ANTIMICROBIAL AGENTS AND RESISTANCE

Increase in incidence of resistance to ampicillin, chloramphenicol and trimethoprim in clinical isolates of *Salmonella* serotype Typhimurium with investigation of molecular epidemiology and mechanisms of resistance

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Antimicrobial resistance patterns of *Salmonella* serotype Typhimurium isolates obtained during the period 1987–1994 were examined and the molecular epidemiology and the mechanisms of resistance to ampicillin, chloramphenicol and trimethoprim were investigated in 24 strains isolated during 1994. Resistance to ampicillin increased from 18% to 78%, to chloramphenicol from 15% to 78%, to tetracycline from 53% to 89% and to co-trimoxazole from 3% to 37%, whereas resistance to norfloxacin remained at 0%. Of *Salmonella* serotype Typhimurium strains isolated during 1994, all ampicillin-resistant strains had an MIC > 256 mg/L, except one strain in which the MIC was 64 mg/L. Twelve strains (52%) had a TEM-type β -lactamase, nine (39%) a CARB-type β -lactamase and two strains (8%) had an OXA-type β -lactamase. Chloramphenicol acetyl-transferase activity was detected in only nine (47%) of 19 chloramphenicol resistant strains, whereas all eight trimethoprim-resistant strains produced a dihydrofolate reductase type Ia enzyme. Three different epidemiological groups were defined by either low-frequency restriction analysis of chromosomal DNA and pulsed-field gel electrophoresis or repetitive extragenic palindromic-PCR. The latter technique provided an alternative, rapid and powerful genotyping method for *S. Typhimurium*. Although quinolones provide a good therapeutic alternative, the multiresistance of *S. Typhimurium* is of public health concern and it is important to continue surveillance of resistance levels and their mechanisms.

Introduction

Gastro-enteritis is the most common clinical manifestation of salmonella infection [1]. The two most common causes of non-typhoidal salmonellosis are serotypes Enteritidis and Typhimurium. Although infection with non-Typhi *Salmonella* often causes mild self-limiting illness, serious sequelae including death may occur, especially in immunocompromised patients [2]. In this sense *Salmonella* serotype Typhimurium is an important cause of diarrhoea and bacteraemia in HIV-positive patients and normally they require antimicrobial treatment [3].

The antimicrobial resistance of *Salmonella* has an impact through the recognised association between drug-resistant salmonellae and the routine clinical use of antimicrobial agents for infections other than salmonellosis. There are three facets of this association: (i) antimicrobial-resistant salmonella infections can complicate antimicrobial treatment of other infections; (ii) prior antimicrobial therapy allows fewer numbers of antimicrobial-resistant *Salmonella* to cause symptomatic infections; (iii) an increase in the proportion of salmonella that are antimicrobial-resistant will increase the overall frequency of salmonellosis [4].

Various typing techniques have been used in epidemiological studies to discriminate strains of Typhimurium. These include biotyping [5], phage typing [6, 7], antimicrobial susceptibility testing [8], plasmid

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analysis [9, 10], multilocus-enzyme electrophoresis [11], restriction endonuclease analysis of chromosomal DNA [12, 13] and restriction fragment length polymorphisms (RFLP) of 16S rRNA and insertion sequence IS200 [14, 15].

In this study, the evolution of antimicrobial resistance to several antimicrobial agents and the molecular basis of ampicillin (Amp), chloramphenicol (Cm) and trimethoprim (Tm) resistance mechanisms in Typhimurium clinical isolates were investigated.

Materials and methods

Bacteria

All clinical isolates of Typhimurium obtained mainly from outpatients attending the Emergency Department of the Hospital Clinic de Barcelona between 1987 and 1994 were analysed in this study. They were identified by conventional methods [16] and serotyping. A strain was defined as multiresistant if it was resistant to two or more antimicrobial agents tested. The 24 multiresistant Typhimurium strains isolated in 1994 were investigated in detail to determine their mechanisms of resistance to ampicillin, chloramphenicol and trimethoprim.

Susceptibility testing

Antimicrobial susceptibility tests were performed by an agar diffusion disk method as recommended by the National Committee for Clinical Laboratory Standards [17]. Mueller-Hinton agar was obtained from Becton Dickinson and antimicrobial disks (ampicillin 10 µg; chloramphenicol 30 µg; tetracycline 30 µg; trimethoprim-sulphamethoxazole 1.25/23.75 µg and norfloxacin 5 µg) were obtained from BBL Microbiology Systems (Becton Dickinson, Cockeysville, MD, USA). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms and tested weekly. Each time a new batch of Mueller-Hinton agar was introduced, *Enterococcus faecalis* ATCC 29212 was tested to detect the presence of inhibitors of trimethoprim-sulphamethoxazole.

The MICs of ampicillin (Antibiotics, SA, Leon, Spain), chloramphenicol (Ifesa, Barcelona, Spain) and trimethoprim alone (not trimethoprim-sulphamethoxazole) (Sigma) were determined by an agar dilution method with an inoculum of 10⁴ cfu/spot in accordance with the guidelines of the National Committee for Clinical Laboratory Standards [18].

β-Lactamase assays

β-Lactamase activity was quantified by the spectrophotometric assay of nitrocefin hydrolysis [19] in 0.1 M phosphate buffer (pH 7.0); β-lactamases were detected

by two different methods – iso-electric focusing and the genes encoding them by PCR. For iso-electric focusing, cell-free lysates were obtained by ultrasonication of exponential cultures grown at 37°C in LB broth containing ampicillin 50 mg/L. Analytical iso-electric focusing was done by applying the crude sonic extracts to Phast gels (pH gradient 3–9) in a Phast System (Pharmacia AB, Uppsala, Sweden). Several strains carrying β-lactamases of known pI were used as controls and focused in parallel with the extracts. Nitrocefin (50 mg/L) was used for detection. Three different sets of primers were used to detect β-lactamase genes by PCR, one specific for *bla*_{TEM} gene amplification and another for *bla*_{CARB} gene amplification [20]. The third set was designed according to the *bla*_{OXA-1} gene and comprised 5'-GGCACCAGATTCAACTTTCAA-3' and 5'-TTTTTCTTGGCTTTTATGCTTG-3'.

Chloramphenicol acetyl-transferase detection

The chloramphenicol acetyl-transferase activity was assayed by the radiometric method of Robinson *et al.* [21] and by a modification of a rapid method developed by Azemun *et al.* [22]. Briefly, the strains were grown overnight on MacConkey agar. A heavy suspension of bacteria in 0.2 ml of 1 M NaCl, 0.01 M EDTA and SDS 0.05% (pH 8.0) was incubated in an eppendorf tube at 37°C for 60 min. After a short centrifugation in a microfuge, 50 µl were transferred to a microtitration plate. Duplicate wells were prepared with each strain and 50 µl of a solution containing two parts of 0.2 M Tris-HCl, pH 8.0, 2 mM acetylco-enzyme A and one part of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M Tris-HCl, pH 8.0, were added to each well. A 50-µl amount of 5 mM sterile chloramphenicol (dissolved in water) was added to one well (test reaction), and an equivalent amount of water was added to the duplicate well (control). The plate was re-incubated at 37°C for 5 min. The reaction was stopped by adding 1 N H₂SO₄ and read spectrophotometrically.

Detection of trimethoprim resistance genes

Samples (1.5 ml) of overnight cultures were centrifuged for 1 min at 12 000 *g* in a microcentrifuge. The cells were resuspended in 100 µl of sterile saline and 5-µl volumes were dotted on to a Qiabran Nylon Plus Membrane (Hybaid, Twickenham) and allowed to dry in air. Cell lysis and denaturation of DNA for subsequent hybridisation experiments were as described by Maniatis *et al.* [23]. The biotin-labelled oligonucleotide used as a probe for the type Ia dihydrofolate reductase (DHFR) gene [24] was as follows: 5'-C-GGGAATGGCCCTGATATTCCATGGAGTGC-3'. The probe was custom-synthesised and 5'-biotinylated by British Biotechnology Products (Abingdon). Pre-treatment of membranes with proteinase K was as described previously [25]. Subsequent pre-hybridisation, hybridisation and washing conditions were those recom-

mended by Heikkilä *et al.* [24]. Positive hybridisation results were detected by the BluGENE Detection System (Gibco/BRL, Paisley) with the conditions and washes recommended by the manufacturer.

Low-frequency restriction analysis of chromosomal DNA by pulsed field gel electrophoresis (PFGE)

Genomic DNA was extracted by a slightly modified version of the method of Matushek *et al.* [26]. Briefly, 3 ml of an overnight culture were centrifuged and resuspended in 0.5 ml of lysis 2× buffer (1×, 6 mM Tris-HCl, pH 7.4, 1 M NaCl, 10 mM EDTA, pH 7.5, Brij 0.5%, deoxycholate 0.2%, sodium lauryl sarcosinate 0.5%) to which lysozyme (Sigma) 0.5 mg/ml and RNAase (Sigma) 10 µg/ml were added. Genomic DNA was prepared in agarose plugs as described previously [27]. It was then incubated with 3 ml of 1× lysis solution at 37°C for 2 h with gentle shaking. After incubation, the lysis solution was replaced with ESP (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), to which proteinase K (Sigma) at a final concentration of 100 µg/ml and SDS 1% were added; the plugs were incubated at 50°C for 1 h. Protein digestion products were removed by washing the plugs three times with TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) at 50°C for 5, 30 and 30 min.

A plug slice 4 mm wide was suspended in 500 µl of restriction buffer for 1 h at 37°C. The buffer was then replaced with 250 µl of fresh restriction buffer and it was incubated overnight with 20 U of *Xho* I (Promega) following the manufacturer's recommendations. The DNA fragments were separated by PFGE in an agarose 1% gel (BioRad) that was prepared and run in Tris-borate-EDTA (TBE) buffer on a contour-clamped homogeneous field apparatus (CHEF-DR2, BioRad). The conditions for electrophoresis were 200 V for 21 h, with pulse time from 5 to 8 s.

Thereafter, the gels were stained with ethidium bromide and photographed.

Repetitive extragenic palindromic (REP) PCR

Bacteria were grown on MacConkey agar overnight. Half a colony of each isolate was suspended in 25 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM KCl, 3.0 mM MgCl₂ and gelatin 0.1%; 400 µM dNTPs and 1 µM primer were added, together with 2.5 U of *Taq* polymerase (Gibco-BRL). The reaction mixture was overlaid with mineral oil and amplified with the following programme: 30 cycles at 94°C for 1 min, 40°C for 1 min and 65°C for 8 min, with a single final extension at 65°C for 16 min. Samples (10 µl) of each PCR end-product were analysed on agarose 1.5% w/v gels. One set of primers was used as indicated by Vila *et al.* [28].

Statistical test

Percentages of antimicrobial resistance among different years were compared by Fisher's exact test with Epi-Info, version 6.02.

Results

During the study period, 292 Typhimurium strains were isolated. The origins of the *Salmonella* isolates were stool (85%), blood (13%) and urine (2%). The increase in the resistance of Typhimurium isolates to several antibiotics during the period 1987–1994 is summarised in Fig. 1. In this period, the resistance of Typhimurium clinical isolates to ampicillin increased from 18% to 78% ($p < 0.00007$), to chloramphenicol from 15% to 78% ($p < 0.00007$), to tetracycline from 53% to 89% ($p < 0.01$) and to co-trimoxazole from 3% to 37% ($p < 0.007$); all these increases in resistances were highly significant. Meanwhile, resistance to norfloxacin

Fig. 1. Antimicrobial resistance, expressed as a percentage, in clinical isolates of *S. Typhimurium* obtained during 1987–1994; *, ampicillin; ●, trimethoprim-sulphamethoxazole; ▲, chloramphenicol; ◆, tetracycline; □, norfloxacin.

remained at 0%. In 1994, 83% of the strains were multiresistant.

Twenty-four Typhimurium strains isolated during 1994 were used for detailed investigations of the mechanisms of resistance to ampicillin, chloramphenicol and trimethoprim alone. Although a strain that is sensitive to co-trimoxazole is not necessarily sensitive to trimethoprim alone, all subsequent studies on trimethoprim resistance mechanisms were performed with strains shown to be resistant to trimethoprim alone in MIC tests. Thirteen of these 24 strains were resistant to ampicillin and chloramphenicol; six were resistant to ampicillin, chloramphenicol and trimethoprim; three were resistant only to ampicillin; one strain was resistant only to trimethoprim; and one strain was resistant to ampicillin and trimethoprim. The MICs of ampicillin, chloramphenicol and trimethoprim for these strains are shown in Table 1. All the ampicillin-resistant strains had an ampicillin MIC > 256 mg/L, except for one strain in which the MIC was 64 mg/L. All the strains resistant to chloramphenicol and trimethoprim also had MICs > 256 mg/L. On the basis of antibiotic susceptibility, five phenotypes could be defined. Thirteen strains belonged to phenotype I (Amp^R, Cm^R, Tm^S), six strains to phenotype II (Amp^R, Cm^R, Tm^R), three to phenotype III (Amp^R, Cm^S, Tm^S), one strain to phenotype IV (Amp^S, Cm^S, Tm^R) and one strain to phenotype V (Amp^R, Cm^S, Tm^R).

Iso-electric focusing was used first to detect β -lactamase production. The results are shown in Table 1. β -Lactamase activity was detected in crude extracts

from 96% of the strains resistant to ampicillin. Twelve strains (52%) showed a β -lactamase with a pI of c. 5.4, nine strains (39%) with a pI c. 5.6 and two (8%) with a pI c. 7.0. To corroborate the results of iso-electric focusing a PCR technique with specific primers for amplification of the genes encoding TEM-, CARB- and OXA-type β -lactamases was used. The PCR results are also shown in Table 1. All the strains that produced a β -lactamase of pI 5.4 showed a positive amplification result with TEM-specific primers, probably corresponding to TEM-1 β -lactamase, whereas all the strains with a β -lactamase of pI 5.6 failed to amplify with the TEM primers, but were positive with CARB-specific primers, corresponding to carbenicillinase and not to TEM-2 β -lactamase as originally thought. The PCR products obtained with both TEM-specific primers and CARB-specific primers had the expected sizes of 503 bp for TEM and 586 bp for CARB (Fig. 2). Both strains with a β -lactamase of pI 7.0 showed a positive reaction (PCR product 598 bp) with the OXA-1 primers.

Chloramphenicol acetyl transferase activity was detected in only 9 (47%) of the 19 chloramphenicol-resistant strains with either the radioactive or spectrophotometric assay (Table 1). All eight strains resistant to trimethoprim gave a positive result with the oligonucleotide probe for DHFR type Ia gene (Table 1).

The 24 clinical isolates were typed by PFGE and REP-PCR. Three completely different restriction fragment patterns were observed among the 24 isolates of Typhimurium studied when the DNA was digested with *Xho*I (Table 2 and Fig. 3a). The profiles

Table 1. Characteristics of *Salmonella* serotype Typhimurium clinical isolates

Strain no.	MIC (mg/L)			β -Lactamases				CAT		
	Amp	Cm	Tm	IEF	PCR _{TEM}	PCR _{CARB}	PCR _{OXA}	Assay A*	Assay B†	DHFR
3	>256	>256	0.5	5.6	—	+	—	—	—	ND
285	>256	4	0.25	5.4	+	—	—	ND	ND	ND
317	>256	8	0.5	5.4	+	—	—	ND	ND	ND
410	>256	>256	>256	5.4	+	—	—	+	+	+
451	1	8	>256	ND	ND	ND	—	ND	ND	+
470	>256	>256	0.25	5.6	—	+	—	+	+	ND
582	>256	>256	0.5	5.4	+	—	—	—	—	ND
1077	>256	>256	0.25	5.6	—	+	—	—	—	ND
1215	256	>256	>256	5.4	+	—	—	+	+	+
1228	>256	256	0.5	5.6	—	+	—	—	—	ND
1282	>256	>256	>256	5.4	+	—	—	+	+	+
1289	>256	>256	>256	5.4	+	—	—	+	+	+
1694	64	8	>256	—	—	—	—	ND	ND	+
1742	>256	>256	0.25	5.6	—	+	—	—	—	ND
1763	256	>256	>256	5.4	+	—	—	+	+	+
1843	>256	>256	0.5	5.6	—	+	—	—	—	ND
2120	256	4	0.25	5.4	+	—	—	ND	ND	ND
2322	>256	>256	0.25	5.6	—	+	—	—	—	ND
2542	>256	>256	0.5	5.4	+	—	—	—	—	ND
2545	>256	>256	0.25	5.6	—	+	—	+	+	ND
2557	>256	>256	0.5	5.4//7.0	+	—	+	—	—	+
2626	256	256	>256	5.4	+	—	—	+	+	+
2675	>256	>256	0.5	7.0	—	—	+	+	+	ND
2781	>256	>256	0.5	5.6	—	+	—	—	—	ND

CAT, chloramphenicol acetyl-transferase; DHFR, dihydrofolate reductase.

*Radiometric assay to detect CAT.

†Spectrophotometric assay to detect CAT.

Fig. 2. Agarose gel electrophoresis of DNA fragments amplified with specific primers to detect *bla*_{OXA} gene (1), *bla*_{CARB} gene (2), and *bla*_{TEM} gene (3). M, mol. wt marker 100-bp Ladder (Life Technologies, Gaithersburg, MD, USA).

generated with REP primers contained several bands of 0.3–2.5 kb. REP-PCR analysis also revealed three different banding patterns (Table 2 and Fig. 3b). There was very good correlation between the techniques and in both cases the clinical isolates studied could be distributed into three different groups with 15, 6 and 3 strains in each group, respectively (Table 2). However, the results with the fingerprinting techniques did not

Fig. 3. Patterns obtained by (a) PFGE (b) REP-PCR.

correlate with the results obtained by antimicrobial susceptibility tests.

Discussion

Salmonella enteritis in man is usually a self-limiting disease and antimicrobial treatment is seldom required; but should spread beyond the intestine occur, effective antimicrobial treatment is essential. Furthermore, HIV-positive patients frequently develop bacteraemia with *S. Typhimurium* [3, 29–34]. Under these circumstances, a knowledge of the likelihood of resistance to commonly available drugs could be of considerable value to the clinician.

The overall increase in resistance observed in Typhimurium is in agreement with that shown in other geographical areas of Spain [35–38] and other countries [39, 40], except that resistance to trimethoprim-sulphamethoxazole in this study was 37%, compared with 2–11% in other developed countries. It should be noted that a strain that is sensitive to co-trimoxazole is not necessarily sensitive to trimethoprim alone, but all strains studied in detail that were resistant to trimethoprim-sulphamethoxazole in disk tests also showed resistance to trimethoprim alone in MIC tests.

Resistance to β -lactam antibiotics in gram-negative bacilli is mainly associated with β -lactamase production [41]. In the present study, the overall percentage (96%) of β -lactamase-producing strains was similar to that found by others [42]. The ampicillin resistance of strain no. 1694 (ampicillin MIC 64 mg/L) could be associated with changes in permeability. Three types of β -lactamases (TEM, CARB and OXA) were detected. The percentage of strains with either TEM or CARB type β -lactamase was very similar; however, the percentage of strains producing TEM-1 was lower

Table 2. Subdivision of *Salmonella* serotype Typhimurium isolates by three different epidemiological markers

Strain no.	Antibiogram	PFGE	REP-PCR
3	I	A	1
285	III	A	1
317	III	B	2
410	II	B	2
451	IV	C	3
582	I	A	1
643	I	A	1
1077	I	A	1
1215	II	C	3
1228	I	A	1
1282	II	B	2
1289	II	B	2
1694	V	A	1
1742	I	A	1
1763	II	B	2
1843	I	A	1
2120	III	C	3
2322	I	A	1
2542	I	A	1
2545	I	A	1
2557	I	B	2
2626	II	A	1
2675	I	A	1
2781	I	A	1

than found in the study by Roy *et al.* [42] who found 27 (87%) TEM-1 β -lactamase-producing strains from a total of 31 ampicillin-resistant strains of *Salmonella* spp. In the present study, a CARB-type β -lactamase originally isolated from *P. aeruginosa* was found in 36% of the strains analysed, and this type of β -lactamase has been shown previously in Typhimurium [43]. A plasmid-mediated OXA-type β -lactamase with a pI of 7.0 has also been described in this micro-organism [44] and it was found in 8% of the strains of the present study.

The percentage of chloramphenicol-resistant strains producing chloramphenicol acetyl-transferase was very low, suggesting the presence of other mechanism(s) of chloramphenicol resistance. Toro *et al.* [45] found that the lack of OmpF played a major role in the high level of chloramphenicol resistance in a *S. Typhi* strain. In this strain, chloramphenicol acetyl-transferase activity was not detected. A similar mechanism could occur in the strains investigated in the present study and further studies to identify possible other mechanism(s) of chloramphenicol resistance are in progress.

All the strains resistant to trimethoprim showed a DHFR type Ia gene, in agreement with the fact that DHFR type Ia is common in trimethoprim-resistant Enterobacteriaceae in many European countries [46, 47], but in contrast to the results presented by Agodi *et al.* [48]. These authors studied the occurrence of trimethoprim resistance in different *Salmonella* species isolated from human patients and water samples in Sicily and found that only four of 14 trimethoprim-resistant Typhimurium clinical isolates analysed produced a type Ia DHFR, whereas the remaining 10 strains did not produce dihydrofolate reductases belonging to groups Ia, II, IV or V.

No resistance was observed to quinolones or third-generation cephalosporins (data not shown). However, recently there has been an increasing number of reports of strains of Typhimurium that are either resistant, or have decreased susceptibility, to these antimicrobial agents. Resistance to quinolones in Typhimurium clinical isolates has been mainly associated with mutations in the *gyrA* gene [49–51] and also to changes in drug accumulation [52]. Detection of extended-spectrum β -lactamases in Typhimurium is rare, although a strain of Typhimurium producing a SHV-2 β -lactamase was detected in France [53, 54].

Typhimurium is commonly associated with cattle, but it has also been associated with sheep, pigs, goats, chickens and turkeys, with an association between illness and the consumption of several food items made from the above-mentioned animals demonstrated in a case-control study [55]. Probably the use of antimicrobial agents in veterinary medicine and animal husbandry selects multiresistant Typhimurium, although it

is surprising that this dramatic increase in the antimicrobial resistance has not been observed in Enteritidis, which also has an animal reservoir.

Phage typing has been the most useful typing technique to investigate outbreaks of Typhimurium infection; however, its requirement for a collection of specialised phages and the appropriate bacterial strains for their propagation means that it is routinely used in only a few reference laboratories. Some genotyping methods, such as RFLP of 16S rRNA and IS200 and low-frequency restriction analysis of chromosomal DNA with PFGE have proved to be helpful for the epidemiological typing of Typhimurium [56]. PFGE may provide evidence of minor genetic heterogeneity which cannot be detected readily by such RFLP-based techniques as ribotyping and IS200 fingerprinting. However, these techniques are time-consuming and labour-intensive procedures and unsuitable for routine epidemiological analysis of a large number of isolates. A PCR-based fingerprinting technique widely applied in epidemiological investigations uses consensus primers to amplify sequences located between successive repetitive extragenic palindromic elements (REP-PCR) [57]. In the present study, REP-PCR had the same power of discrimination as PFGE.

The strains could be distributed into three different groups based on REP-PCR and PFGE analysis. However, if the different mechanisms of resistance to ampicillin, chloramphenicol and trimethoprim shown by the strains studied are considered, together with the epidemiological markers, 13 different groups were defined. Two groups (Amp^R , Cm^R , Tm^S , CARB+, REP-PCR-1, PFGE-A and Amp^R , Cm^R , Tm^R , TEM+, CAT+, DHFR+, REP-PCR-2, PFGE-B) were the most frequent with seven and four strains, respectively. Some strains belonging to a different group showed the same susceptibility pattern and the same mechanism of resistance to β -lactam antibiotics, whereas other strains from the same group showed different mechanisms of antimicrobial resistance. The evolutionary divergence observed is probably due to the acquisition of different mobile genetic elements, such as plasmids, transposons or integrons carrying resistance genes.

In conclusion, REP-PCR analysis provides an alternative, rapid and powerful genomic typing method for Typhimurium. The multiresistance in this micro-organism is of public health concern and although third-generation cephalosporins and quinolones currently provide a very good alternative for clinical treatment it is important to continue surveillance of resistance levels and mechanisms of resistance in this species.

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References

- Miller SI, Hohmann EL, Pegues DA. *Salmonella* (including *Salmonella typhi*). In: Mandell GL, Bennett JE, Dolin R (eds) *Mandell, Douglas and Bennett's Principles and practice of infectious diseases*, vol. 2, 4th edn. 1995. New York, Churchill Livingstone. 1995: 2013–2033.
- Hook EW. Salmonellosis: certain factors influencing the interaction of *Salmonella* and the human host. *Bull NY Acad Med* 1961; **37**: 499–512.
- Glaser JB, Morton-Kute L, Berger SR *et al.* Recurrent *Salmonella typhimurium* bacteremia associated with the acquired immunodeficiency syndrome. *Ann Intern Med* 1985; **102**: 189–193.
- Cohen ML, Tauxe RV. Drug-resistant *Salmonella* in the United States: an epidemiologic perspective. *Science* 1986; **234**: 964–969.
- Duguid JP, Anderson ES, Alfredsson GA, Barker R, Old DC. A new biotyping scheme for *Salmonella typhimurium* and its phylogenetic significance. *J Med Microbiol* 1975; **8**: 149–166.
- Anderson ES, Ward LR, de Saxe MJ, de Saxe JDH. Bacteriophage-typing designations of *Salmonella typhimurium*. *J Hyg* 1977; **78**: 297–300.
- Guinée PAM, Jansen WH, van Schuylenberg A, van Leeuwen WJ. Bacteriophage typing of *Salmonella typhimurium* by use of a mechanized technique. *Appl Microbiol* 1973; **26**: 474–477.
- Anderson ES. Drug resistance in *Salmonella typhimurium* and its implications. *BMJ* 1968; **3**: 333–339.
- Brunner F, Margadant A, Peduzzi R, Piffaretti JC. The plasmid pattern as an epidemiologic tool for *Salmonella typhimurium* epidemics: comparison with the lysotype. *J Infect Dis* 1983; **148**: 7–11.
- Taylor DN, Wachsmuth IK, Shangkuang Y-H *et al.* Salmonellosis associated with marijuana. A multistate outbreak traced by plasmid fingerprinting. *N Engl J Med* 1982; **306**: 1249–1253.
- Kapperud G, Lassen J, Dommarsnes K *et al.* Comparison of epidemiological marker methods for identification of *Salmonella typhimurium* isolates from an outbreak caused by contaminated chocolate. *J Clin Microbiol* 1989; **27**: 2019–2024.
- Platt DJ, Brown DJ, Old DC, Barker RM, Munro DS, Taylor J. Old and new techniques together resolve a problem of infection by *Salmonella typhimurium*. *Epidemiol Infect* 1987; **99**: 137–142.
- Threlfall EJ, Frost JA. The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. *J Appl Bacteriol* 1990; **68**: 5–16.
- Nastasi A, Mammola C. Epidemiological evaluation by PCR ribotyping of sporadic and outbreak-associated strains of *Salmonella enterica* serotype typhimurium. *Res Microbiol* 1995; **146**: 99–106.
- Stanley J, Baquar N, Threlfall EJ. Genotypes and phylogenetic relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS200 and 16S *rrn* loci. *J Gen Microbiol* 1993; **139**: 1133–1140.
- Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover FC (eds). *Manual of clinical microbiology*, 6th edn. Washington, DC, ASM. 1995.
- National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. M2-A4, 4th edn. Villanova, PA, National Committee for Clinical Laboratory Standard. 1990.
- National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd edn. Approved standard M7-A2. Villanova, PA, National Committee for Clinical Laboratory Standard. 1990.
- O'Callaghan CH, Morris A, Kirby SM, Shingler AH. Novel method for detection of β -lactamase by using a chromogenic cephalosporin substrate. *Antimicrob Agents Chemother* 1972; **1**: 283–288.
- Arlet G, Philippon A. Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable beta-lactamases (TEM, SHV, CARB). *FEMS Microbiol Lett* 1991; **82**: 19–26.
- Robison LR, Seligsohn R, Lerner SA. Simplified radioenzymatic assay for chloramphenicol. *Antimicrob Agents Chemother* 1978; **13**: 25–29.
- Azemun P, Stull T, Roberts M, Smith AL. Rapid detection of chloramphenicol resistance in *Haemophilus influenzae*. *Antimicrob Agents Chemother* 1981; **20**: 168–170.
- Maniatis T, Fritsch EF, Sambrook J. In: *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 1982: 314.
- Heikkilä E, Sundström L, Skurnik M, Huovinen P. Analysis of genetic localization of the type I trimethoprim resistance gene from *Escherichia coli* isolated in Finland. *Antimicrob Agents Chemother* 1991; **35**: 1562–1569.
- Carter GI, Townner KJ, Slack RCB. Detection of TEM beta-lactamase genes by non-isotopic spot hybridisation. *Eur J Clin Microbiol* 1987; **6**: 406–409.
- Matushek MG, Bonten MJM, Hayden MK. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *J Clin Microbiol* 1996; **34**: 2598–2600.
- Marcos MA, Jimenez de Anta MT, Vila J. Correlation of six methods for typing nosocomial isolates of *Acinetobacter baumannii*. *J Med Microbiol* 1995; **42**: 328–335.
- Vila J, Marcos MA, Jimenez de Anta MT. A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus*-*A. baumannii* complex. *J Med Microbiol* 1996; **44**: 482–489.
- Blaser MJ, Feldman RA. *Salmonella* bacteremia: Reports of the Centers for Diseases Control, 1968–1979. *J Infect Dis* 1981; **143**: 743–746.
- Bottone EJ, Wormser GP, Duncanson FP. Nontyphoidal salmonella bacteremia as an early infection in the acquired immunodeficiency syndrome. *Diagn Microbiol Infect Dis* 1984; **2**: 247–250.
- Celum CL, Chaisson RE, Rutherford GW, Barnhart JL, Echenberg DF. Incidence of salmonellosis in patients with AIDS. *J Infect Dis* 1987; **156**: 998–1002.
- Cherubin CE, Neu HC, Imperato PJ, Harvey RP, Bellen N. Septicemia with non-typhoid salmonella. *Medicine* 1974; **53**: 365–376.
- Sperber SJ, Schleupner CJ. Salmonellosis during infection with human immunodeficiency virus. *Rev Infect Dis* 1987; **9**: 925–934.
- Smith PD, Macher AM, Bookman MA *et al.* *Salmonella typhimurium* enteritis and bacteremia in the acquired immunodeficiency syndrome. *Ann Intern Med* 1985; **102**: 207–209.
- Alós JI, Gómez Garcés JL, Cogollos R, Amor E, Pérez-Rivilla A. Susceptibilities of ampicillin-resistant strains of *Salmonella* other than *S. typhi* to 10 antimicrobial agents. *Antimicrob Agents Chemother* 1992; **36**: 1794–1796.
- Muñoz P, Díaz MD, Rodríguez-Crèixems M, Cercenado E, Peláez T, Bouza E. Antimicrobial resistance of *Salmonella* isolates in a Spanish hospital. *Antimicrob Agents Chemother* 1993; **37**: 1200–1202.
- Ramos JM, Alés JM, Cuenca-Estrella M, Fernandez-Roblas R, Soriano F. Changes in susceptibility of *Salmonella enteritidis*, *Salmonella typhimurium*, and *Salmonella virchow* to six antimicrobial agents in a Spanish hospital, 1980–1994. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 85–88.
- Reina J, Gomez J, Serra A, Borell N. Analysis of the antibiotic resistance detected in 2043 strains of *Salmonella enterica* subsp. *enterica* isolated in stool cultures of Spanish patients with acute diarrhoea (1986–1991). *J Antimicrob Chemother* 1993; **32**: 765–769.
- Lee LA, Puhf ND, Maloney EK, Bean NH, Tauxe RV. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989–1990. *J Infect Dis* 1994; **170**: 128–134.
- Ward LR, Threlfall EJ, Rowe B. Multiple drug resistance in salmonellae in England and Wales: a comparison between 1981 and 1988. *J Clin Pathol* 1990; **43**: 563–566.
- Liu PYF, Gur D, Hall LMC, Livermore DM. Survey of the prevalence of β -lactamase amongst 1000 gram-negative bacilli isolated consecutively at the Royal London Hospital. *J Antimicrob Chemother* 1992; **30**: 429–447.
- Roy C, Foz A, Segura C, Tirado M, Fuster C, Reig R. Plasmid-determined β -lactamases identified in a group of 204 ampicillin-resistant Enterobacteriaceae. *J Antimicrob Chemother* 1983; **12**: 507–510.
- Medeiros AA, Hedges RW, Jacoby GA. Spread of a “*Pseudomonas*-specific” β -lactamase to plasmids of enterobacteria. *J Bacteriol* 1982; **149**: 700–707.
- Dale JW, Godwin D, Mossakowska D, Stephenson P, Wall S. Sequence of the OXA-2 β -lactamase: comparison with other penicillin-reactive enzymes. *FEBS Lett* 1985; **191**: 39–44.

45. Toro CS, Lobos SR, Calderón I, Rodríguez M, Mora GC. Clinical isolate of a porinless *Salmonella typhi* resistant to high levels of chloramphenicol. *Antimicrob Agents Chemother* 1990; **34**: 1715–1719.
46. Amyes SGB. The success of plasmid-encoded resistance genes in clinical bacteria. *J Med Microbiol* 1989; **28**: 73–83.
47. Goldstein FW, Papadopoulos B, Acar JF. The changing pattern of trimethoprim resistance in Paris with a review of worldwide experience. *Rev Infect Dis* 1986; **8**: 725–737.
48. Agodi A, Marranzano M, Jones CS, Threlfall EJ. Molecular characterization of trimethoprim resistance in salmonellas isolated in Sicily, 1985–1988. *Eur J Epidemiol* 1995; **11**: 33–38.
49. Brown JC, Thomson CJ, Amyes SGB. Mutations of the *gyrA* gene of clinical isolates of *Salmonella typhimurium* and three other *Salmonella* species leading to decreased susceptibilities to 4-quinolone drugs. *J Antimicrob Chemother* 1996; **37**: 351–356.
50. Hof H, Ehrhard I, Tschape H. Presence of quinolone resistance in a strain of *Salmonella typhimurium*. *Eur J Clin Microbiol Infect Dis* 1991; **10**: 747–749.
51. Ruiz J, Castro D, Goñi P, Santamaria JA, Borrego JJ, Vila J. Analysis of the mechanism of quinolone resistance in nalidixic acid-resistant clinical isolates of *Salmonella* serotype Typhimurium. *J Med Microbiol* 1997; **46**: 623–628.
52. Hirai K, Aoyama H, Irikura T, Iyobe S, Mitsuhashi S. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrob Agents Chemother* 1986; **29**: 535–538.
53. Garbarg-Chenon A, Godard V, Labia R, Nicolas J-C. Nucleotide sequence of SHV-2 β -lactamase gene. *Antimicrob Agents Chemother* 1990; **34**: 1444–1446.
54. Vu Thien H, Baudon JJ, Rolland M. *Salmonella typhimurium*: résistance aux céphalosporines de troisième génération. *Med Mal Infect* 1987; **6/7**: 398–400.
55. Wall PG, Morgan D, Lamden K *et al.* A case-control study of infection with an epidemic strain of multiresistant *Salmonella typhimurium* DT 104 in England and Wales. *Commun Dis Rep CDR Rev* 1994; **4**: R130–135.
56. Murase T, Okitsu T, Suzuki R *et al.* Evaluation of DNA fingerprinting by PFGE as an epidemiologic tool for *Salmonella* infections. *Microbiol Immunol* 1995; **39**: 673–676.
57. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991; **19**: 6823–6831.