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Class 1 integrase and genetic cassettes *bla*$_{oxa}$ and *bla*$_{tem}$ among multi-drug resistant *Shigella* isolates in Costa Rica

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Abstract
Shigella is an important enteropathogen and the major cause of dysentery and diarrheal disease around the world. There are no studies related to the molecular detection of integrons among *Shigella spp.* isolates in Costa Rica. Here we are reporting for the first time the presence of class 1 integrase (*intI-1*) and extended spectrum β-lactamases genetic cassettes, *bla*$_{OXA}$, and *bla*$_{TEM}$ among multidrug resistant *Shigella* isolates in Costa Rica. The antibiotic resistance pattern and presence of integrase class 1(*intI-1*) and genetic cassettes *bla*$_{OXA}$ and *bla*$_{TEM}$ were analyzed in 30 *Shigella* isolates (*S. flexneri* (83%) and *S. sonnei* (17%). Bacteria were collected at hospitals and cliniques in Costa Rica and exclusively from stools of individuals with diarrhea. from Costa Rica. Twenty nine out of 30 isolates were resistant to at least, one antibiotic. Multidrug resistance was observed in 26 out of 30 isolates (87%), and found to carry integrase class 1 (93%), and genetic cassettes *bla*$_{OXA}$ (76%) and *bla*$_{TEM}$ (7%).

1. Introduction

Shigella spp is a virulent bacterium and a major cause of dysentery throughout the world. This pathogen is associated with 5% to 15% of cases of diarrhea and 30% to 50% of cases of dysentery, particularly in developing countries (1).

In Costa Rica, *Shigella spp.* has been isolated from fecal, water and food samples (2-6). It is considered an endemic pathogen in Central American countries, causing severe diarrheal diseases that require hospitalization in young children (2).

The emergence of antibiotic resistance among the bacterial enteropathogens, such as *Shigella,* is considered a global problem that requires urgent actions (1).

Antimicrobial resistance is not a new phenomenon and it is considered as a major threat to effective treatment outcome (1,7).

Multidrug resistance is associated with mobile genetic elements including transmissible plasmids, transposons and insertion sequences (i.e. integrons). The importance of integrons in the acquisition of resistance genes, and therefore to emergence of multidrug resistant strains, was only recognized late the 1980's

decade (7,8). Mobile genetic elements may facilitate the dissemination of multidrug resistance genes among species and even genera (8,9).

Integrons are assembly plataforms that incorporate genetic determinants or cassettes by site-specific recombination and convert them to functional genes (8,10).

They are composed of three key elements: a tyrosine-recombinase gene or integrase (*intI*), a recombination site (*attI*) and a promoter site (P_c) (7,9, 10). All integrons analyzed today are composed by an *intI* gene encoding an integrase. This enzyme belongs to the tyrosine-recombinase family, which can recombine discrete unites of circularized DNA known as genetic cassettes (8,10). The other integron component is the primary recombination site (*attI*). A wide variety of genetic cassetes that confers resistance to antibiotics, are found to insert this region (8). And the third important component is a strong promoter (Pc, P_{ant}) that directs transcription of the captured genes or genetic cassettes (11).

Recent studies have detected class 1 and clasee 2 integrons among multidrug-resistant *Shigella spp.* (12-14). However, there are no studies related to the molecular detection of integrons among *Shigella spp.* isolates in Costa Rica.

In this study, we report for the first time the presence of class 1 integrase and the extended spectrum β-lactamase genetic cassette, bla_{OXA}, and bla_{TEM} among multidrug resistant isolates in Costa Rica.

2. Methodology

Bacterial strains:

Thirty isolates were obtained from INISA bacterial collection. Bacteria were collected at hospitals and cliniques in Costa Rica and exclusively from stools of individuals with diarrhea. All of the strains were *S. flexneri* (83%) and *S. sonnei* (17%).

Antibiotic susceptibility testing:

Antibiotic susceptibility testing was determined by disc diffusion assay, according to Clinical and Laboratory Standards Institute 2012 Guide (CLSI).

The isolates were tested for susceptibility to: amoxicillin (AML), ampicillin (AMP), ceftazidime (CAZ), cephalotin (KF), chloramphenicol (C), co-trimoxazole (SXT), gentamicin (CN), nalidixic acid (NA), tetracycline (TE) and norfloxacin (NOR).

DNA extraction and PCR analysis:

Preparation of the bacterial DNA samples and PCR conditions have been described previously (Barrantes et al. 2010).

Class 1 integrase (*intI1*), bla_{OXA} and bla_{TEM} genetic cassettes were detected by PCR. The sequences of *intI1* 899 bp product primer pair (fw 5'-ATGGCCGAGCAGATCCTGCACG -3' and rv 5'-GCCACTGCGCCGTTACCACCGC -3') (12), bla_{OXA} 820 bp product primer pair (fw 5'-ATGAAAAACACAATACATATCAACTTCGC-3' and rv 5'-GG GTGTGTTTAGAATGGTGATCGCATT -3'), bla_{TEM} 859 bp product primer pair (fw 5'-ATGAGTATTCAACATTTCCG- 3' and rv 5'-ACCAATGCTTAATCAGTGAG- 3') (14), and amplification control 16S 585 bp product primer pair (fw 5'- GGGAGTAAAGTTAATACCTTTGCTC-3' and rv 5`-TTCCCGAAGGCACATTCT-3`) were prepared by Invitrogen (USA).

The PCR products were detected by 2% agarose gel electrophoresis (140V, at 45 min) in 1X TBE Buffer at pH 8.3 and stained with GelRed (Biotium). Gen Ruler 50 bp DNA ladder (Thermo Scientific) was used as DNA size marker. Strains E705 and EIEC were used as *intI-1* and bla_{TEM} positive controls, respectively.

The products were visualized as 899 bp (*intI-1*), 820 bp (bla_{OXA}), 859 (bla_{TEM}) and 585 bp (*16S*) bands using an UVITEC transiluminator (Cambridge, UK).

DNA sequences of these products were determined (310 ABI Genetic Analyzer, Applied Biosystems) to confirm amplification of the correct genes.

Statistical analysis:

Statistical calculations were performed with SPSS software v. 17.0 (SPSS, Chicago, IL. USA). Student T-Test was used for statistical comparisons.

3. Results and Conclusions

Twenty nine out of 30 isolates were resistant to at least, one antibiotic. Multidrug resistance was observed in 26 out of 30 isolates (87%) (Table 1).

Most of the isolates were resistant to β-lactamic antibiotics, amoxicillin (24/30, 80%) and ampicillin (23/30, 77%), followed by tetracycline (22/30, 73%), cotrimoxazole (19/30, 63%), chloramphenicol (13/30, 43%), cephalotin (9/30, 30%) and gentamicin (2/30, 7%). No resistant phenotypes to ceftazidime, nalidixic acid and norfloxacin were observed.

It is well recognized that *Shigella* have progressively become resistant to most of the widely used antibiotics, resulting in treatment failure and increased mortality (7).

Ampicillin and trimethoprim–sulfamethoxazole have been recommended for treating shigellosis, but according to this study, the observed resistance profile of almost all *Shigella* isolates confirmed the recommendation that these antibiotics are considered ineffective for empirical therapy (7, 15).

Multidrug resistance dissemination in Gram negative pathogens by mobile elements such are integrons and gene cassettes is a well known and frequently reported phenomenon (8,11-15). Those resistance determinants are frequently carry within R plasmids, transposons and genomic islands, which facilitate the dissemination of resistance genes among bacterias (7). Horizontal transfer of these resistance determinants is possible between species in *Shigella* and also in other enteropathogens (10, 11).

In this study, Class I integrase (*intI-1*) was detected in 28 (93%) isolates and extended spectrum β-lactamases, bla_{OXA}, in 23 (76%) and bla_{TEM} in one isolate (7%). (Figure 1).

Presence of *intI-1* correlates with the zone inhibition diameter in amoxicillin and ampicillin (Table 2). *Shigella* isolates which were positive for *intI-1*, showed shorter diameters comparing to *intI-1* negative isolates ($p<0.05$). This situation was also observed for bla_{OXA} positive and negative isolates and the antibiotics chloramphenicol, co-trimaxole and tetracycline ($p<0.05$).

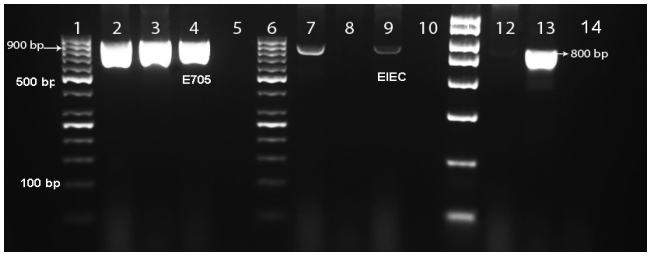

Figure 1. *PCR results of intI-1, bla_{OXA} and bla_{TEM} detection in Shigella isolates: Lane 1: molecular marker; lane 2: INISA-02; lane 3: INISA-07; lane 4: E705(intI-1 positive control); lane 5:negative control; PCR bla_{TEM} lane 6: molecular marker; lane 7: INISA-02; lane 8: INISA-07; lane 9: EIEC(bla_{TEM} positive control); lane 10: negative control; PCR bla_{OXA} lane 11: molecular marker; lane 12: INISA-02; lane 13: INISA-07; lane 14: negative control.*

On the other hand, *intI-1* and genetic cassettes bla_{OXA} and bla_{TEM} were not detected in one *Shigella* isolate susceptible to antibiotics.

In *S. flexneri and S. dysenteriae*, resistance to some antibiotics, such as ampicillin, streptomycin and/or trimethoprim is mostly associated with the presence of class 1 integrons, meanwhile, class 2 integrons are detected more frequently in *S.sonnei* and *S. boydii* (8,11).

Table 1. *Antibiotic resistant phenotype and number of resistant isolates of Shigella spp.*

Phenotype	Number of isolates
SXT	2
TE	1
SXT-TE	2
AML-KF	1
AML-AMP-TE	1
AML-AMP-KF	1
AML-AMP-SXT	1
AML-AMP-KF-SXT	2
AML-AMP-SXT-TE	1
AML-AMP-C-TE	6
AML-AMP-KF-SXT-TE	3
AML-AMP-C-SXT-TE	6
AML-AMP-KF-SXT-CN-TE	1
AML-AMP-C-KF-SXT-CN-TE	1
Total	29

AML=amoxiciliin; AMP=ampicillin; C=chloramphenicol; CN=gentamicin; KF=cephalotin; SXT=co-trimaxole; TE=tetracycline

In Latin America, classes 1 and 2 integrons has been detected more frequently in *S. flexneri,* resistant to trimethoprim, sulfamethoxazole, streptomycin, ampicillin, chloramphenicol and tetracyclin (8, 13, 14). Class 2 integrons were mainly tested in *S.sonnei* strains in earlier reports. This observation is consistent with results of class 1 and class 2 integrases detection mainly in *S. flexneri* isolates in this study (data not shown).

Table 2. *Antibiotic zone diameter (ZD)means (disk diffusion method) and presence of intI-1 and bla_{OXA} in Shigella spp isolates.*

	intI-1		***bla_{OXA}***	
Antibiotic*	**Positive isolates ZD mean (SD)**	**Negative isolates ZD mean (SD)**	**Positive isolates ZD mean (SD)**	**Negative isolates ZD mean (SD)**
AML	6.4(9.7)**	23.5(2.1)**	6.0(10.2)	12.4(9.7)
AMP	6.6(8.5)**	22.0(0.0)**	6.5(9.2)	11.3(8.5)
CAZ	29.7(3.0)	34.0(5.6)	29.6(2.9)	31.6(4.1)
KF	19.0(3.7)	21.0(2.1)	19.5(3.2)	18.7(4.9)
C	19.0(13.1)	37.0(7.0)	16.2(12.7)**	33.6(5.1)**
CN	20.0(3.2)	21.0(6.3)	20.4(3.2)	22.6(3.7)
SXT	10.1(14.1)	31.0(11.3)	7.8(12.2)**	23.6(16.8)**
TE	6.7(11.0)	13.0(18.3)	4.2(9.6)**	16.7(11.6)**
NA	26.6(2.3)	32.0(9.9)	26.3(2.3)	29.0(4.9)
NOR	32.3(4.4)	36.0(8.5)	31.9(3.9)	34.9(6.3)

* AML= amoxicillin; AMP=ampicillin; CAZ=ceftazidime; KF=cephalotin; C=chloramphenicol; CN= gentamicin; SXT= cotrimaxole; TE= tetraciclycline; NA= nalidixic acid; NOR= norfloxacin.
** $p < 0.05$

Gene cassettes that encoded resistance to β-lactamic antibiotics are more related to integrons class 1, and they are detected in the bacterial chromosome and plasmids (8). The bla_{OXA} gene has often been identified in ampicillin resistant enterobacterial strains such as isolates of *Escherichia coli, Shigella flexneri*, and *Salmonella* spp. This gene encoded for a class D β-lactamase, is also known as oxacillinase or OXA type β-lactamase (11).

Antimicrobial susceptibility of *Shigella* strains is related to general use of antimicrobials in population. This study provided evidence that in Costa Rica, β-lactams antibiotics such as ampicillin, amoxicillin and cephalotin are not effective against shigellosis and it seems that this is the situation worldwide (16). Also, tetracycline, cotrimaxole (trimetoprim-sulphametoxazole), chloramphenicol and gentamicin showed important resistance rates among *Shigella* isolates. Nevertheless, third generation cephalosporin (i.e. ceftazidime) and quinolons, such as nalidixic acid and norfloxacin, could be considered still helpful tools in the management of shigellosis, at least, in Costa Rica.

We are reporting for the first time the presence of class 1 integrase and extended spectrum β-lactamase genetic cassettes, bla_{OXA} and bla_{TEM} among multidrug resistant *Shigella spp.* isolates in Costa Rica. The importance of integrons in the acquisition of resistance genes, and therefore the emergence of multidrug resistant strains, is a major public health problem. Information about epidemiology and molecular mechanisms of multidrug resistance determinants in *Shigella spp.* and other enteropathogens is important to develop intervention strategies. Regional and local antimicrobial resistance

pattern in *Shigella* should be considered as a part of control strategies. (7)

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