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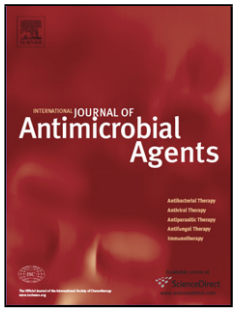

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Strategies to overcome extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in shigellae

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ABSTRACT

Oral cephalosporins and mecillinam are used to treat *Shigella* infections but are compromised by extended-spectrum β-lactamases (ESBLs) and plasmid AmpC β-lactamases. Potential solutions include combining an oral or intravenous cephalosporin with a β-lactamase inhibitor (BLI) or using an oral penem. These strategies were examined using *Escherichia coli* transconjugants and clinical isolates with ESBLs or AmpC as a proxy for shigellae. The Clinical and Laboratory Standards Institute agar dilution method was used with inocula of 10^4 and 10^6 colony-forming units/spot. ESBLs conferred resistance to the cephalosporins and mecillinam, at least at high inoculum, although: (i) ceftibuten was significantly compromised only by SHV and CTX-M-15 ESBLs, but not by TEM or CTX-M-9 and -14; (ii) cefdinir was little affected by TEM-type ESBLs and mecillinam was little affected by CTX-M-9 group enzymes. The BLI clavulanic acid reduced the minimum inhibitory concentrations (MICs) of cephalosporins and mecillinam to ≤2 mg/L for ESBL-producers, even at high inocula; sulbactam in particular and tazobactam were less effective, especially against SHV types. Strains with AmpC were resistant to all cephalosporins ± inhibitors, but mecillinam remained active (MIC = 1 mg/L) against a strain with AmpC alone, where strains with TEM-1 + AmpC were susceptible to mecillinam + clavulanic acid at ≤2 mg/L. Faropenem was active against all ESBL- and AmpC-producers at 4 mg/L, with little inoculum effect or inhibitor potentiation. In conclusion, cephalosporin + clavulanic acid combinations overcame ESBLs, with ceftibuten + clavulanic acid being particularly promising. Mecillinam + clavulanic acid and faropenem overcame both ESBLs and AmpC enzymes. Clinical utility will depend also on the drug's ability to reach intracellular shigellae in the intestinal epithelium and this deserves exploration for clavulanic acid and faropenem.

1. Introduction

Shigellosis remains a major cause of morbidity and mortality in the developing world, with ca. 165 million cases and 1.1 million deaths annually, most of them in children aged ≤5 years [1,2]. The infective dose is small and in severe disease mortality can reach 15%. Epidemics occur in settings of poverty and poor hygiene, including refugee camps.

The World Health Organization (WHO) recommends that shigellosis is treated with antimicrobials. The benefits of chemotherapy were confirmed by a Cochrane review [2] highlighting two placebo-controlled trials which showed that antibiotics reduced the risk of continued diarrhoea by 70–79% [3,4]. Ampicillin, co-trimoxazole (trimethoprim/sulfamethoxazole) and fluoroquinolones were effective but are now compromised by resistance [2], with therapy increasingly shifting to oral oxyimino-cephalosporins (ceftibuten, cefixime or cefdinir) or to intravenous (i.v.) ceftriaxone. Disturbingly, however, cephalosporin resistance is emerging in *Shigella* spp. associated with plasmid-mediated AmpC enzymes in Argentina [5] and Taiwan [6] or, more often, with extended-spectrum β-lactamases (ESBLs), which have been reported in shigellae from East Asia [7–9], the Middle East [10–13] and Argentina [14]. Most ESBLs in shigellae are now CTX-M types and, as shown by a Korean study, these enzymes are associated with cephalosporin failure [7]. If ESBLs or AmpC enzymes occur in strains already resistant to fluoroquinolones and co-trimoxazole, azithromycin may be the sole option for oral therapy.

In this study, we aimed to explore whether faropenem or combinations of cephalosporins or mecillinam with a β-lactamase inhibitor (BLI) might overcome these resistances. Emphasis was placed on oral antibiotics or combinations as these are more suitable for outbreaks, especially in developing countries, but i.v. cephalosporin + BLI combinations were also considered as appropriate for the most serious cases; the WHO recommendations currently include the use of ceftriaxone in shigellosis [1]. For safety reasons, *Escherichia coli* was used as a proxy for shigellae; taxonomists view the two genera as a continuum [15].

2. Materials and methods

2.1. Strains

Strains were *E. coli* transconjugants and transformants with a diversity of different ESBL types [16] or were clinical isolates with AmpC activity as evidenced by strong synergy between cefotaxime and cloxacillin and polymerase chain reaction (PCR) evidence of an acquired *ampC* gene [17].

2.2. Antibiotics and susceptibility tests

Cefdinir, cefotaxime, ceftazidime, ceftriaxone and tazobactam were from Sigma (Poole, UK); cefpodoxime and cefixime were from Aventis (Hounslow, UK); ceftibuten and faropenem were from Onbio Inc. (Richmond Hill, Ontario, Canada); clavulanic acid was from GlaxoSmithKline (Brentford, UK); mecillinam was from Leo Pharma (Princes Risborough, UK); and sulbactam was from Pfizer (Sandwich, UK). Minimum inhibitory concentrations (MICs) were determined by the Clinical and

Laboratory Standards Institute (CLSI) agar dilution method [18], but with inocula of 10^4 and 10^6 colony-forming units (CFU)/spot. BLIs were tested at 4 mg/L.

3. Results

3.1. Oral cephalosporins and combinations

All of the ESBL-producing transconjugants and transformants were clearly resistant to cefpodoxime (MICs ≥ 64 mg/L), even with low inocula of 10^4 CFU/spot (Table 1). The degree of resistance to the other oral cephalosporins varied with the enzyme type. For cefixime, TEM-6, TEM-10, SHV-4, SHV-5 and CTX-M-15 enzymes were associated with MICs ≥ 16 mg/L even at low inocula, whereas TEM-3, SHV-2 and CTX-M-3 and -14 enzymes only gave MICs > 4 mg/L at high inoculum, and even at high inoculum the MIC for the transconjugant with CTX-M-9 enzyme was only 4 mg/L. MICs of cefdinir were raised to ≥8 mg/L even at low inoculum by all CTX-M and SHV ESBLs, but the drug was less affected by TEM types and, among these, only TEM-3 raised the MIC above 4 mg/L even at high inocula. Ceftibuten was the cephalosporin least affected by ESBLs; even at high inoculum only SHV-4 and -5 and CTX-M-15 enzymes raised its MIC above 4 mg/L, whilst producers of CTX-M-9 and -14 were susceptible at ≤1 mg/L. The BLI clavulanic acid reduced the MICs of all four oral cephalosporins to ≤2 mg/L for all the ESBL-producing transconjugants, with MICs for ceftibuten + clavulanic acid consistently ≤1 mg/L (Table 1). Potentiation by sulbactam was less impressive; MICs of nearly all of the cephalosporin + sulbactam combinations remained ≥8 mg/L at low inocula for transconjugants with SHV-4 and -5 and CTX-M-15 enzymes, and all did so at high inoculum.

The four isolates with AmpC β-lactamases were resistant to all four oral cephalosporins (MICs ≥ 64 mg/L), irrespective of the inoculum and the addition of inhibitors (Table 1).

3.2. Faropenem

Faropenem had consistently good activity against all of the ESBL- and AmpC-producing transconjugants and isolates, with MICs ≤ 4 mg/L and no inoculum effect (Table 1). At most its MIC (e.g. for the transconjugant with SHV-5) was two doubling dilutions higher than for the corresponding recipient strain, with a one dilution reduction achieved by clavulanic acid.

3.3. Mecillinam

Mecillinam MICs for the ESBL-producing transconjugants and transformants were mostly >8 mg/L at the 10^4 CFU inoculum and ≥128 mg/L at 10^6 CFU inoculum (Table 1). The major exception was that even at the high inoculum, MICs for the transformants with CTX-M-group 9 enzymes (i.e. CTX-M-9 and -14) were ≤1 mg/L. Addition of clavulanic acid reduced the MICs for all the ESBL-producers to ≤1 mg/L, except for the transconjugant with SHV-4 (MIC = 128 mg/L). The reason for the failure with this latter strain is unclear but, in any event, SHV-4 enzyme has been predominantly associated with a *Klebsiella* clone in France and Belgium [19] and has never been reported in shigellae. As with the cephalosporins, potentiation of mecillinam by sulbactam was less effective than by clavulanic acid.

Among the four isolates with AmpC activity, one isolate, which lacked any other β-lactamase, was susceptible to mecillinam at 1 mg/L (low inoculum) or 4 mg/L (high), whereas the other three isolates, which additionally had classical TEM activity, as revealed by isoelectric focusing (not shown), were resistant to mecillinam, with MICs of 32 mg/L to >128 mg/L at low inoculum and >128 mg/L at high inoculum. These latter organisms became susceptible to mecillinam when clavulanic acid was added, with high-inoculum MICs ≤ 2 mg/L, except for one isolate with a CIT-type enzyme for which the high-inoculum mecillinam MIC remained at 4 mg/L. Once again, potentiation by sulbactam was less impressive, with MICs of 8–64 mg/L.

3.4. Injectable cephalosporins

TEM-3, SHV-2, -4 and -5, and CTX-M-3 enzymes were associated with cefotaxime, ceftriaxone and ceftazidime MICs ≥ 16 mg/L even at low inocula (Table 2), whereas TEM-6 and -10 enzymes predominantly conferred ceftazidime resistance at low inocula, although they also raised cefotaxime and ceftriaxone MICs to ≥32 mg/L at high inocula. Even at high inocula, CTX-M-3, -9 and -14 enzymes only raised the MICs of ceftazidime to 0.5–2 mg/L.

As with the oral analogues, there was good synergy with clavulanic acid against the ESBL-producers, and MICs of all the cephalosporin + clavulanic acid combinations were ≤2 mg/L even at high inocula. Synergy with sulbactam in particular and tazobactam was less consistent, especially for the transconjugants and transformants with SHV ESBLs, although MICs of cephalosporin + tazobactam combinations were all <1 mg/L for transconjugants with CTX-M ESBLs. None of the

injectable cephalosporins were significantly potentiated against the isolates with AmpC enzymes.

4. Discussion

The emergence of cephalosporin resistance in *Shigella* spp. presents a clinical challenge, since many of the β-lactamase-producing isolates are already resistant to established therapies for shigellosis, including ampicillin, co-trimoxazole and fluoroquinolones [2]. So far, shigellae with ESBLs have been repeatedly reported from the Far and Middle East as well as Argentina. The enzymes are mostly group 1 (i.e. CTX-M-3 and -15) [10–12] and group 9 (i.e. CTX-M-9, -14 and -24) [9] CTX-M types, although CTX-M-2 is recorded from shigellae in Argentina and Israel [13,14], SHV-2 in Argentina [14] and a TEM-116 variant in China [8]. Plasmid AmpC enzymes are rarer, but have been found in shigellae in Argentina and Taiwan [5,6]. Cephalosporin-resistant shigellae are circulating on the Indian subcontinent, but their prevalence and the enzyme types are uncertain [20].

Azithromycin is often the only active therapy against multiresistant ESBL- or AmpC-producing shigellae, and we sought to explore alternatives including faropenem and combinations of mecillinam or oral cephalosporins with BLIs. Although no cephalosporin–BLI combination is marketed in the West, many such combinations are available in India. Similarly, although faropenem was denied a license by the US Food and Drug Administration (FDA), it is licensed for respiratory infections in, for example, India and Japan. We also considered the potential of injectable cephalosporin + BLI combinations as being appropriate in severe disease.

Based on microbiological activity, the most promising drugs identified were ceftibuten + clavulanic acid, faropenem alone or mecillinam + clavulanic acid. Other cephalosporin + clavulanic acid combinations, including those involving injectable compounds, were only marginally less active than ceftibuten + clavulanic acid and might be preferred if they offer a better pharmacodynamic match with clavulanic acid; combinations of sulbactam or tazobactam with cephalosporins were less active than clavulanic acid combinations. Faropenem and mecillinam + clavulanic acid had the additional advantage of overcoming AmpC enzymes. Although these are presently less reported than ESBLs in shigellae, there must be a concern that they will spread in the future.

Whether or not these proposed treatments will also prove clinically effective will depend on the drugs' penetration to intracellular bacteria in the intestinal mucosa. Ceftibuten, cefdinir, cefotaxime, ceftriaxone and mecillinam are active in shigellosis caused by susceptible strains [7,21,22] and so must achieve adequate penetration. However, for reasons that remain unclear, cefixime was found to be effective in children but not adults [23,24]. There are no convincing data that we are aware of on whether clavulanic acid and faropenem reach this site, although there are old data to indicate that clavulanic acid has some intracellular penetration [25]. In view of the mortality associated with *Shigella* infections and the erosion of existing therapies by resistance [1,2], we would urge urgent investigation of these aspects along with clinical trials to explore the efficacy these regimens.

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Competing interests: DML has shareholdings in GlaxoSmithKline, Merck, Dechra, Pfizer and AstraZeneca within a diversified portfolio and acts as Enduring Attorney for a close relative, managing further holdings in GlaxoSmithKline and Eco Animal Health. TN is an employee of the Institute for OneWorld Health. All others authors declare no competing interests.

Ethical approval

Not required.

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Table 1

Minimum inhibitory concentrations (mg/L) of oral β-lactams and combinations

Inoculum/strain	Cefpodoxime			Cefixime			Cefdinir			Ceftibuten			Faropenem			Mecillinam		
	Alone	+CLA	+SUL	Alone	+CLA	+SUL	Alone	+CLA	+SUL	Alone	+CLA	+SUL	Alone	+CLA	+SUL	Alone	+CLA	+SUL
10^4 CFU/spot																		
J62 TEM-3	128	1	1	4	0.5	0.25	4	0.25	0.5	0.5	0.25	0.25	1	1	1	8	0.5	0.25
J53 TEM-6	128	1	1	16	1	1	2	0.25	0.25	1	0.5	0.25	2	1	2	64	0.5	0.5
J53 TEM-10	64	1	1	16	1	0.5	1	0.5	0.5	1	0.5	0.25	2	1	2	>128	0.5	0.5
J53 SHV-2	>128	1	128	4	1	4	8	0.25	4	2	0.5	1	2	1	2	128	0.25	32
J53 SHV-4	>128	0.25	>128	>128	0.125	>128	32	0.125	32	16	0.06	8	2	2	2	>128	128	>128
J53 SHV-5	>128	1	>128	>128	2	>128	64	1	32	32	0.5	32	4	2	4	>128	0.5	64
DH5α CTX-M-3	128	0.5	2	4	0.25	1	64	0.25	4	1	0.5	0.25	2	1	2	8	0.25	4
DH5α CTX-M-15 (TR516)	>128	0.5	16	128	0.5	32	>128	0.25	16	8	0.25	1	0.5	1	1	8	0.25	4
DH5α CTX-M-9	128	0.5	1	2	0.25	0.25	64	0.25	0.5	0.5	0.125	0.125	1	0.5	0.5	0.25	0.125	0.125
DH5α CTX-M-14	>128	0.5	32	4	0.5	1	128	0.25	32	0.5	0.25	0.25	0.5	0.5	0.5	0.5	0.25	0.25
Isolate CIT AmpC	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	2	2	2	>128	1	64
Isolate ACC AmpC	>128	>128	>128	>128	>128	>128	>128	>128	>128	64	64	64	2	2	2	32	0.125	16

Isolate CIT AmpC	>128	>128	>128	>128	>128	>128	128	128	128	>128	>128	>128	2	2	2	32	0.5	8
Isolate CIT AmpC	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	4	4	4	1	0.5	0.5
DH5α	0.5	0.5	0.5	0.25	0.25	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.5	0.5	0.5	0.25	0.125	0.125
J53	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.25	0.125	0.5	1	0.5	0.25	0.125	0.25
J62	2	0.5	1	1	0.5	0.25	0.5	0.25	0.25	0.25	0.25	0.25	1	0.5	0.5	0.25	0.25	0.25
ATCC 25922	1	1	0.5	1	1	0.25	0.5	0.5	0.5	0.5	1	0.25	0.5	0.5	0.5	0.25	0.125	0.125
10^6 CFU/spot																		
J62 TEM-3	>128	1	1	16	1	1	64	0.5	0.25	1	0.5	0.25	1	1	1	>128	0.5	0.5
J53 TEM-6	>128	1	4	64	1	1	2	0.5	0.5	2	0.5	0.5	2	1	1	>128	0.5	0.5
J53 TEM-10	>128	2	4	128	1	1	4	0.5	0.5	4	1	1	2	2	2	>128	0.5	0.5
J53 SHV-2	>128	1	64	8	1	8	>128	0.5	8	2	0.5	1	2	1	2	>128	0.25	64
J53 SHV-4	>128	0.5	>128	>128	0.125	>128	32	0.125	32	32	0.06	8	2	2	2	>128	128	>128
J53 SHV-5	>128	2	>128	>128	2	>128	64	1	32	128	1	32	4	2	4	>128	0.5	128
DH5α CTX-M-3	>128	1	8	8	0.5	1	128	0.5	4	1	0.5	0.5	1	0.5	2	128	0.25	4
DH5α CTX-M-15 (TR516)	>128	1	>128	>128	1	64	>128	1	>128	32	0.5	8	2	0.5	2	>128	0.25	16
DH5α CTX-M-9	>128	1	4	4	0.25	0.5	128	0.25	1	1	0.125	0.125	1	0.5	0.5	0.5	0.125	0.25
DH5α CTX-M-14	>128	1	64	16	0.5	2	>128	0.5	32	1	0.5	0.5	1	0.5	1	0.5	0.25	0.25

Isolate ACC AmpC	>128	>128	>128	>128	>128	>128	>128	>128	>128	128	64	64	4	2	4	>128	0.5	32
Isolate CIT AmpC	>128	>128	>128	>128	>128	>128	128	64	128	>128	>128	>128	2	2	2	>128	4	64
Isolate CIT AmpC	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	4	4	4	4	1	0.5
DH5α	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.125	0.125	1	0.5	0.5	0.5	0.125	0.125
J53	1	1	0.5	1	0.5	0.25	0.5	0.5	0.25	0.5	0.5	0.25	1	1	0.5	0.25	0.25	0.25
J62	2	1	1	1	1	0.5	0.5	0.25	0.5	0.5	0.25	0.25	1	0.5	1	0.5	0.25	0.25

CFU, colony-forming units; CLA, clavulanic acid; SUL, sulbactam.

Table 2

Minimum inhibitory concentrations (mg/L) of injectable cephalosporin combinations

Inoculum/strain	Ceftriaxone				Cefotaxime				Ceftazidime			
	Alone	+CLA	+TAZ	+SUL	Alone	+CLA	+TAZ	+SUL	Alone	+CLA	+TAZ	+SUL
10^4 CFU/spot												
J62 TEM-3	16	0.03	0.06	0.03	16	0.06	0.125	0.06	16	0.25	0.25	0.25
J53 TEM-6	8	0.03	0.06	0.06	2	0.06	0.125	0.06	>128	0.5	1	0.5
J53 TEM-10	2	0.06	0.06	0.06	1	0.06	0.125	0.06	>128	0.5	0.5	2
J53 SHV-2	64	0.06	4	8	64	0.125	8	8	16	0.25	8	8
J53 SHV-4	128	0.03	16	32	128	0.03	16	16	>128	0.25	>128	>128
J53 SHV-5	128	0.125	32	32	128	0.125	16	32	>128	2	>128	>128
DH5α CTX-M-3	64	0.03	0.06	0.125	32	0.06	0.06	0.125	1	0.125	0.25	0.125
DH5α CTX-M-15 (TR516)	>128	0.03	0.06	1	>128	0.06	0.125	2	16	0.25	0.5	4
DH5α CTX-M-9	32	0.015	0.03	0.125	32	0.03	0.03	0.125	0.5	0.06	0.125	0.125
DH5α CTX-M-14	64	0.03	0.03	4	32	0.03	0.03	2	1	0.125	0.125	0.25
Isolate CIT AmpC	64	64	16	64	64	32	16	64	64	32	8	64
Isolate ACC AmpC	64	64	64	64	64	64	64	64	128	>128	>128	>128
Isolate CIT AmpC	32	32	16	32	16	16	16	16	64	64	16	64
Isolate CIT AmpC	128	64	64	64	64	64	64	64	128	128	64	128
DH5α	0.015	0.015	0.015	0.015	0.03	0.015	0.03	0.03	0.06	0.06	0.125	0.06

J53	0.03	0.03	0.03	0.03	0.06	0.03	0.06	0.03	0.13	0.125	0.125	0.06
J62	0.06	0.03	0.06	0.06	0.06	0.03	0.06	0.06	0.25	0.125	0.25	0.25
10^6 CFU/spot												
J62 TEM-3	>128	0.06	0.06	0.06	>128	0.125	0.125	0.125	32	0.25	0.5	0.25
J53 TEM-6	32	0.125	0.125	0.06	32	0.06	0.125	0.125	>128	1	1	1
J53 TEM-10	64	0.125	0.125	0.06	32	0.125	0.125	0.125	>128	0.5	2	1
J53 SHV-2	>128	0.06	8	16	>128	0.06	8	16	16	0.5	8	16
J53 SHV-4	>128	0.03	16	16	>128	0.03	16	32	>128	0.5	>128	>128
J53 SHV-5	>128	0.125	32	64	>128	0.125	32	64	>128	1	>128	>128
DH5α CTX-M-3	>128	0.06	0.06	1	>128	0.06	0.125	1	2	0.125	0.25	0.25
DH5α CTX-M-15 (TR516)	>128	0.06	0.06	64	>128	0.06	0.25	32	32	0.25	0.5	8
DH5α CTX-M-9	>128	0.015	0.03	0.25	>128	0.06	0.06	0.5	0.5	0.06	0.125	0.06
DH5α CTX-M-14	>128	0.06	0.06	8	>128	0.06	0.06	8	2	0.125	0.25	0.5
Isolate CIT AmpC	128	64	32	128	64	128	16	64	64	128	16	128
Isolate ACC AmpC	128	128	64	128	64	64	64	64	>128	>128	>128	>128
Isolate CIT AmpC	64	64	32	64	32	32	32	16	>128	128	32	128
Isolate CIT AmpC	128	128	128	128	128	128	128	128	>128	128	>128	>128
DH5α	0.015	0.015	0.03	0.015	0.06	0.03	0.06	0.06	0.06	0.125	0.25	0.06
J53	0.03	0.03	0.06	0.03	0.125	0.06	0.125	0.06	0.25	0.25	0.25	0.25
J62	0.125	0.06	0.125	0.06	0.125	0.06	0.125	0.125	0.5	0.25	0.5	0.25

CFU, colony-forming units; CLA, clavulanic acid; TAZ, tazobactam; SUL, sulbactam.