ANTIMICROBIAL RESISTANCE

Association between cyclohexane resistance in *Salmonella* of different serovars and increased resistance to multiple antibiotics, disinfectants and dyes

LUKE P. RANDALL, SUE W. COOLES, ANTONY R. SAYERS and MARTIN J. WOODWARD

Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey KT15 3NB

A panel of 388 salmonellas of animal and human origin, comprising 35 serotypes, was tested for resistance to cyclohexane and to a range of antibiotics, disinfectants and dyes. Cyclohexane resistance was detected in 41 isolates (10.6%): these comprised members of the serovars Binza (1 of 15), Dublin (1 of 24), Enteritidis (1 of 61), Fischerkietz (4 of 5), Livingstone (9 of 11), Montevideo (1 of 32), Newport (4 of 23), Saint-paul (1 of 3), Senftenberg (10 of 24) and Typhimurium (9 of 93). Most (39 of 41) of the cyclohexane-resistant isolates were from poultry. Statistical analysis showed that the cyclohexane-resistant strains were significantly more resistant than the cyclohexane-susceptible strains to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid, tetracycline, trimethoprim, ceftriaxone and triclosan. The multiresistance patterns seen were typical of those caused by efflux pumps, such as AcrAB. The emergence of such resistance may play an important role in the overall antibiotic resistance picture of *Salmonella*, with particular effect on ciprofloxacin.

Introduction

In recent studies (unpublished), cyclohexane resistance was observed in *Salmonella* isolates from animals between 1994 and 1998, and this trait was associated with low-level resistance to multiple antibiotics and disinfectants and with reduced expression of outer-membrane protein F (OmpF). Such cyclohexane resistance was observed in 6 of 124 Dublin isolates, 2 of 142 Enteritidis and 1 of 137 Typhimurium isolates.

Cyclohexane resistance in *Escherichia coli* has been associated with up-regulation of the *acrAB*, *marA*, *soxS* or *rob* genes [1–3], which are also involved in low-level resistance to multiple antibiotics [2–5] and disinfectants [6, 7]. Up-regulation of *mar*, *rob* or *sox* genes confers resistance to organic solvents by up-regulation of the efflux pump AcrAB [3], which has been identified as the major mechanism responsible for the multi-drug resistance in Mar mutants [8]. Many efflux pumps have a wide substrate specificity, and AcrAB is involved in the efflux of β -lactam antibiotics,

chloramphenicol, erythromycin, fluoroquinolones, fusidic acid, nalidixic acid, rifampicin, tetracycline, acriflavin, deoxycholate, ethidium bromide, crystal violet and sodium dodecyl sulphate [9–12].

Oethinger *et al.* [13] showed an association between cyclohexane tolerance and fluoroquinolone resistance in clinical isolates of *E. coli* and Goldman *et al.* [14] showed that expression of *mar* protected *E. coli* from rapid killing by fluoroquinolones. Moreover, *E. coli* strains with gyrase mutations are not clinically resistant to fluoroquinolones in the absence of the AcrAB pump [15] and there is evidence that active efflux is the primary mechanism of resistance to ciprofloxacin in *S. Typhimurium* [16]. The aim of this study was to test a wide range of *Salmonella* serotypes isolated from man and animals for cyclohexane resistance and to relate cyclohexane resistance to resistance to those antibiotics (e.g., fluoroquinolones and β -lactams) and dyes (acridine orange and ethidium bromide) that are substrates for AcrAB [11, 12, 16].

Materials and methods

Bacterial isolates

Strains of animal or environmental origin were from the culture collection at the Veterinary Laboratories

Received 4 Dec. 2000; revised version accepted 9 April 2001.

Corresponding author: Dr M. J. Woodward (e-mail: M.J. Woodward@vla.defra.gsi.gov.uk).

Agency (VLA), Weybridge (Table 1). Poultry isolates were from turkeys, laying hens and broilers; human strains were obtained either from Kingston Hospital (Surrey) or from the Central Public Health Laboratory (CPHL), Colindale, London (Table 1). Most strains were isolated in 1998 and 1999 (Table 1) and were chosen to represent most common serotypes (Table 2). The organisms were stored on Dorset's egg slopes and frozen on MicroBank^{TM} (Pro-Lab Diagnostics) beads at -70°C before use. *E. coli* K12 strains AG100 and AG102 (a *marR* mutant of strain AG100 selected on tetracycline, [17]) and NCTC 10418 [18] were used as controls.

Antibiotics, disinfectants and chemicals

Antibiotics were obtained from Sigma-Aldrich (Poole, Dorset) except ciprofloxacin, which was donated by Bayer (Newbury, Berkshire). Acridine orange, ethidium bromide, cetrimide (hexadecyltrimethyl-ammonium bromide), cyclohexane and hexane were from Sigma-Aldrich; triclosan (Irgasan DP300) was donated by Ciba Consumer Care (Macclesfield, Cheshire).

Antibiotic susceptibility

Isolates were screened for susceptibility to a panel of 16 antibiotics by a disk diffusion method similar to that

Table 1. Sources of bacterial isolates*

Year isolated	Origin	Number of isolates	Number cyclohexane-resistant (%)
Unknown	Human	14	0
	Unknown	2	0
1995	Poultry	1	0
	Unknown	1	0
1996	Other animals	2	0
	Poultry	2	0
	Unknown	5	0
1997	Cattle	1	0
	Environment	5	0
	Other animals	1	0
	Poultry	2	0
	Unknown	2	0
1998	Cattle	5	0
	Environment	18	0
	Feed	4	0
	Other animals	4	0
	Pig	7	0
	Poultry	49	15 (31)
	Sheep	1	0
	Cattle	31	0
	Environment	14	1 (7) [†]
1999	Feed	1	0
	Human	40	0
	Other animals	1	0
	Pig	31	0
	Poultry	114	24 (21)
	Sheep	25	1 (4)
	Environment	4	0
	Human	1	0

*The panel was selected to contain *Salmonella* isolates from animal sources, but also those from human and environmental sources.

[†]The only environmental isolate that was cyclohexane-resistant was from a chicken farm.

Table 2. Frequency of serotypes* for panel and cyclohexane resistance

Serotype	Number of isolates in panel	Number cyclohexane-resistant (%)
Agama	1	0
Agona	1	0
Albany	1	0
Binza	15	1 (7)
Blockley	1	0
Cubana	1	0
Derby	3	0
Dublin	24	1 (4)
Emek	1	0
Enteritidis	61	1
Fischerkietz	5	4 (80)
Gold-coast	1	0
Haardt	1	0
Hadar	19	0
Haifa	2	0
Heidelberg	10	0
Indiana	6	0
Kedougou	2	0
Kisangani	1	0
Liverpool	1	0
Livingstone	11	9 (82)
Manhattan	2	0
Mbandaka	11	0
Montevideo	32	1 (3)
Muechen	1	0
Newport	23	4 (17)
Ohio	3	0
Reading	1	0
Saint-paul	3	1 (33)
Senftenberg	24	10 (42)
Stanley	1	0
Thompson	3	0
Typhimurium	93	9 (10)
Urettevreden	1	0
Virchow	12	0
Structure only	10	0

*The panel was selected to contain representatives of the more commonly isolated serotypes.

described previously [19]. The following disks were used (μg): amikacin (10), amoxycillin/clavulanic acid (20 + 10), ampicillin (10), apramycin (15), chloramphenicol (10), cefoperazone (30), cefuroxime (30), colistin (25), furazolidone (15), gentamicin (20), naladixic acid (30), neomycin (10), streptomycin (25), sulphamethoxazole/trimethoprim (25), tetracycline (10) and triple sulphonamide (500). Strains with a zone diameter of <13 mm were classified as resistant.

Cyclohexane resistance

Cyclohexane resistance was determined by the method of Asako *et al.* [1]. Bacteria grown overnight at 30°C in Luria Bertani (LB) broth were inoculated, with a multi-point inoculator, on to four LB agar plates in glass petri dishes. Duplicate plates were then flooded with 6 ml of hexane or cyclohexane, sealed with Saran-Wrap^{TM} and incubated overnight at 30°C . Colonies that grew in the presence of cyclohexane were deemed cyclohexane-resistant.

Minimum inhibitory concentrations (MICs)

MICs were determined by an agar doubling dilution method similar to that recommended by the NCCLS [20], the main exception being that Iso-Sensitest agar (Oxoid CM471) was used instead of Mueller Hinton agar. Bacteria grown overnight at 37°C in LB broth were diluted 1 in 10 in normal saline and inoculated, with a multi-point inoculator, on to agar containing suitable dilutions of the antibiotics ampicillin, ciprofloxacin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, tetracycline or trimethoprim; or the disinfectants triclosan or cetrimide; or the dyes acridine orange or ethidium bromide. The plates were incubated overnight at 37°C and MICs were recorded at the lowest concentrations inhibiting growth.

Statistical analysis

MIC distributions were compared by Wilcoxon-Mann-Whitney non-parametric tests. Exact two-sided probabilities were calculated with the statistical package StatXact 4 for Windows [21] (Cytel Software Corporation, Cambridge, MA, USA).

Results

Source of isolates and cyclohexane resistance

It was not possible to compare directly the numbers of cyclohexane-resistant strains from the different sources, because a comprehensive collection strategy was not employed (Table 1); however, the cyclohexane-resistant organisms comprised 23.4% poultry isolates, 3.8% ovine isolates and 2.4% environmental isolates. Most of the resistant isolates were obtained in 1998 and 1999 (Table 1), but it was not possible to draw any conclusions from this as lower numbers of organisms were tested from the other years.

Antibiotic and cyclohexane susceptibilities of the isolates

The *Salmonella* panel was selected mainly to contain isolates that were susceptible, by disk diffusion, to all 16 antimicrobial agents or which were nalidixic acid-resistant but included 69 isolates without disk susceptibility test data. Among the isolates for which disk diffusion susceptibility data was available (82.2% of the complete panel) only 6.7% of the 104 fully antibiotic-susceptible organisms were cyclohexane-resistant compared with 18.1% of the 144 nalidixic acid-resistant organisms. Cyclohexane resistance was observed in 9.9% of 71 organisms that were nalidixic acid-susceptible but resistant to other antimicrobials. In total, 41 (10.6%) of the 388 organisms tested were cyclohexane-resistant.

Serotypes and cyclohexane resistance

Comprehensive comparison was not possible for all 35 serotypes tested, because 23 of these were represented by fewer than 10 strains, with only single strains tested for 14 serotypes (Table 2). However, cyclohexane resistance was observed in 1 (7%) of 15 representatives of serovar Binza, 1 (2%) of 24 Dublin, 1 (1.6%) of 61 Enteritidis, 4 (80%) of 5 Fischerkietz, 9 (82%) of 11 Livingstone, 1 (3%) of 32 Montevideo, 4 (17%) of 23 Newport, 1 (33%) of 3 Saint-paul, 10 (42%) of 24 Seftenberg and 9 (10%) of 93 Typhimurium.

MICs and cyclohexane resistance

The frequencies of antibiotic and non-antibiotic MICs for cyclohexane-susceptible and cyclohexane-resistant strains, are shown in Tables 3 and 4 respectively with the MIC₅₀, MIC₇₅ and MIC₉₀ values indicated. The MIC₅₀, MIC₇₅ and MIC₉₀ values of ciprofloxacin were raised 16–32-fold for the cyclohexane-resistant isolates. For all the other antimicrobial agents except kanamycin and the disinfectants, MICs for cyclohexane-resistant organisms tended to be *c.* 2–8 fold higher than for cyclohexane-susceptible organisms. Some cyclohexane-resistant organisms had high-level antibiotic resistance. For the dyes acridine orange and ethidium bromide, there were only small differences between the MIC distribution for the cyclohexane-susceptible and -resistant organisms.

Statistical comparisons were performed by the Wilcoxon-Mann-Whitney test to determine if the cyclohexane-susceptible organisms were more resistant to compounds tested than cyclohexane-resistant organisms (results not shown). For all substances tested except kanamycin ($p = 0.356$), the cyclohexane-resistant strains were significantly more resistant with p values of <0.001 recorded, except for acridine orange ($p = 0.068$) and ethidium bromide ($p = 0.002$). The MIC₅₀, MIC₇₅ and MIC₉₀ values for ciprofloxacin against cyclohexane-susceptible organisms were 0.03, 0.13 and 0.13 mg/L, respectively, compared with 0.5, 1 and 2 mg/L for cyclohexane-resistant organisms.

Discussion

We previously have noted a low prevalence (~ 1 –5%) of cyclohexane resistance in Dublin, Enteritidis and Typhimurium strains from animals (unpublished observations) and have found that their resistance correlated well with low-level resistance to multiple antibiotics and reduced expression of OmpF, but not with the higher-level antibiotic resistance seen in many *S. Typhimurium* isolates. It was interesting to note that reduced OmpF was associated with this phenotype, because whilst down-regulation of OmpF is reported to be involved in the multiple antibiotic resistance phenotype in Mar mutants of *E. coli* [22, 23],

Table 3. MIC frequency (%) of antibiotics against cyclohexane-susceptible and resistant salmonellas

Antibiotic	Susceptibility*	Percentage of isolates with MIC (mg/L)												
		0.004	0.008	0.015	0.03	0.06	0.13	0.25	0.5	1	2	4	8	>8
Ciprofloxacin	S	0.6	0.03	15.9	<i>46.4</i>	0.06	<u>7.2</u>	19	8.6	1.2	0.3			
	R			2.4	2.4	24.4	7.3	12.2	7.3	<u>29.3</u>	14.6			
	MIC	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	>128
Ampicillin	S		0.3	0.3	8.1	<i>41.8</i>	<u>25.9</u>	0.6					0.6	22.5
	R				2.4	14.6	12.2	<i>24.4</i>	19.5					<u>26.8</u>
Chloramphenicol	S					0.6	0.6	<i>49.3</i>	<u>29.7</u>	2.6		0.6	7.2	9.5
	R							9.8	12.2	<i>34.1</i>	<u>24.4</u>			19.5
Kanamycin	S				0.3	5.2	42.8	<u>46.7</u>	1.2		0.6	0.3	0.3	3.2
	R				2.4	2.4	<i>48.8</i>	<u>46.6</u>						
Nalidixic acid	S					0.6			35.2	0.6	0.6		0.3	<u>35.4</u>
	R								4.9	26.8	2.4			<u>65.9</u>
Tetracycline	S				1.4	38.9	<i>30.8</i>			0.3	2.3	<u>10.7</u>	8.9	6.6
	R					14.6	19.5	29.3	<u>14.6</u>			7.3	14	
Trimethoprim	S	6.3	34	<u>44.1</u>	2								0.6	13
	R		4.9	<u>46.3</u>	<u>31.7</u>	9.8	7.3							
	MIC	8	16	32	64	128	256	512	1024	>1024				
Erythromycin	S		0.3		<i>52.4</i>	<u>46.1</u>		0.3	0.3	0.6				
	R				14.6	29.3	<u>56.1</u>							

Italic text denotes MIC50 values, underlined text denotes MIC75 values and bold text denotes MIC90 values.

*Salmonella strains susceptible (S; n = 347) and resistant (R; n = 41) to cyclohexane.

Table 4 MIC frequency of disinfectants and dyes against cyclohexane-susceptible and resistant salmonellas

Compound	Susceptibility*	Percentage of isolates with indicated MIC (mg/L)									
		0.03	0.06	0.13	0.25	0.5	1	2	4	8	>8
Triclosan	S	0.6	8.9	<i>82.7</i>	6.3	1.2					0.3
	R			<u>17.1</u>	<u>63.4</u>	19.5					
		MIC 16	32	64	128	256	512	1024	2048	4096	>4096
Acridine orange	S			0.3				<u>86.3</u>	13.3	0.3	
	R							<u>75.6</u>	24.4	2.4	
Cetrimide	S		0.9		63.7	34.6	0.9				
	R				19.5	22	<u>41.5</u>	17.1			
Ethidium bromide	S		0.3			1.4	5.2	65.4	<u>27.7</u>		
	R					2.4		46.3	<u>51.2</u>		

Italic text denotes MIC50 values, underlined text denotes MIC75 values and bold text denotes MIC90 values. *Salmonella* strains susceptible (S; n = 347) and resistant (R; n = 41) respectively to cyclohexane.

cyclohexane resistance in *E. coli* has been shown to be dependent on AcrAB [3] but independent of the level of OmpF in the membrane [24]. In this study, a higher incidence (10.6%), of cyclohexane-resistance was seen than previously, possibly because of the inclusion of many (144) nalidixic acid-resistant organisms. Specifically, 26 (18.1%) of 118 of the nalidixic acid-resistant organisms were cyclohexane-resistant. Previous workers have shown a close relationship between cyclohexane resistance and fluoroquinolone resistance in clinical isolates of *E. coli* [13].

In this study, most (39 of 41: 95%) cyclohexane-resistant isolates were from poultry, although such isolates have also been obtained from cattle (unpublished observations). The cyclohexane-resistant *S. Newport* strains were all from turkeys owned by a large turkey-rearing company, but were isolated from two different sites at different times. It is not clear why such a large proportion of the cyclohexane-resistant isolates were from poultry, but the antibiotics and disinfectants used in poultry rearing may select for these types of *Salmonella*. Of the 144 nalidixic acid-resistant organisms, 96 (67%) were from poultry. It would be disturbing if the environment of poultry specifically selected for cyclohexane-resistant strains, allowing the increase in resistance of these organisms to ciprofloxacin, which is the drug of choice for treating invasive human salmonellosis [25–27]. The veterinary fluoroquinolone enrofloxacin has been used in mycoplasma eradication programmes in turkeys and to aid the control of salmonella and other bacterial infections in commercial poult [28].

In a recent study [29] *S. Typhimurium* isolates from a patient treated with ciprofloxacin showed increased resistance to multiple antibiotics including ciprofloxacin associated with reduced accumulation of antibiotics. Three of the post-treatment isolates showed increased expression of *acrB*. Thus the quinolone treatment may select for variants with gyrase mutations [30, 31] or efflux mutations, or perhaps both [29].

The cyclohexane-resistant organisms were significantly

more resistant ($p < 0.001$) to all the antibiotics tested except kanamycin. All of these antibiotics except kanamycin and trimethoprim are substrates for AcrAB [9–12]. The most pronounced differences between cyclohexane-susceptible and -resistant organisms were for ciprofloxacin. Other workers have observed a correlation between cyclohexane resistance and fluoroquinolone resistance in *E. coli* [13].

The cyclohexane-resistant strains were also significantly more resistant to triclosan and cetrimide ($p < 0.001$) than the cyclohexane-susceptible strains. Triclosan is a broad-spectrum antibacterial agent used in hand soaps, lotions, fabrics, plastics and toothpaste [6]; previous studies have demonstrated resistance to be associated with over-expression of the *marA*, *soxS* or *acrAB* genes in *E. coli* [6]. Ethidium bromide is a substrate of AcrAB in *E. coli* and *Salmonella* [9, 16], therefore it was surprising that the cyclohexane-resistant organisms did not show such an increased resistance to ethium bromide as to many antibiotics. Nevertheless, the observed patterns of resistance to antibiotics and disinfectants were consistent with over-expression of AcrAB [9–12, 16].

These findings, and previous unpublished data, suggest that cyclohexane resistance may be a useful marker for the emergence of antibiotic resistance. Such monitoring becomes more relevant in view of recent findings that active efflux is the primary mechanism of resistance to ciprofloxacin in *S. Typhimurium* [16], and that gyrase mutations in *E. coli* only mediate clinically relevant fluoroquinolone resistance in the presence of AcrAB [15, 32].

We would like to thank Dr Laura Piddock for advice concerning this work and the Ministry of Agriculture, Fisheries and Food, UK for funding this work (MAFF Project code OZO132).

References

- Asako H, Nakajima H, Kobayshi K, Kobayashi M, Aono R. Organic solvent tolerance and antibiotic resistance increased by over-expression of *marA* in *Escherichia coli*. *Appl Environ Microbiol* 1997; **63**: 1428–1433.

2. Nakajima H, Kobayashi K, Kobayashi M, Asako H, Aono R. Overexpression of the *roxA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. *Appl. Environ Microbiol* 1995; **61**: 2302–2307.
3. White DG, Goldman JD, Demple B, Levy SB. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *roxA* in *Escherichia coli*. *J Bacteriol* 1997; **179**: 6122–6126.
4. Gambino L, Gracheck SJ, Miller PF. Overexpression of the *marA* positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. *J Bacteriol* 1993; **175**: 2888–2894.
5. Miller PF, Gambino LF, Sulavik MC, Gracheck SJ. Genetic relationship between *soxRS* and *mar* loci promoting multiple antibiotic resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 1994; **38**: 1773–1779.
6. McMurtry LM, Oethinger M, Levy SB. Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol Lett* 1998; **166**: 305–309.
7. Moken MC, McMurtry LM, Levy SB. Selection of multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. *Antimicrob Agents Chemother* 1997; **41**: 2770–2772.
8. Okuso H, Ma D, Nikaido H. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *J Bacteriol* 1996; **178**: 306–308.
9. Nikaido H. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 1996; **178**: 5853–5859.
10. Nikaido H, Basina M, Nguyen V, Rosenberg EY. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β -lactam antibiotics containing lipophilic side chains. *J Bacteriol* 1998; **180**: 4686–4692.
11. Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE. The local repressor AcrR plays a modulating role in the regulation of the *acrAB* genes of *Escherichia coli* by global stress signals. *Mol Microbiol* 1996; **19**: 101–112.
12. Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol Microbiol* 1995; **16**: 45–55.
13. Oethinger M, Kern WV, Goldman JD, Levy SB. Association of organic solvent tolerance and fluoroquinolone resistance in clinical isolates of *Escherichia coli*. *J Antimicrob Chemother* 1998; **41**: 111–114.
14. Goldman JD, White DG, Levy SB. Multiple antibiotic resistance (*mar*) locus protects *Escherichia coli* from rapid cell killing by fluoroquinolones. *Antimicrob Agents Chemother* 1996; **40**: 1266–1269.
15. Oethinger M, Kern WV, Jellen-Ritter AS, McMurtry LM, Levy SB. Ineffectiveness of topoisomerase mutations in mediating clinically-significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. *Antimicrob Agents Chemother* 2000; **44**: 10–13.
16. Giraud E, Cloeckaert A, Kerboeuf D, Chaslus-Dancla E. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2000; **44**: 1223–1228.
17. George AM, Levy SB. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. *J Bacteriol* 1983; **155**: 531–540.
18. Wray C, Beedell YE, McLaren IM. A survey of antimicrobial resistance in salmonellae isolated from animals in England and Wales during 1984–1987. *Br Vet J* 1991; **147**: 356–369.
19. Phillips I. A report of the working party on antibiotic sensitivity of the British Society for Antimicrobial Chemotherapy London, Academic Press. 1991.
20. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically, Villanova, PA, National Committee for Clinical Laboratory Standards 6th edn. 1998.
21. Cytel Software Corporation. StatXact 4 for Windows User Manual. Cambridge, MA.
22. Cohen SP, McMurtry LM, Hooper DC, Wolfson JS, Levy SB. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (*mar*) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to *OmpF* reduction. *Antimicrob Agents Chemother* 1989; **33**: 1318–1325.
23. Cohen SP, McMurtry LM, Levy SB. *marA* locus causes a decreased expression of *OmpF* porin in multiple antibiotic resistant (Mar) mutants of *Escherichia coli*. *J Bacteriol* 1988; **170**: 5416–5422.
24. Asako H, Kobayashi K, Aono R. Organic solvent tolerance of *Escherichia coli* is independent of *OmpF* levels in the membrane. *Appl Environ Microbiol* 1999; **65**: 294–296.
25. Dutta P, Rasaily R, Saha MR *et al*. Ciprofloxacin for treatment of severe typhoid fever in children. *Antimicrob Agents Chemother* 1993; **37**: 1197–1199.
26. Smith MD, Duong NM, Hoa NT *et al*. Comparison of ofloxacin and ceftriaxone for short-course treatment of enteric fever. *Antimicrob Agents Chemother* 1994; **38**: 1716–1720.
27. Wang F, Gu XJ, Zhang MF, Tai TY. Treatment of typhoid fever with ofloxacin. *J Antimicrob Chemother* 1989; **23**: 785–788.
28. Davies RH, Teale CJ, Wray C *et al*. Development of quinolone resistance in salmonellas in Great Britain and the need for standardised surveillance methods. In: Cost Action 97 – Pathogenic micro-organisms in poultry and eggs. 10. Development of monitoring procedures, rapid detection methods and techniques. Nice, France, 13–15 Dec. 1998: 59–64.
29. Piddock LJV, White DG, Gensberg K, Pumbwe L, Griggs DJ. Evidence for an efflux pump mediating multiple antibiotic resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2000; **44**: 3118–3121.
30. Griggs DJ, Gensberg K, Piddock LJV. Mutations in *gyrA* gene of quinolone-resistant salmonella serotypes isolated from humans and animals. *Antimicrob Agents Chemother* 1996; **40**: 1009–1013.
31. Piddock LJV, Griggs DJ, Hall MC, Jin YF. Ciprofloxacin resistance in clinical isolates of *Salmonella typhimurium* obtained from two patients. *Antimicrob Agents Chemother* 1993; **37**: 662–666.
32. Oethinger M, Podglajen I, Kern WV, Levy SB. Overexpression of the *marA* or *soxS* regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* 1998; **42**: 2089–2094.