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Characterization of sulphonamide resistance genes and class 1 integron gene cassettes in Enterobacteriaceae, Central African Republic (CAR)

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***Objectives*: The aim of this study was to characterize genes encoding sulphonamide resistance and gene cassettes associated with class 1 integrons in trimethoprim-sulphamethoxazole resistant Enterobacteriaceae recovered from Bangui, Central African Republic (CAR).**

***Methods*: We studied 78 clinical Enterobacteriaceae isolates, including 16 extended-spectrum β-lactamases producers, 10 *Salmonella* and 9 *Shigella*, resistant to trimethoprim-sulphamethoxazole as assessed by the disc diffusion method. PCR was used to test for *sul1* and *sul2* genes. Class 1 integron resistance gene cassettes were characterized by directly sequencing PCR products obtained with primers recognising 5′ and 3′ conserved regions.**

***Results*: The *sul1* gene was found in 67 isolates, the *sul2* gene in 72 isolates and both genes in 62 isolates, while the *int1* gene was found in 74 isolates. The most prevalent *dfr* genes were *dfrA7* (49%), *dfrA1* (17%) and *dfrA2d* (13%).**

***Conclusion*: These results illustrate the wide distribution of sulphonamide and trimethoprim resistance genes among Enterobacteriaceae in Bangui (CAR).**

Keywords: co-trimoxazole resistance, gene cassettes, antimicrobial agents

Introduction

Sulphonamides and trimethoprim are inexpensive antibiotics that have a synergistic effect.[1] Consequently, they have been used in combination (co-trimoxazole) since 1968 for a wide range of clinical indications including uncomplicated urinary tract infections, enteric bacterial diseases and respiratory tract infections.[1] Plasmid-mediated resistance to sulphonamides and trimethoprim is normally due to the acquisition of novel target enzymes that are naturally resistant: dihydropteroate synthases for sulphonamides and dihydrofolate reductases for trimethoprim.[2] Three resistance genes, *sul1*, *sul2* and *sul3* encoding dihydropteroate synthases and more than 20 dihydrofolate reductase (*dfr*) genes have been described. Both groups of genes are associated with class 1 integrons residing in plasmids and/or the bacterial chromosome.[2–4]

Our knowledge of resistance to sulphonamides and trimethoprim in developing countries is not extensive. Some reports indicate that the prevalence of resistance in enterobacterial pathogens isolated in these countries is very high (33–96%) compared to isolates from developed countries (7–24%).[3,5–7]

Recent studies in the Central African Republic (CAR) showed that more than 76% of Enterobacteriaceae recovered from urinary tract infections in 2000–2002 and from bloodstream infections in 1999 were resistant to co-trimoxazole.[8,9]

Here, we report the molecular characterization of sulphonamide resistance genes and gene cassettes associated with class I integrons in various Enterobacteriaceae including extended spectrum β-lactamase (ESBL) producers and enteric pathogens such as *Salmonella* and *Shigella* recovered at the Pasteur Institute, Bangui (CAR).

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

Sixty-one ESBL-negative-Enterobacteriaceae isolated between October 2004 and June 2005 and 17 ESBL-producing

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Enterobacteriaceae isolated between January 2003 and October 2005, were recovered at Pasteur Institute in Bangui (CAR) from clinical samples sent by four public health centres, one paediatric hospital and ambulatory medical visits; all these centres are located in Bangui, the capital of Central African Republic. The isolates (one isolate/patient) were associated with urinary tract infections ($n = 47$), pneumonia in an AIDS patient ($n = 1$), wound infections ($n = 5$), vaginal colonizations ($n = 5$), ear infection ($n = 1$), meningitis infection ($n = 1$), bacteraemia ($n = 2$) and diarrhoeal diseases ($n = 16$). These pathogen isolates included several bacterial species: *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Shigella spp.* and *Salmonella spp* (Table 1).

All isolates were tested for their susceptibility to antimicrobial agents including trimethoprim-sulphamethoxazole (1.25/23.75 μg) using a standard disc diffusion method on Mueller–Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM at www.sfm.asso.fr). The isolates were scored as resistant and were selected if their inhibition zone diameter for co-trimoxazole was <10 mm. *E. coli* ATCC 25922 was used as control.

During this period, 73% of the ESBL-negative Enterobacteriaceae recovered from urinary tract infections were resistant to co-trimoxazole.

The ESBL-negative isolates included: *E. coli*, *K. pneumoniae*, *Enterobacter cloacae* and *Enterobacter aerogenes* (Table 1). The ESBLs were previously characterized (except one) as CTX-M-3, CTX-M-15, SHV-12 and SHV-2a.[10]

PCR screening of sul genes, class 1 integrase gene and characterization of class 1 integron resistance gene cassettes

All the PCR assays were carried out in a total volume of 50 μL mixture containing the following reagents: DNA (100 ng), Primers (1 μM), dNTP (200 μM), Tris-HCl (10 mM; pH 8.3), KCl (50 mM), $MgCl_2$ (1.5 mM) and 1 U of *Taq* DNA polymerase.

The strains were screened for *sul1* and *sul2* by a multiplex PCR using specific oligonucleotide primers (Table 2) as previously described.[11] PCR using specific primers (Table 2) used to detect the *sul3* gene involved an annealing temperature at 53°C. Three *E. coli* clinical isolates (strains 02-57295, 02-58161 and 03-709) obtained in December 2002 and January 2003, in Tenon Hospital (France) and harbouring the *intI1* gene and the *sul1* gene, the *sul2* gene and the *sul3* gene, respectively were used as positive controls (A. Doloy, G. Arlet, personal data). *E. coli* DH10B was used as negative control for PCR assays.

The integrase gene (*int1*) was detected by PCR using specific primers that amplified an amplicon of 899 bp (Table 2). The PCR conditions were as follows: initial denaturation (94°C for 5 min) followed by 35 cycles (94°C for 30 s, 60°C for 40 s and 72°C for 1 min). A final extension was performed at 72°C for 7 min.

Table 1. Characterization of sulphonamide resistance markers and antibiotic resistance gene cassettes inserted in class1 integron detected in Enterobacteriaceae isolated in CAR

Clinical isolates[a]	Isolates number[b]	Sulphonamide resistance markers			Integrase gene (*int1*)	Antibiotic resistance gene cassettes						
		sul1	*sul2*	both		*dfrA2d*	*dfrA5*	*dfrA7*	*dfrA1-aadA1*	*dfrA5-ere(A)*	*dfrA12-ORF-aadA8*	*dfrA17-aadA5*
E. coli (ESBLs)	9	8	7	6	8	4	1	2	0	0	0	2
E. coli (no ESBLs)	34	29	33	28	32	1	1	18	6	2	2	3
K. pneumoniae (ESBLs)	5	5	4	4	5	2	0	1	2	0	0	0
K. pneumoniae (no ESBLs)	2	2	2	2	2	0	0	2	0	0	0	0
C. freundii	6	6	6	6	6	0	0	1	1	0	4	0
E. cloacae (ESBLs)	2	1	2	1	2	0	0	2	0	0	0	0
E. aerogenes (ESBL)	1	1	1	1	1	1	0	0	0	0	0	0
Shigella spp.	9	7	9	7	8	2	0	4	2	0	0	0
Salmonella sp.	10	9	8	7	10	0	0	8	2	0	0	0
Total	78	68	72	62	74	10	2	38	13	2	6	5

[a]The enteric pathogen isolates included several species of *Shigella* (*S. dysenteriae A2*, *S. flexneri 1b*, *S. flexneri* and *S. boydii*) and *Salmonella* (*S.* Enteritidis, *S.* Stanleyville and *S.* Typhimurium).
[b]All isolates were not found clonally related by rep-PCR or ERIC-PCR.
[c]All isolates carried one *dfr* gene (except one *Shigella* sp).

Table 2. Sequences of the primers used to amplify the sulphonamide resistance genes, class 1 integrase gene and resistance gene cassettes

Primer name	Primer sequence	Primer position	PCR target	Reference
Sul1 upper	CGGCGTGGGCTACCTGAACG	1709–1728 (AY655485.1)	*sul1* gene	11
Sul1 lower	GCCGATCGCGTGAAGTTCCG	2122–2141 (AY655485.1)	*sul1* gene	11
Sul2 upper	GCGCTCAAGGCAGATGGCATT	244–264 (AY360321.1)	*sul2* gene	11
Sul2 lower	GCGTTTGATACCGGCACCCGT	508–528 (AY360321.1)	*sul2* gene	11
Sul3 forward	GGAAGAAATCAAAAGACTCAA	3155–3175 (AJ459418.2)	*sul3 gene*	this study
Sul3 reverse	CCTAAAAAGAAGCCCATACC	3517–3498 (AJ459418.2)	*sul3 gene*	this study
Int1 upper	ATGGCCGAGCAGATCCTGCACG	105–126 (DQ393784.1)	integrase gene	this study
Int1 lower	GCCACTGCGCCGTTACCACCGC	983–1004 (DQ393784.1)	integrase gene	this study
5'CS (*intI1*)	AAACGGATGAAGGCACGAAC	24–43 (AY152821.1)	variable region of class 1 integron	this study
3'CS (*qacEΔ1*)	ATTGCGATAACAAGAAAAAGCC	2160–2181 (AY152821.1)	variable region of class 1 integron	this study
dfrA1 upper	AGCATTACCCAACCGAAAGT	273–292 (DQ875875.1)	gene cassette *dfrA1-aadA1*	this study
aadA1 lower	TGTCAGCAAGATAGCCAGAT	1072–1091 (DQ875875.1)	gene cassette *dfrA1-aadA1*	this study
dfrA2d upper	CGGTTCGCATTCCCATCAA	168–186 (AY973253.1)	*dfrA 2d* gene	this study
dfrA2d lower	GGACTGAGCCTGGGTGAGA	324–306 (AY973253.1)	*dfrA 2d* gene	this study
dfrA7 upper	AAATGGCGTAATCGGTAATG	151–170 (AJ884724.1)	*dfrA7* gene	this study
dfrA7 lower	GTGAACAGTAGACAAATGAAT	475–455 (AJ884724.1)	*dfrA7* gene	this study
dfrA12 upper	CAACGCTGTCGCACGCTATC	557–576 (Z21672.1)	gene cassette *dfrA12*-orfF-*aadA8*	this study
aadA8 lower	CTACCAAGGCAACGCTATGT	430–449 (DQ149925)	gene cassette *dfrA12*-orfF-*aadA8*	this study
dfrA5 upper	TGGCGGGGAGATTTACAGA	410–428 (AB188269.1)	gene cassettes *dfrA5-ereA2*	this study
ere(A) lower	TTTCAGGACAGGGGCAAGC	1361–1343 (AB188269.1)	gene cassettes *dfrA5-ereA2*	this study

The variable region of class 1 integrons was amplified by PCR using primers (Table 2) specific for the 5′ conserved segment (5′CS) and 3′ conserved segment (3′CS). Thermal cycler conditions were 94°C for 5 min followed by 35 cycles (94°C for 30 s, 60°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 7 min.

PCR products were subjected to DNA sequencing using PCR primers with an ABI PRISM 3100 Genetic Analyser sequencing (Applied Biosystems). Additional sequencing primers (Table 2) were designed using Oligo4 software and used for DNA sequencing.

Sequences obtained were analysed by comparison with the sequences in databases by BLASTN (www.ncbi.nlm.nih.gov) and Clustal W (http://www.ebi.ac.uk/).

Results and discussion

The PCR analysis indicated that 72 isolates (92%) carried the *sul1* gene, 67 (86%), the *sul2* gene and 62 (80%) carried both *sul1* and *sul2* genes while *sul3* was not found (Table 1). The frequencies of *sul1* and *sul2* genes found among our isolates are in conformity with the data previously reported.[3,11] However, both *sul1* and *sul2* gene frequencies were higher in the current isolates than has been previously published from European countries.[11–14]

Seventy-four isolates (95%) were positive for the class 1 integrase gene while nine isolates (11%) were negative for *sul1* but positive for *sul2* as has previously described in Portuguese *Salmonella* and London *E. coli* strains.[12,14] Three other *E. coli* isolates were *sul1* positive and *int1* negative. Previously, one plasmid has been described carrying a class 1 integron and a truncated *int1* gene which could have given similar results.[15]

Six *dfr* genes encoding dihydrofolate reductase were identified in 76 isolates (97%) with *dfrA7* frequencies being the most common (38 isolates; 48%) (Table 1). This correlates with a previous study of Senegalese isolates.[16] The *dfrA1* gene was the second most common, in 13 strains (17% of isolates) (Table 1) and has previously been associated with blood culture isolates in European hospitals.[17] *dfrA2d* was the third most common gene, in 10 strains (13% of isolates) (Table 1) and in the CAR isolates is associated with ESBLs. Two *E. coli* isolates carried *dfrA5* gene.

We detected three linkages between streptomycin resistance and *dfr* genes and one linkage with erythromycin esterase [*ere*(A)] and *dfr* genes (Table 1). All four of these linkages have previously been described.[18–22]

In CAR, the combinations of trimethoprim-sulfamethoxazole and pyrimethamine-sulfadoxine (Fansidar®) has been extensively used for bacterial treatment and antimalarial prophylaxis, respectively.[8,23] Thus, it is not surprising that 73% of urinary pathogen Enterobacteriaceae were resistant to co-trimoxazole and that 90% of the isolates examined carried *sul* and *int1* genes and >95% carried *dfr* genes, similar results have been found in other studies of African isolates but this carriage rate is higher than reported in Europe or America.[16, 18–22,24]

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Transparency declarations

None to declare.

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