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Resistance to third-generation cephalosporins in human non-typhoidal *Salmonella enterica* isolates from England and Wales, 2010-2012.

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Running Title:

ESBLs and AmpCs in UK Salmonella

Keywords:

ESBL, AmpC, surveillance

Objectives: To identify the mechanism(s) underlying cefotaxime resistance in 118 of 21,641 (0.55%) non-typhoidal *Salmonella enterica* collected from humans throughout England and Wales from January 2010 to September 2012.

Methods: Non-duplicate isolates (*n*=118) resistant to cefotaxime (MICs >1 mg/L) were screened by PCR for genes encoding CTX-M extended-spectrum beta-lactamases (ESBLs) and associated IS*Ecp1*-like elements, and for genes encoding acquired AmpC, SHV, TEM, VEB, PER and GES beta-lactamases. Sequencing was used to identify specific alleles in selected isolates. Carbapenem resistance was sought by ertapenem disc screening.

Results: Seventy-nine isolates (0.37% of all referred *S. enterica*) produced ESBLs, 37 (0.17%) produced CMY-type AmpC enzymes, and one had both enzyme types; the mechanism of cefotaxime resistance in three isolates could not be identified. Group 1 CTX-M genes were identified in 57 isolates belonging to 22 serotypes, with CTX-M-1 (*n*=11), -15 (*n*=9) and -55/57 (*n*=8) the most prevalent alleles amongst the 29 (49%) investigated. CTX-M-2 (*n*=5), -14 (*n*=5), -8 (*n*=1) and -65 (*n*=1) were also identified. TEM-52 was identified in two isolates and SHV-12 in seven isolates. There was no evidence of carbapenem resistance. ESBL and AmpC genes were detected in both domestically-acquired and travel-associated salmonellae. Eighty-nine isolates (75%) were multidrug-resistant (resistant to ≥3 antimicrobial classes) and 42 (36%) had decreased susceptibility to ciprofloxacin (MICs 0.25 – 1 mg/L), with a further 13 isolates (11%) resistant (MICs >1 mg/L).

Conclusion: The prevalence of CTX-M and acquired AmpC genes in human non-typhoidal *S. enterica* from England and Wales is still low, but has increased from 0.03% in 2001-2003 to 0.49% in 2010-12. Resistance to third-generation cephalosporins requires monitoring as it may reduce therapeutic options.

Introduction

Non-typhoidal *Salmonella enterica* (NTS) frequently cause mild gastrointestinal infections that normally resolve without the need for antimicrobials. However, invasive infections can occur in vulnerable patients, where treatment with a fluoroquinolone or third-generation cephalosporin can be life-saving. Resistance to third generation cephalosporins is increasing in *Salmonella* spp. and is mainly due to production of acquired AmpC and extended-spectrum beta-lactamases (ESBLs). [1] The increased occurrence of these enzymes in *Salmonella* spp., coupled with decreased susceptibility to quinolones compromises the use of these drugs and is a serious public health issue. [1, 2]

The prevalence of cephalosporin resistance was very low (0.04%) in clinical NTS isolates collected in England and Wales between 1992-2003, with only 14 CTX-M and nine AmpC enzymes detected over the eleven-year period. [3, 4] At present carbapenem resistance in *S. enterica* is extremely rare, although isolates expressing different acquired carbapenemases have been reported. [5-7] This study aimed to determine the prevalence of cephalosporin resistance in NTS isolates collected throughout England and Wales from January 2010 to September 2012. We also sought to identify the underlying ESBL, AmpC genes and screened cephalosporin-resistant isolates for carbapenem resistance.

Methods

Selection and phenotypic characterization of *Salmonella* isolates

Non-duplicate isolates (*n*=118) resistant to 1 mg/L cefotaxime were selected from all NTS isolates causing human salmonellosis in England and Wales between January 2010 and mid-September 2012 (*n*=21,641). Isolates were recovered from faeces (*n*=116), blood (*n*=1) and wound swab (*n*=1) samples. Resistance to antimicrobials was determined using breakpoint concentrations and methodology based on long-term studies within the Gastrointestinal Bacteria Reference Unit, Public Health England. [8] Isolates were screened for carbapenem resistance as described by Lolans *et al.* with

modifications. [9] Briefly, 0.5 McFarland suspensions in Iso-Sensitest broth were used to inoculate Mueller-Hinton agar plates and a 10 μg ertapenem disc (Oxoid, Basingstoke, UK) was added. Inhibition zone diameters ≤ 27 mm after 18 h incubation at 37°C were considered 'resistant' by comparison with positive control strains (not salmonellae) producing NDM-1, KPC, VIM, IMP and OXA-48 carbapenemases.

Determination of β-lactamase genotypes

Isolates were screened by PCR for the presence of CTX-M, AmpC, TEM, SHV, VEB, PER and GES beta-lactamase genes (primer sequences in Table S1, available as Supplementary data at JAC Online). Where SHV or TEM enzymes were the sole mechanism identified to explain cefotaxime resistance, ESBL production was confirmed by double-disc synergy test and the alleles were identified by sequencing in most cases. All group 2, 8 and 9 CTX-M genes were identified to allele level by sequencing. Group 1 CTX-M alleles and their upstream genetic environments were investigated by PCR and sequencing using primers specific for IS*Ecp1*-like and IS*26*-like elements (Table S1) in 29 (49% of CTX-M producers) isolates representing diverse serotypes. Isolates positive for CIT group genes were subsequently screened for CMY genes by PCR (Table S1). OXA-1-like, OXA-2-like, OXA-10-like and PSE-1 genes were sought in isolates for which no other cephalosporin resistance mechanism was detected (Table S1).

Results and Discussion

One hundred and eighteen (0.55%) human NTS isolates from England and Wales in January 2010 – mid-September 2012 were resistant to cefotaxime (MICs >1 mg/L) (Table 1). This indicates a significant increase (P<0.0001 by χ^2 test with Yates correction) since the last prevalence study in 2003. [3, 4] However, this represents the 2010 European average amongst countries using low breakpoints; Netherlands (0.3%) and Denmark (0.5%) (breakpoint >0.5 mg/L), Ireland (3.1%) and France (4.3%) (breakpoint >2 mg/L). [10] Resistance to third-generation cephalosporins in 2010-12 in England and Wales was due primarily to production of CTX-M-type ESBLs and AmpC beta-

lactamases, which were found in 69 (58%) and 37 (31%) of cefotaxime-resistant isolates, respectively. The prevalence of these genes has increased from 0.03% (15/45,318) in human isolates from 2001-2003 to 0.49% (107/21,641) in isolates from 2010 to 2012. [3, 4] Where a travel history was known (74 of 118 isolates), ESBL and AmpC genes were associated with both domestically-acquired (*n*=16, 22%) and travel-associated (*n*= 58, 78%) NTS infections (Table 1).

CTX-M genes in *S. enterica*

The occurrence of β-lactamase genes in NTS serotypes are detailed in Table 1 and in Supplementary Table S2 and Figure S1 (available at JAC online). The most common resistance mechanisms detected were group 1 CTX-M genes, which were identified in 57 isolates, 29 of which were *S.* Typhimurium of various phage types and which were often associated with travel to Asia. The other 28 isolates represented 21 serotypes. PCR revealed that group 1 CTX-M genes were linked to an upstream IS*Ecp1*-like element in 27 of 29 isolates investigated, representing diverse serotypes. IS*26* elements were sought but not found in the two remaining isolates, one of which contained CTX-M-15/28. The remaining group 1 CTX-M alleles sequenced comprised 11 CTX-M-1, 8 CTX-M-55/57 and 9 CTX-M-15.

CTX-M-1 is the most common food animal-associated CTX-M enzyme in EU countries and is circulating throughout Europe on IncN plasmids in *E. coli* and *Salmonella* from human, animal and environmental sources. [11-13] CTX-M-15 is widespread in clinical enterobacterial isolates worldwide and has been identified in human NTS isolates throughout Europe and Asia, while CTX-M-55/57 has almost exclusively been reported in human and animal enterobacterial isolates from Asian countries. [3, 12, 14-16] Transfer of resistance plasmids encoding group 1 CTX-M enzymes from *E. coli* to *S. enterica* has been demonstrated previously and this may have contributed to the increased prevalence of these genes in NTS. [17]

CTX-M-2 alleles were identified in five isolates including 3/6 *S.* Heidelberg isolates with the same multidrug-resistance type (ASSu). CTX-M-2 was previously described in *S.* Virchow isolates common to poultry and humans in Europe. [18, 19] CTX-M-14, which was previously described in

Spanish NTS, [20] was identified in five isolates from diverse serotypes, one of which was associated with travel to Spain. Multiple cefotaxime resistance mechanisms were identified in three isolates, including one *S.* Concord isolate that expressed a group 1 CTX-M gene in combination with TEM and SHV-12 and resembled a multidrug-resistant strain associated with Ethiopian adoptees that was previously identified in England and Wales (Figure S1 and Table S2). [21] One *S.* Enteritidis isolate contained CTX-M-8 and one *S.* Infantis isolate contained a group 9 CTX-M-65 gene. To our knowledge this is the first description of either gene in *S. enterica*.

Other β-lactamases in *S. enterica*

TEM-type genes were identified as the sole resistance mechanism potentially explaining cefotaxime resistance in three isolates, which were confirmed as ESBL producers by double-disc synergy test. The most common European TEM ESBL, $bla_{\text{TEM-52}}$,[22] was identified in two of these isolates that were investigated by sequencing. SHV-12 was found in nine isolates in the present study and was the sole ESBL in seven isolates, including both *S.* Virchow isolates. This ESBL was first found in human NTS isolates from Africa [23] and has since occurred in NTS from Europe,[20] the USA [24] and more recently India. [16]

Thirty-seven isolates were positive for CMY-type AmpC enzymes by PCR. Similar to the situation reported in America, CMY enzymes were found in a wide range of *S. enterica* serotypes. [24] The mechanism(s) of cefotaxime resistance in three isolates could not be identified, although two of them were phenotypically positive for an ESBL by the double-disc synergy test. None of the isolates produced PCR products with primers specific for VEB, PER, GES, OXA-1-like, OXA-2-like, OXA-10-like and PSE-1 genes.

None of the isolates was considered resistant to carbapenems by the ertapenem disc screen used.

147 Resistance to non-beta-lactam antibiotics

148 Eighty-nine (75%) cefotaxime-resistant isolates representing 25 serotypes were multidrug-resistant
149 (MDR; resistant to ≥3 antimicrobial classes, Tables 1 and S2). NTS isolates were resistant to
150 sulphonamides (MICs >64 mg/L; 72%), tetracycline (MICs >8 mg/L; 68%), gentamicin (MICs >4
151 mg/L; 35%), amikacin (MICs >4 mg/L; 2%), kanamycin (MICs >16 mg/L; 18%), neomycin (MICs
152 >8 mg/L; 16%), streptomycin (MICs >16 mg/L; 92%), nalidixic acid (MICs >16 mg/L; 31%),
153 chloramphenicol (MICs >8 mg/L; 39%), trimethoprim (MICs >2 mg/L; 25%), furazolidone (MICs >8
154 mg/L; 11%) and colistin (MICs >8 mg/L; 4%).

155 Forty-two NTS (36%) had decreased susceptibility to ciprofloxacin (DSC) (MICs of 0.25 to 1
156 mg/L), with 22 of these retaining susceptibility to nalidixic acid (MICs ≤16 mg/L), indicating the
157 likely presence of a plasmid-mediated quinolone resistance determinant, based on the findings of a
158 previous study. [25] Thirteen NTS (11%) were resistant to ciprofloxacin (MICs >1 mg/L) including four
159 of five *S.* Kentucky isolates, three of which were associated with travel to Egypt. These isolates are
160 likely to belong to the ciprofloxacin-resistant ST198-X1 international clone, which originated in this
161 country. [26] Three ciprofloxacin-resistant isolates were *S.* Agona associated with travel to Thailand or
162 the Asian continent (Table S2). Co-resistance to fluoroquinolones and extended-spectrum
163 cephalosporins is already a major public health problem in Asia where 9.3% of NTS isolates sampled
164 from 2003-2005 had dual resistance to ciprofloxacin (MICs >0.125 mg/L) and ceftriaxone (MICs of
165 2-8 mg/L). [27] In the present study dual resistance was found in 0.25% of UK NTS isolates. Where
166 travel history was recorded 58% of these isolates had ties to Asia. The high rate (25%) of reduced
167 susceptibility to ceftriaxone in *S.* Typhimurium throughout Asia was not evident in England and
168 Wales (0.8%). [27] In this study all 13 isolates associated with travel to Thailand were resistant to ≥5
169 antimicrobial classes. *S.* Typhimurium isolates (88% MDR) were significantly more resistant than *S.*
170 Enteritidis isolates (35% MDR) (P=0.0004 by Fisher's exact test). Thirty-four percent of all NTS had
171 penta-resistance type ACSSuT including 16 (39%) *S.* Typhimurium, 15 of which had a group 1 CTX-
172 M gene. Twenty-seven (23%) NTS also had decreased susceptibility or resistance to ciprofloxacin
173 (ACSSuTCp), including 12 (29%) *S.* Typhimurium.

In conclusion, resistance to third-generation cephalosporins in NTS is a growing concern that requires monitoring. The high degree of co-resistance to fluoroquinolones in cefotaxime-resistant isolates compromises treatment of vulnerable patients, although resistance to carbapenems in NTS remains rare. Our data support travel-associated spread of resistant strains to the UK from locally endemic areas. However the epidemiology of resistance is clearly complex and may involve the spread of multidrug resistance plasmids expressing ESBLs and AmpCs between enterobacterial strains and species. [1, 17] The most commonly identified ESBL and AmpC genes in this study have all been identified in *E. coli* and *Salmonella* from food animals in Europe and control measures to limit the dissemination of these strains through the food chain are necessary. [12]

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Transparency Declaration

Nothing to declare

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278 **Table 1.** Genotypic, phenotypic and epidemiological features of 3GC-resistant non-typhoidal *S. enterica*

	Genotypic Features			Antimicrobial Resistance			Foreign Travel history		
β-Lactamase group(s)	**N**	**Alleles (n)**	**Serovars**	**MDR**	**DSC**	**CIP**	**Country (n)**	**None**	**Unknown**
			n	**n (%)**	**n (%)**	**n (%)**			
Grp1 CTX-M	56	CTX-M-1(11), CTX-M-15(9), CTX-M-15/28[a](1), CTX-M-55/57[b](8)	21	46 (82)	23 (41)	3 (5)	Thailand (8), Pakistan (4), Morocco (3), Egypt (2), Cambodia (2) India (1), Portugal (1), United Arab Emirates (1), Qatar (1), Unspecified (4)	8	21
Grp1 CTX-M & SHV	1	SHV-12(1)	1	1 (100)	0	0	0	0	1
Grp 2 CTX-M	4	CTX-M-2(4)	3	4 (100)	2 (50)	0	Saudi Arabia (1)	1	2
Grps2 & 9 CTX-M	1	CTX-M-2(1)	1	1 (100)	0	0	0	0	1
Grp8 CTX-M	1	CTX-M-8(1)	1	0	0	0	Spain (1)	0	0
Grp9 CTX-M	5	CTX-M-14(4), CTX-M-65(1)	4	4 (80)	3 (60)	2 (40)	China (1), Peru (1), Egypt (1), Thailand (1)	0	1
Grp 9 CTX-M & CMY	1	CTX-M-14(1)	1	1 (100)	0	1 (100)	Spain (1)	0	0
SHV	7	SHV-12(6)	6	6 (86)	4 (57)	3 (43)	Egypt (2), Spain (1), India (1), Unspecified (2)	0	1
TEM	3	TEM-52(2)	3	2 (66)	1 (33)	1 (33)	Asia (1), China (1)	0	1
CMY	36	ND	16	24 (67)	9 (25)	3 (8)	Thailand (4), Mexico (3), Egypt (3), India (1), Gambia (1), Jamaica (1), Pakistan (1)	7	15
None found	3	N/A	3	0	0	0	Turkey (1), China (1)	0	1
All isolates	118	N/A	32	89 (75)	42 (36)	13 (11)	58	16	44

279 [a]not possible to differentiate between these two alleles with DNA sequence obtained; [b]share identical DNA sequences; MDR = resistant to 3 or more
280 antimicrobial classes; DSC = decreased susceptibility to ciprofloxacin (MICs 0.25 to 1 mg/L); CIP = resistant to ciprofloxacin (MICs >1 mg/L); ND = not
281 determined; N/A = not applicable