

# Quinolone Resistance of *Salmonella enterica* Serovar Virchow Isolates from Humans and Poultry in Israel: Evidence for Clonal Expansion<sup>∇</sup>

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***Salmonella enterica* serovar Virchow is highly prevalent in humans and farm animals in Israel. In addition to high rates of resistance to multiple antibiotics, this serovar exhibits a high incidence of resistance to nalidixic acid. More than 90% of *Salmonella* serovar Virchow isolates of human and poultry origin obtained from 1997 to 2004 were resistant to nalidixic acid (MIC  $\geq$  128  $\mu$ g/ml), with reduced susceptibility to ciprofloxacin (MIC between 0.125 and 0.250  $\mu$ g/ml). Most isolates belonged to two predominant, closely related pulsed-field gel electrophoresis image types. Investigation of the mechanisms of quinolone resistance revealed that this pathogen probably emerged from a parental clone that overproduced the AcrAB efflux pump and had a single point mutation in *gyrA* leading to the Asp87Tyr substitution. The close resemblance between human and poultry isolates points to poultry as a likely source of *Salmonella* serovar Virchow in the food chain.**

The global increase in the prevalence of *Salmonella* strains with a reduced susceptibility to quinolones constitutes a major concern, since these pathogens have been associated with a significant burden of hospitalization and mortality (18, 19, 30) and with clinical failures of therapy (6–8, 11, 23, 30, 36, 38, 39). Quinolone resistance in gram-negative pathogens is usually acquired by chromosomal mutations, primarily in the genes that encode DNA gyrase and topoisomerase IV (22). Additionally, mutations affecting the uptake or efflux of the quinolones result in decreased accumulation of the antibiotics in the bacterial cell (32). Plasmids that harbor *qnr* genes that encode fluoroquinolone-inactivating enzymes have also been discovered (15, 37).

*Salmonella enterica* serovar Virchow, a serovar that is rare in the United States and uncommon in Europe, has emerged and spread among humans and farm animals in Israel to become one of the three ranking *Salmonella* serovars. The higher proportion of *Salmonella* serovar Virchow infections among blood isolates reflected its increased invasiveness in young children (43, 44). In addition to high rates of resistance to multiple antibiotics, this serovar displays notable rates of nalidixic acid resistance (about 90%) (44). The unique epidemiology in Israel, where *Salmonella* serovar Virchow is both highly prevalent among humans and poultry and unusually highly resistant to nalidixic acid, presents an opportunity to investigate the existence and evolution of the various mechanisms of quinolone resistance.

## MATERIALS AND METHODS

**Bacterial strains.** A total of 336 isolates of *Salmonella* serovar Virchow were available for our studies. Human stool and blood isolates ( $n = 243$ ) collected between 1997 and 2004 were obtained from the Government Central Laborato-

ries (44), and poultry isolates ( $n = 93$ ) collected between 2001 and 2003 from the Veterinary Services (random samples).

**Typing by PFGE.** Typing of the isolates was carried out by pulsed-field gel electrophoresis and analyzed as previously described (44).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed by the disk diffusion technique according to the disk manufacturer's (Oxoid) guidelines, which, except for guidelines pertaining to colistin and polymyxin B, are based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (31). Guidelines for colistin and polymyxin B are based on the approval of the U.S. Food and Drug Administration.

MICs of nalidixic acid and ciprofloxacin were determined by the broth dilution method according to the CLSI guidelines (10).

**Cyclohexane resistance.** Cyclohexane resistance was determined by the method of Asako et al. (1). *Escherichia coli* K-12 AG100, AG102, (resistant strain) and AG100-A (sensitive strain) (16, 45) were used for controls.

**Determination of mutations within *gyrA*.** Mutations in *gyrA* were determined by using pyrosequencing and/or sequencing of the *gyrA* amplicon. For sequence analysis, the *gyrA* gene was amplified by PCR from the extracted genomic DNA of the isolates using the primers CACCCGAATAAAGCATTGTCTGG/ACGGACGCGAAATCAGCG. Primers were designed based on the *gyrA* sequence of *Salmonella* serovar Typhimurium (accession no. X78977). Pyrosequencing of the locus encoding the codons of amino acids 83 to 88 of GyrA was performed on an additional 87 isolates to expand the sampling. The experiment was performed and data were analyzed as previously described (21) with the PCR primers 5'-AAAATCTGCCGTGTCGT-3' and 5'-Biotine-C-TGCGCCATACGAACGAT-3' and the sequencing primer 5'-ATCCCCACGCGATT-3'. Controls were serovar Virchow isolates that had been sequenced and clinical isolates of serovars Agona and Typhimurium with known mutations.

**PCR screening for the *qnr* genes.** The presence of *qnrA*, *qnrB*, and *qnrS* genes was studied by PCR as previously described (15). Positive controls were *E. coli* strains with the plasmids pMG252, pMG298, and pMG306 (15).

**Determination of transcription levels of *acrAB*, *marA*, and *soxS*.** Transcription analysis of *acrAB*, *marA*, and *soxS* was carried out using the green fluorescent protein (GFP) signals expressed from the *gfp* gene that was fused to the promoter/operator regions of these genes. Construction of the plasmids was previously described (E. Hartog, L. Ben-Shalom, D. Shachar, K. R. Matthews, and S. Yaron, presented at the 2nd ASM Conference on *Salmonella*: From Pathogenesis to Therapeutics, Victoria, Canada, 9 to 13 September 2006). *E. coli* K-12 AG100 and AG102 and *Salmonella* serovar Typhimurium ATCC 14028 were used for comparison. Each isolate was also transformed with a promoterless plasmid for control. Growth, fluorescence detection, and data analysis were carried out as described previously (3).

**Statistical methods.** Proportions were compared using the chi-square test, and two-tailed *P* values were reported. Analyses were performed using SPSS version 13.

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TABLE 1. Frequency of resistance to antimicrobial agents in *Salmonella* serovar Virchow isolates from human and poultry sources

Resistance to <sup>a</sup>	Human isolates (n = 243)		Chicken isolates (n = 93)		P value <sup>b</sup>
	No.	%	No.	%	
No antibiotic agents	13	5.3	2	2.2	NS
Colistin	4	1.6	15	16.1	<0.001
Polymyxin B	0	0	14	15.1	<0.001
Ampicillin	31	12.8	19	20.7	NS
Neomycin	1	0.4	4	4.3	NS
Gentamicin	1	0.4	0	0.0	NS
Nalidixic acid	221	90.9	86	92.5	NS
Trimethoprim-sulfamethoxazole	113	46.5	52	55.9	NS
Tetracycline	124	51.0	59	63.4	0.05
Chloramphenicol	85	35.0	37	39.8	NS
Streptomycin	125	51.4	48	51.6	NS
Ciprofloxacin	0	0	0	0	NS
Ceftriaxone	0	0	0	0	NS
≥4 Antibiotic agents	113	46.5	52	55.9	NS

<sup>a</sup> Antibiotics and the amounts in disks were as follows: chloramphenicol, nalidixic acid, neomycin, ceftriaxone, tetracycline, and polymyxin B, 30 µg each; ampicillin, streptomycin, and colistin, 10 µg each; ciprofloxacin and gentamicin, 5 µg each; trimethoprim-sulfamethoxazole 1:19, 25 µg. The quality control strains that were used for the susceptibility testing are *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

<sup>b</sup> NS, not significant. For all NS cell entries, the difference between human isolates and chicken isolates is not statistically significant ( $P > 0.05$ ).

## RESULTS AND DISCUSSION

A high rate of resistance was detected when we determined the susceptibility of the *Salmonella* serovar Virchow isolates to 12 antibiotics (Table 1). Overall, the resistance rates were comparable between the two sources, poultry and humans, except for the excessive frequency of resistance to colistin and polymyxin B among the poultry isolates. The highest resistance rate was associated with nalidixic acid—more than 90%. However, all the tested isolates were susceptible to ciprofloxacin according to the CLSI criteria (31). There was no change in the resistance pattern to nalidixic acid during the study period.

PFGE analysis showed 20 pulsotypes. Most isolates from human and poultry sources belonged to two predominant pulsotypes, A1 (43%) and B1 (36%), which differ by two bands only (44). Other pulsotypes (see Table 3) were also closely related. Based on published PFGE images, the predominant pulsotypes in Belgium, France, and Australia were similar to the less common A4 pulsotype in Israel (4, 5). Resistance to nalidixic acid was not associated with a particular PFGE profile.

Approximately one-third of the nalidixic acid-resistant isolates from both sources (105/307) and four susceptible isolates were randomly selected for further testing of their resistance to nalidixic acid and ciprofloxacin by the broth dilution method. Resistance to ciprofloxacin was not detected, but the ciprofloxacin MIC for the nalidixic acid-resistant isolates was usually higher than that for the sensitive isolates, and a significant correlation between nalidixic acid resistance and an elevated ciprofloxacin MIC was observed ( $P < 0.001$ ) (Table 2). To the

TABLE 2. Correlation of MICs between nalidixic acid and ciprofloxacin for *Salmonella* serovar Virchow isolates<sup>a</sup>

NAL MIC (µg/ml) <sup>b</sup>	CIP MIC (µg/ml) <sup>c</sup>		
	0.250	0.125	≤0.03
≤4			3
8			1
16			
32			
64			
128		11	2
256	1	74	
512	1	16	

<sup>a</sup> Numbers in table cells represent numbers of isolates.

<sup>b</sup> NAL, nalidixic acid.

<sup>c</sup> CIP, ciprofloxacin.

best of our knowledge, a stable frequency of more than 90% nalidixic acid resistance with MIC ≥ 128 µg/ml and with elevated ciprofloxacin MIC in human and veterinary isolates over an 8-year study period had not been described with *Salmonella* (22). This rate of quinolone resistance was by far higher than the rates reported for *Salmonella* serovar Virchow in Europe (40). However, nalidixic acid resistance among isolates of *Salmonella* serovar Virchow in Belgium doubled during the period of 2000 to 2003 to 84% (5). Resistance to nalidixic acid, among other prevalent *Salmonella* serovars in Israel, was also less common: 37% in serovar Typhimurium, 17% in serovar Hadar, and 1% in serovar Enteritidis (29).

It was previously shown that mutations in *gyrA* can be sufficient to cause high-level resistance to nalidixic acid in *Salmonella* (17, 22). We screened for mutations in *gyrA* to target the reason for the high incidence of resistance among the isolates. Initially we sequenced the *gyrA* gene of 27 nalidixic acid-resistant isolates and 3 susceptible isolates. The same single-point mutation was observed in all resistant isolates: GAC was switched to TAC (Asp87Tyr). The same mutation was also found in a susceptible isolate, while the *gyrA* gene of the other susceptible isolates was identical to the *gyrA* of *Salmonella* serovar Typhimurium (accession no. X78977). Pyrosequencing was performed on an additional 87 isolates to expand the sampling. The only substitution that was detected in the serovar Virchow isolates was Asp87Tyr. Table 3 summarizes the sequence results of all isolates with regard to their resistance and PFGE typing. Overall, there was a significant correlation between the presence of the Asp87Tyr substitution and resistance to nalidixic acid ( $P = 0.013$ , chi-square test), as well as an elevated ciprofloxacin MIC ( $P < 0.001$ ). No correlation was observed between the presence/absence of the Asp87Tyr substitution and any PFGE profile.

The Asp87Tyr substitution was reported with *Salmonella* serovar Virchow as well as with other serovars. Similar to the results of this study, for the different serovars with the Asp87Tyr substitution, the nalidixic acid MICs were between 64 and 512 µg/ml and ciprofloxacin MICs were between 0.125 and 1 µg/ml (12, 20, 24, 27, 42), lower MICs than for strains with other mutations in the codons of Asp87 or Ser83 (26).

A high incidence of nalidixic acid resistance could arise from either rare mutation events with subsequent clonal expansion and dissemination or from frequent mutation and selection

TABLE 3. Mutation in *gyrA* gene in *Salmonella* serovar Virchow isolates

Nalidixic acid MIC (μg/ml)	Ciprofloxacin MIC (μg/ml)	No. of isolates	PFGE result	GyrA mutation
Resistant isolates 128–512	0.125–0.25	78	A1 (43%), B1 (30%), A4 (8%), A3 (5%), B2 (4%), A2 (4%), B6, B7, A6, A9 (1.3%)	Asp87-Tyr
128	0.125	7	A1 (29%), B1 (42%), A4 (29%)	No mutation
Resistant <sup>a</sup>	≤0.03	2	A1 (100%)	No mutation
	Susceptible <sup>a</sup>	26	B1 (58%), A1 (23%), B4 (12%), A10 (4%), A4 (4%)	Asp87-Tyr
Sensitive isolates				
≤8	≤0.03	3	B1 (33%), A1 (33%), A3 (33%)	No mutation
4	≤0.03	1	A1	Asp87-Tyr

<sup>a</sup> MICs were not determined; disk diffusion was performed.

events. Clonal expansion would result in relatively homogeneous resistant organisms, whereas the frequent mutation hypothesis should result in greater heterogeneity (24). The observation that most of the resistant isolates from 1997 to 2004 shared the same mutation is indicative of the spread of a single resistant clone of *Salmonella* serovar Virchow. This is also supported by the observations that all isolates from human and poultry sources had closely related PFGE profiles. Evidence for the spread of a resistant clone with the Asp87Tyr switch was also described for *Salmonella* serovar Enteritidis (24). Sim-

ilarly, a single substitution (Ser83Phe) was found in a clone of extended-spectrum beta-lactamase-producing *Salmonella* serovar Virchow with an elevated MIC of ciprofloxacin (5).

To examine whether quinolone resistance of *Salmonella* serovar Virchow is also plasmid encoded, we screened the isolates for the presence of *qnrA*, *qnrB*, and *qnrS*. All PCRs were negative, in contrast to those of the *E. coli* strains which served as positive controls.

Mechanisms resulting in a decreased quinolone accumulation may also contribute to the elevated resistance. The major

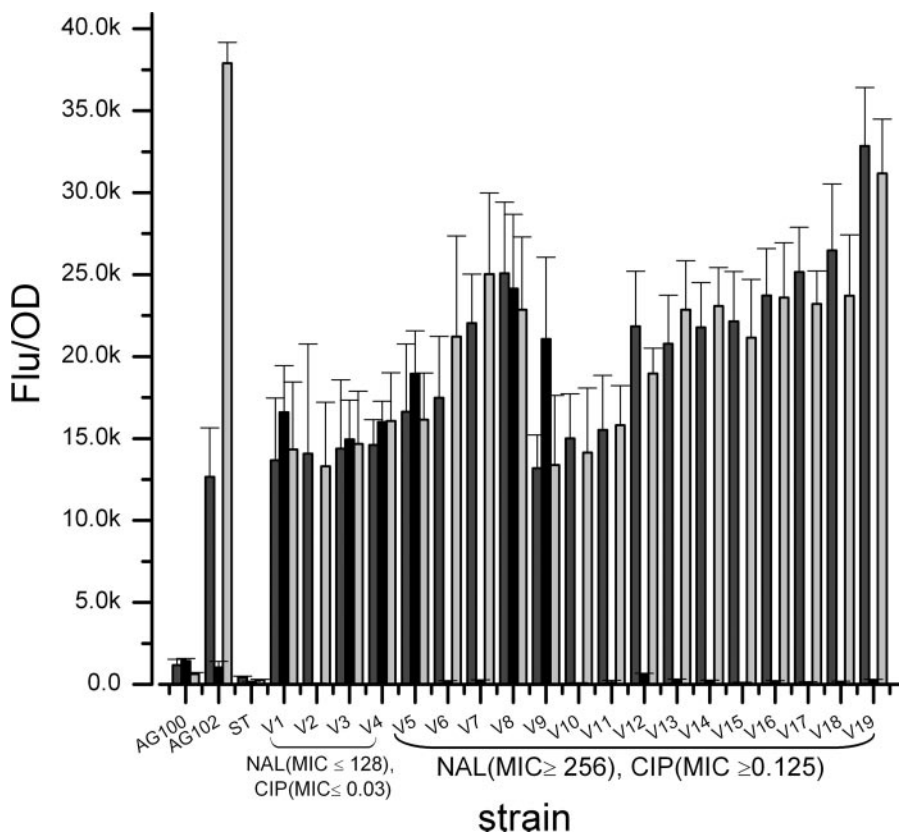


FIG. 1. Transcription of *acrAB*, *marA*, and *soxS* in *E. coli* K-12 strains AG100 and AG102, *Salmonella* serovar Typhimurium ATCC 14028 (ST), and representative isolates of *Salmonella* serovar Virchow (V1-V19). Numbers represent the means of normalized GFP fluorescence intensities of cultures with the following promoter-*gfp* fusions: *acrAp::gfp* (dark gray), *soxSp::gfp* (black), and *marAp::gfp* (light gray). The bars represent the standard errors of the means of the results of three experiments; each experiment was conducted in triplicate. SV10 and SV11 are cyclohexane-resistant isolates.

mechanism identified in *Salmonella* is active efflux mediated by AcrAB-TolC (2, 14). This can be achieved by overexpression of AcrAB or the activators MarA and SoxS (25, 28, 35). Using the GFP reporting system, we compared the transcription levels of *soxS*, *marA*, and *acrA* in 19 isolates with different resistance properties. A high correlation was observed between the transcription of *acrA* and that of *marA* ( $P < 0.0001$ ); however, the transcription of neither *acrA* nor *marA* correlated with resistance to nalidixic acid or ciprofloxacin (Fig. 1). The transcription of *acrA* and *marA* was significantly higher in all tested *Salmonella* serovar Virchow isolates than in *Salmonella* serovar Typhimurium or *E. coli* AG100 isolates. *E. coli* AG102 is a mutant that overexpresses MarA and AcrAB due to mutation in the repressor MarR (16). The transcription of *acrA* in AG102 was in the range of the *Salmonella* serovar Virchow isolates. A notable variability was observed in the transcription of *soxS*, and a high proportion (3/6) of isolates that overexpressed *soxS* were susceptible to nalidixic acid. This agrees with the previous reports that fluoroquinolone-resistant isolates of *Salmonella* displayed increased expression of *marA* and *acrA* but not *soxS* (13, 41). The reason for the high transcription levels of *acrA* and *marA* in the Virchow isolates has not been elucidated, as no mutation was detected in the sequence of these genes or their promoters. All these loci were found to be identical to *Salmonella* serovar Typhimurium (GenBank accession no. NC\_003277). The high transcription levels of *marA* and *acrA* in all isolates (including the sensitive ones) could suggest that this overproduction appeared prior to the mutation in *gyrA*.

Recent reports pointed to a possible correlation between antibiotic resistance and cyclohexane resistance (9, 33, 34). Since the isolates produced high levels of *acrA* and *marA* transcripts, we hypothesized that they would be resistant to cyclohexane. All but two tested isolates were found to be sensitive to cyclohexane. The two isolates (one from humans and one from poultry) that were resistant to cyclohexane and to nalidixic acid had the Asp87Tyr exchange in *gyrA* (Fig. 1, SV10 and SV11). The infrequent resistance to cyclohexane indicates a lack of correlation between resistance to cyclohexane and antibiotic resistance or the transcription levels of *acrA* and *marA*. Similar observations were also reported for other *Salmonella* serovars (13).

Ciprofloxacin is routinely used in Israel for the treatment of severe gastrointestinal infections in adults. Other fluoroquinolones like enrofloxacin, norfloxacin, danofloxacin, and ofloxacin are widely used with farm animals. Despite the persistent antibiotic pressure, we observed a high stability of a single type of mutation over the 8-year period studied without the development of ciprofloxacin resistance. Our data suggest that the high prevalence of nalidixic acid resistance with increased ciprofloxacin MICs among *Salmonella* serovar Virchow isolates in Israel probably emerged from the spread of a parental clone that overproduced the AcrAB efflux pump and had a single mutation in the *gyrA* gene. The same clone emerged in both humans and poultry, indicating that poultry was probably the main source for *Salmonella* serovar Virchow in the food chain. The veterinary and health authorities should target this clone for the application of more intensive and efficacious control measures.

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