV. Recently two other plasmid-mediated quinolone resistance genes, namely, *qnrB* and *qnrS* have been identified that code for QnrB (six variants) and QnrS (two variants) also belonging to the pentapeptide repeat family and sharing 41 and 60% amino acid identity with QnrA, respectively. QnrA confers resistance to quinolones such as nalidixic acid and increases MIC values of fluoroquinolones up to 20-fold (Robicsek et al., 2006).

Raised MICs to quinolones and fluoroquinolones are associated with mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene. Less frequently, quinolone resistance is associated with point mutations in the type IV topoisomerase. The QRDR of DNA *gyrA*se A in *S. enterica* is between amino acids 67

and 122 encoded by nucleotides 185 to 361 in the *gyrA* gene. The most common amino acid substitutions in the *GyrA* subunit associated with quinolone resistance occur at codons Ser83 and Asp87 (Kilmartin et al., 2005). Mutations in *gyrB* of *Salmonella* spp. are rarely detected, even in strains exhibiting high level fluoroquinolone resistance. Previous studies have shown that topoisomerase IV is not as sensitive to quinolones as DNA *gyrA*se and is therefore, a secondary target for quinolones. Mutations in *parC* of salmonellae are not as frequent as in *E. coli* and studies suggest that they do not play an important role in quinolone resistance or they may only be required to achieve high level resistance. Several studies have failed to detect any mutations in the *parE* gene of salmonellae (Hopkins et al., 2005).

The high resistance to nalidixic acid and reduced susceptibility to ciprofloxacin of *Salmonella* spp. isolated from stool samples of neonates presenting with acute diarrhea in 2001 at the King Edward VIII hospital in Durban, South Africa, prompted this study to determine if there were any mutations in the QRDR of these *Salmonella* spp. and to search for the *qnrA* gene.

MATERIALS AND METHODS

Bacterial strains

Fifty nine putative ESBL positive isolates of *Salmonella* spp. were obtained from stool samples of neonates presenting with acute diarrhea in 2001 at the King Edward VIII hospital in Durban. The ESBL study is published elsewhere (Govinden et al., 2008).. All isolates that were resistant to nalidixic acid (n = 22) and 7 isolates that were susceptible to nalidixic acid were selected for QRDR analysis in this study. *E. coli* 25922 was used as the control for susceptibility testing.

The isolates were serotyped using commercially available antisera (Bioweb, South Africa) according to the Kauffman–White scheme for *Salmonella* serotyping (Kauffman, 1972; Popoff, 2001).

Susceptibility testing

Disc diffusion susceptibility tests for nalidixic acid and ciprofloxacin, were performed according to NCCLS guidelines (2003). Results were read with the Biomic automated reading system (Giles Scientific, New York).

PCR detection of *gyrA* and *qnrA* genes

Amplification of the *gyrA* and *QnrA* genes were done with primers *gyrA*- F 5' TGTCCGAGATGGCCTGAAGC 3', *gyrA*- R 5' CGTTGATGACTTCCGTCAG 3' (Giraud et al., 1999) and QP1- 5' GATAAAGTTTTTCAGCAAGAGG 3' and QP2 -5' ATCCAGATCGGCAAAGGTTA 3' (Jacoby et al., 2003) respectively. Strains were grown overnight at 37°C in Mueller-Hinton broth. 1.5 ml of each culture was pelleted and cells were boiled in 200 µl of water. After centrifugation the supernatants were kept at -20°C. PCR was performed in a total volume of 50 µl, which contained 5 µl of supernatant, 25 µl of master mix (Applied Biosystems, Johannesburg, South Africa)), 25 pmol of each primer and water. After an initial denaturation of 3 min at 94°C, amplification was performed over 30 cycles, each one consisting of 1 min at 94°C, 1 min at hybridization temperature 55°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C (Giraud et al., 1999). 5 µl aliquots of PCR product were analysed by gel electrophoresis with 1% agarose. Negative controls were PCR mixtures with the addition of water in place of template DNA. Gels were stained with ethidium bromide at 10 µg/ml and photographed with UV illumination. Sequencing of the amplified products was performed with the SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequences were analysed using the BLAST 2.0 (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.mih.gov/Blast/; accessed September 2007.

RESULTS AND DISCUSSION

Of the 29 isolates studied, 37% (22/59) were resistant to nalidixic acid with MICs > 48 µg/ml, whilst 63% (37/59) fell in the susceptible range with MICs from 4 to 10 µg/ml. All the isolates were susceptible to ciprofloxacin with MIC ranging from 0.125 to 0.5 µg/ml. 16 resistant and 5 susceptible isolates to nalidixic acid were ESBL positive. Sequencing identified mutations in the QRDR of *gyrA* as per Table 1. Mutations in *gyrA* were noted for ESBL positive and negative strains with resistance to nalidixic acid; the exception was isolate 376 that was ESBL positive, susceptible to nalidixic acid and had a S83T mutation. No positive amplification product was obtained for the *qnrA* gene.

All isolates with nalidixic acid MICs > 48 had the single mutation D87N, or D87G in the QRDR of the *gyrA* gene, and only 2 strains; 580 and 695 had an additional mutation; S83L and S83F respectively. The mutation S83T was present in only one strain that had the nalidixic acid MIC of 10 µg/ml whilst the 6 other strains with nalidixic acid MICs < 10 µg/ml had no changes in the QRDR of the *gyrA* gene. An increase in the MIC to ciprofloxacin was noted in most strains with ciprofloxacin MIC > 0.125 µg/ml. Although ciprofloxacin MICs of < 1 µg/ml and > 4 µg/ml are accepted breakpoints for susceptibility and resistance to salmonellae, it has been suggested that fluoroquinolone-susceptible strains that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis (CLSI, 2008). Susceptibility testing for nalidixic acid is therefore encouraged although this drug is not used for the treatment of extraintestinal *Salmonella* infections (Rodriguez et al., 2005). A single mutation in *gyrA* of *Salmonella* may be sufficient to cause high-level resistance to nalidixic acid but additional mutations may be required to attain high level fluoroquinolone resistance. Levy et al. (2004) showed that the relative frequency of mutations depended on the particular fluoroquinolone used for selection. Selection with enrofloxacin was more likely to yield S83F mutations, while selection with ciprofloxacin or nalidixic acid favoured recovery of D87G mutations (Hopkins et al., 2005). The most frequent mutations noted in this study were D87G and D87N. There is also a speculation that the reduced

Table 1. Mutations in *gyrA* of *Salmonella* isolates.

Isolate	Serotype	MIC (µg/ml)		Mutations In *gyrA*	ESBL + (n = 21) ESBL - (n = 8)
		Nalidixic acid	Ciprofloxacin		
216	*S. isangi*	>48	0.25	D87N	+
218	*S. isangi*	>48	0.25	D87N	-
259	*S. isangi*	>48	0.5	D87N	+
262	*S. isangi*	>48	0.25	D87N	+
296	*S. isangi*	>48	0.25	D87N	+
370	*S. kissi*	>48	0.25	D87N	+
493	*S. reading*	>48	<0.125	D87N	+
509	*S. typhimurium*	>48	0.25	D87N	+
606	*S. typhimurium*	>48	0.25	D87N	-
580	*S. kivu*	>48	0.5	D87N; S83L	+
15	*S. typhimurium*	>48	<0.125	D87G	+
256	*S. isangi*	>48	0.25	D87G	+
464	*S. isangi*	>48	0.125	D87G	+
518*	*ND*	>48	0.25	D87G	-
565	*S. typhimurium*	>48	<0.125	D87G	+
586	*S. typhimurium*	>48	0.25	D87G	-
620	*S. typhimurium*	>48	<0.25	D87G	+
695	*S. typhimurium*	>48	0.25	D87G; S83F	-
709	*S. typhimurium*	>48	<0.125	D87G	+
873	*S. typhimurium*	>48	<0.125	D87G	+
951	*S. typhimurium*	>48	0.25	D87G	+
978	*S. typhimurium*	>48	<0.125	D87G	-
376	*S. typhimurium*	10	<0.25	S83T	+
31	*S. typhimurium*	8	0.125	No change	-
2	*S. typhimurium*	8	<0.125	No change	-
420	*S. isangi*	6	<0.125	No change	+
467	*S. kissi*	10	0.125	No change	+
611	*S. typhimurium*	6	0.125	No change	+
669	*S. typhimurium*	4	<0.125	No change	+

*Serotype could not be determined.

quinolone susceptibility may be due to decreased permeability or the presence of efflux pump mechanisms as exposure to low level quinolones can lead to inactivation of the efflux pump system and a reduction in susceptibility, even when there is no mutation in *gyr*A (Cebrian et al., 2005). Quinolone resistance in Enterobacteriaceae appears to be a staggered process, where an initial mutation in *gyr*A produces nalidixic acid resistance and decreased susceptibility to fluoroquinolones and facilitates the occurrence of a second mutation in the same gene or in other quinolone target encoding genes that will lead to full resistance. Therefore resistance to naldixic acid could be a good predictor for the emergence of fluoroquinolone resistance (Aznar et al., 2007). These findings indicate that there are mutations in the *gyrA* of *Salmonella* isolates which could contribute to resistance to nalidixic acid with increased MICs to ciprofloxacin. In addition there is the co-expression of quinolone and extended-spectrum ß-lactam resistance among *Salmonella* spp. The continued use of nalidixic acid and ciprofloxacin could result in further mutations in the DNA *gyrA*se and increasing resistance to these antibiotics.

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