

SHORT REPORT

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Genetic characterization of antimicrobial resistance of *Shigella flexneri* 1c isolates from patients in Egypt and Pakistan

Salwa F Ahmed^{1*}, John Klena¹, Tupur Husain¹, Jesse Monestersky¹, Amel Naguib² and Momtaz O Wasfy¹

Abstract

Background: *Shigella flexneri* serotype 1c emerged as a critical isolate from children in Egypt and Pakistan. The pattern of antimicrobial susceptibility (AMS) and resistance genes of this serotype have yet to be characterized.

Findings: Sixty nine *S. flexneri* 1c isolates were identified from both Egypt (n=46) and Pakistan (n = 23) and tested for AMS by disk diffusion method and minimal inhibitory concentrations were also determined. Isolates were genotyped by pulsed field gel electrophoresis (PFGE) and five relevant resistance genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, *sulI* and *sulII*) were detected by polymerase chain reaction (PCR) and confirmed by DNA sequencing. High resistance was observed in all isolates for ampicillin (AM >96%); trimethoprim-sulphamethoxazole and tetracycline (>88%). Most AM-resistant isolates from Egypt (70%) harbored *bla*_{TEM} resistance, while 52% of isolates from Pakistan expressed *bla*_{OXA}. All isolates were closely related by PFGE, irrespective of source or time of collection. The *sulII* gene was present in 100% of isolates from pediatric cases in Egypt, 65% of Pakistan isolates, and 53% of isolates from older Egyptian patients.

Conclusions: While different *Shigella* serotypes gathered in specific genotypic groups, 1c serotype isolates formed multiple clusters. Although AMS was considerably high to most commonly used drugs, genetic determinants were variable between countries over time. The data stress the need for a more careful selection of antibiotics in the treatment of shigellosis.

Keywords: *S. flexneri* 1c, Genetic diversity, Antibiotic resistance, Oxa1, PFGE, Egypt, Diarrhea, Pakistan

Findings

While *Shigella sonnei* is the main cause in industrialized countries, *S. flexneri* is predominant in developing countries (particularly serotype 2a) [1-4]. In a recent study, *S. flexneri* serotype 1c emerged as an important isolate (17%) in three pediatric populations in Egypt [5]. The isolates were highly resistant to AM, tetracycline and sulfatrimethoxazole, with some showing simultaneous resistance to two or more antibiotics (multi-drug resistance phenotypes, MDR). Outside Egypt, this serotype has been described in Southeast Asia and the Indian subcontinent [5-8]. Also, many untypeable *S. flexneri* isolates from Pakistan belonged to serotype 1c [9]. The purpose of the present study was to characterize this

serotype in a collection of isolates from Egypt and Pakistan for phenotypic and molecular markers of resistance to β -lactams and sulfonamides.

A total of 46 *S. flexneri* serotype 1c isolates from Egypt were used; 10 from pediatric patients (EP, <5 years) examined between 1990 and 1994, 17 between 2000 and 2004 and 19 between 2001 and 2003. The last group came from non-pediatric patients (NEP; 9–19 years). Nineteen isolates were also obtained between 2002 and 2004 from Pakistani pediatric (PP) clinics located in the urban slums of Karachi [9,10]. All isolates were cultured from rectal swabs and/or stool samples and identified at NAMRU-3 using standard microbiological and biochemical procedures [11]. Informed consents were obtained from all involved adults and guardians of minors. The study was approved by the Institutional Review Board (IRB Protocol No 96) of NAMRU-3, in compliance with Helsinki Declaration and US Federal regulations gover-

* Correspondence: salwa.fouad.eg@med.navy.mil

¹Research Science Directorate, United States Naval Medical Research Unit No. 3, Cairo, Egypt

Full list of author information is available at the end of the article

Table 1 Antibiotic resistance profile of *S. flexneri* 1c isolates from Egypt and Pakistan using both disk diffusion and E-test methods

Antibiotics	AM ¹			C			TE			SXT			CET			CIP			CRO			FEB			CAZ			CTX		
	S ²	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
CLSI MIC ³ breakpoints µg/mL	≤8	16	≥32	≤ 8	16	≥ 32	≤4	8	≥16	≤2/ 38		≥4/ 76	≤8	16	≥32	≤1	2	≥4	≤1	2	≥4	≤8	16	≥32	≤4	8	≥16	≤1	2	≥4
EP ⁴ isolates (n = 10) (1990–1994)	0	0	10 (100) ⁷	0	0	10 (100)	0	0	10 (100)	6 (60)	2 (20)	2 (20)	0	2 (20)	8 (80)	10 (100)	0	0	ND ⁸			ND			ND			ND		
EP isolates (n = 17) (2000–2004)	1 (6)	0	16 (94)	16 (94)	0	1 (6)	2 (12)	0	15 (88)	2 (12)	0	15 (88)	1 (6)	14 (82)	2 (12)	17 (100)	0	0	17 (100)	0	0	17 (100)	0	0	17 (100)	0	0	17 (100)	0	0
NEP ⁵ isolates (n = 19) (2001–2003)	0	0	19 (100)	16 (84)	0	3 (16)	0	0	19 (100)	2 (11)	0	17 (89)	1 (5)	12 (63)	6 (32)	19 (100)	0	0	19 (100)	0	0	19 (100)	0	0	19 (100)	0	0	19 (100)	0	0
PP ⁶ isolates (n = 23) (2002–2003)	1 (4)		22 (96)	4 (17)		19 (83)	2 (9)		21 (91)	1 (4)		22 (96)	6 (26)	17 (74)	0	10 (100)	0	0	23 (100)	0	0	23 (100)	0	0	23 (100)	0	0	23 (100)	0	0

¹ AM Ampicillin, C Chloramphenicol, TE Tetracycline, SXT Trimethoprim sulfamethoxazole, CET Cephalothin, CIP Ciprofloxacin, CRO Ceftriaxone, FEB Cefepime, CAZ Ceftazidime, CTX Cefotaxime.

² S, I and R Susceptible, Intermediate and Resistant.

³ MIC Minimum inhibitory concentrations.

⁴ EP Egyptian pediatric isolates (<5 years).

⁵ NEP Non-pediatric Egyptian isolates (9–19 years).

⁶ PP Pakistan pediatric isolates (<5 years).

⁷ () % of isolates.

⁸ ND Not done.

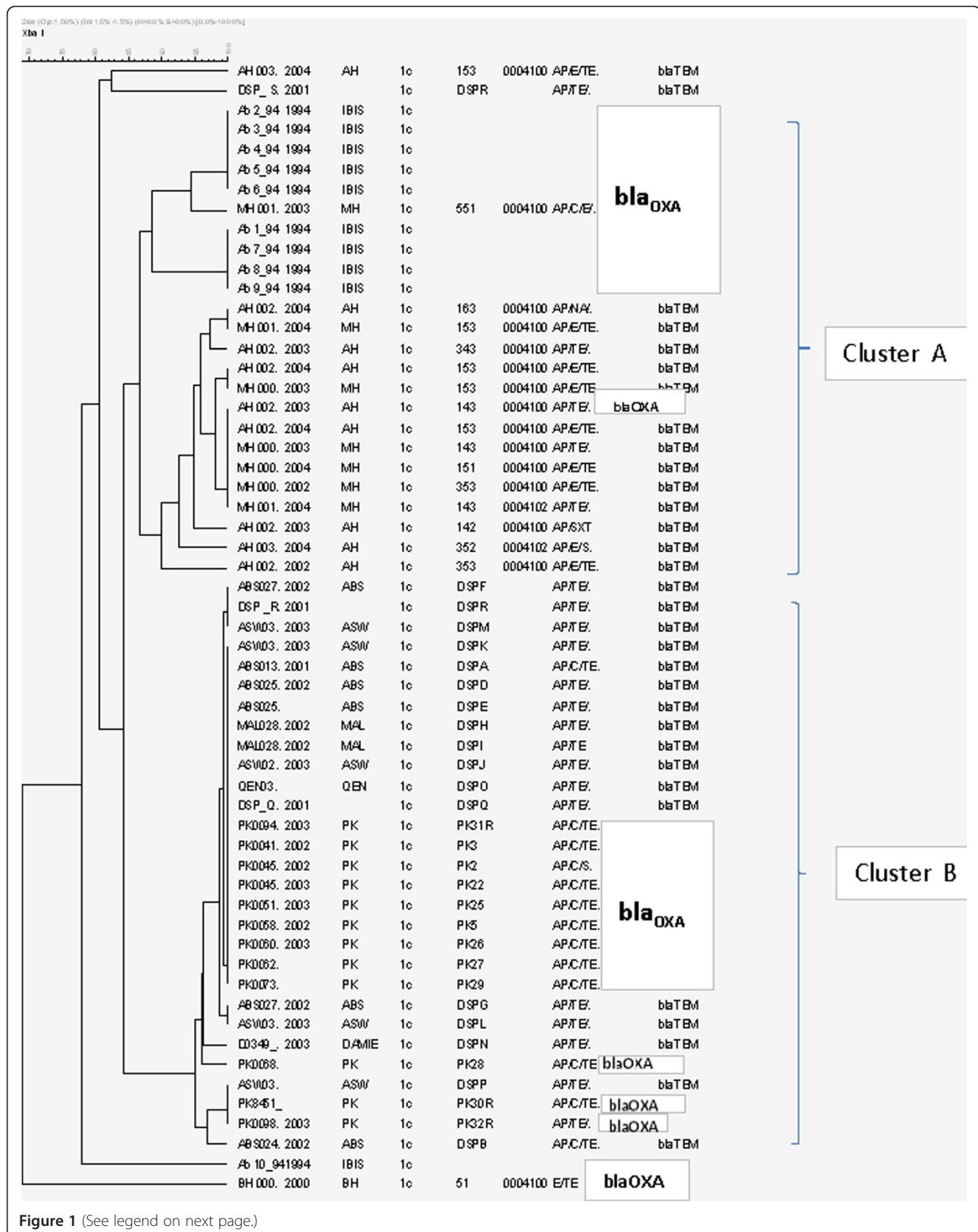


Figure 1 (See legend on next page.)

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Figure 1 The source of the isolate is provided by a two letter code followed by a unique identifying number; an underscore separates the year of isolation from the identifying number. Source abbreviations: AH, Abu Homos, BH, Benha; MH, Mokattum Hills. PK, Pakistan; QEN, ASW, MAL, ABS, P, D, Q,R, S, different regions from upper Egypt and Cairo. Antibiotic resistance profile abbreviations are as follows: AM, ampicillin resistance; C, chloramphenicol resistance; E, erythromycin resistance; TE, Tetracycline resistance; SXT, trimethoprim-sulfamethoxazole resistance. Clonal distance is shown after whole genomic digestion using the restriction enzyme *xbaI*. All samples were normalized against the pattern of *Salmonella enterica* ss. *Enterica* serotype Braenderup strain H9812. Band pattern analysis was performed using Bionumerics vs 4.5 and dendrogram was calculated using Dice similarity coefficients, based on the unweighted pair group method with arithmetic averages (UPGMA).

ning the protection of human subjects. Species serotypes were determined using commercially available serotyping kits [5], whereas the identity of serotype 1c was confirmed using monoclonal antibodies specific for *S. flexneri* 1c (MASF) (obtained through the courtesy of Dr. Carlin, 1989). Antimicrobial susceptibility testing (AMS) was performed against 9 types of antibiotics ampicillin (AM, 10 ug), tetracycline (TE, 30 ug), trimethoprim-sulphamethoxazole (SXT, 25 ug), chloramphenicol (C, 30 ug), ciprofloxacin (CIP, 5 ug), ceftriaxone (CRO, 30 ug), cefepime (FEB, 30 ug), ceftazidime (CAZ, 30 ug) and cefotaxime (CTX, 30 ug) by the disk diffusion and E-strips methods as previously described [12]. MDR isolates were defined as those showing simultaneous resistance to at least three antibiotics [5]. Detection of β -lactam genes was performed by amplifying specific regions of the *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA} genes and the mechanism of resistance to sulfa compounds was determined by amplifying the genes *sulI* and *sulII* [13]. Testing for class 1 and 2 integrons was also conducted [13,14]. Primers were commercially purchased from Sigma-Genosys (St Louis, MO, USA). PCR amplicons were sequenced and confirmed as shown previously [13]. Genomic DNA was prepared to conduct Pulsed Field Gel Electrophoresis (PFGE) [15] using *XbaI* *mrp* and the obtained phylogenetic tree was analyzed at a similarity level of 95% [5].

Availability of supporting data

High levels of drug resistance were found among and within patient groups in both Egypt and Pakistan (Table 1). The majority of isolates ($\geq 88\%$) were resistant to AM and TE, with expected minimal inhibitory concentrations > 16 ug/ml [3]. Also, 83% of PP isolates were resistant to C (≥ 32 ug/ml), while recent EP and NEP isolates were susceptible. Resistance to SXT was only 20% in EP 1990–1994 isolates and increased considerably to 88% in EP 2000–2004 and NEP isolates (89%) ($p > 0.05$). In PP isolates, however, resistance to this drug was 96%. MDR phenotype AM/C/SXT reached 83% in PP isolates and ranged from 11 to 20% in EP 1990–1994 and NEP isolates. Other MDR phenotypes (AM/TE/SXT) were found in 79–82% of EP 2000–2004, NEP and PP isolates. While all isolates from both countries were suscep-

tible to CRO, FEB, CAZ and CTX, some (average = $31 \pm 35\%$) showed resistance to cephalothin.

Of Egypt's EP and NEP AM-resistant isolates, 70% harbored *Bla*_{TEM-1} resistance gene and the remaining carried *Bla*_{OXA-1} gene. None of the PP isolates harbored the *Bla*_{TEM-1} gene while only 52% of AM-resistant isolates expressed *bla*_{OXA-1} gene. The gene *sulII* was detected in 10% of EP 1990–1994 isolates, 53% of NEP and 65% of PP isolates. However, it was found in all EP 2000–2004 isolates. The *sulI* gene was associated with *bla*_{OXA-1} gene in 48% of the isolates. Out of the *bla*_{TEM} positive isolates, 72% (23/32) were positive for class 2 integron and 6% were positive for class 1 integron. The proportions of integrons among *bla*_{OXA}-positive cases were relatively similar. EP and PP isolates that failed to amplify the *Bla*_{OXA} and *Bla*_{TEM} genes were negative for *Bla*_{CTXM}, *bla*_{CMY}, and *bla*_{SHV} [13]. *XbaI* *mrp*-PFGE grouped the majority of isolates in two major clusters; cluster A included 90% of EP isolates positive for *bla*_{OXA} and cluster B covered most NEP isolates that harbored *bla*_{TEM} gene and PP isolates with *bla*_{OXA} gene (Figure 1).

The recent emergence of *S. flexneri* 1c as a dominant serotype of this species was reported in Southeast Asia and the Indian subcontinent [6–8]. In Egypt, this serotype was identified in three different pediatric populations and their antimicrobial susceptibility patterns demonstrated a trend towards multi-drug resistance, with a dominant resistance phenotype against AM, TE, and SXT. In the present study, more *S. flexneri* serotype 1c isolates from Egypt were characterized by phenotypic and molecular methods in comparison with a group of isolates obtained from a pediatric study in Pakistan.

While the obtained data may provide a useful baseline for AMS patterns and underlying genes, further studies to evaluate more recent isolates are underway. The high levels of antimicrobial resistance to many drugs in Egypt and Pakistan is in consent with previous reports [16–18] and reflects common medical treatment practices in both countries. Accordingly, CRO has been recommended as a first-line parenteral therapy for children with shigellosis [19] and CIP was used as an alternative when CRO is contraindicated [20,21]. The increased SXT resistance in EP and PP isolates suggests a wider use of SXT treatment during the late 90s in Egypt and Pakistan [18] and exemplifies a dynamic selective drug pressure influence on AMS pat-

terns [22]. However, a trend towards intermediate resistance to cephalothin has been noticed among all *Shigella* isolates in this study, which is probably an early alert against the development of resistance to third generation cephalosporins. This observation may suggest leaving this line of drug for empirical use in serious cases of shigellosis only [10]. While EP isolates collected between 1990 and 1994 showed 20% of MDR phenotype AM/C/SXT, those collected between 2000–2004 demonstrated 82% of MDR phenotype AM/TE/SXT (Table 1), suggesting that some of the tested drugs may be of limited use as first line therapy [10]. Although little has been known about the prevalence of extended spectrum beta lactamases in *S. flexneri* serotype 1c isolates, molecular analysis of resistance genes revealed substantial differences among the studied populations with a predominance of bla_{TEM} in EP and NEP strains, possibly due to a single gene copy on the large conjugatively transferable plasmid known to endow resistance to AM and SXT [23]. However, the remaining EP isolates carried the bla_{OXA} gene, similar to approximately half of the Pakistan isolates (12/23). Previous studies have demonstrated the presence of bla_{OXA} gene in AM resistant *S. flexneri* in different geographic areas including Denmark [24] and Tanzania [25].

In developing countries, SXT is still used as a first-line antibiotic. Yet, 79% (55/69) of the current 1c isolates were resistant to this drug. The *dhfr*1a gene which is transferred through a class 2 integron was implicated in SXT resistance [26]. The detection of class 2 integron in 63% of all isolates probably portrays its role as a mobile element in the dissemination of antibiotic resistance to SXT and C [23]. In the present study, nucleotide sequence analyses revealed that all the bla_{OXA} genes produced OXA-1 type B-lactamase. Interestingly, 88% of the bla_{OXA}-1 isolates from NPE and PP demonstrated resistance to C, although some studies have reported that C resistance is chromosomally mediated through the *catA1* gene (8). An association between bla_{OXA}-1 gene and AM resistance among *Shigella* isolates has been documented [24,25] owing to the presence of a class 1 integron that may be placed on the chromosomes to counteract the tendency of losing the virulence/resistance plasmid [8]. However, the susceptibility of isolates to CRO, FEB, CAZ and CTX provides promising treatment options.

Regardless of time, genetic elements mediating resistance and location, XbaI mrp-PFGE profiles placed the majority of *S. flexneri* 1c isolates in more than one cluster, unlike other *S. flexneri* serotypes 1b, 2a, 3, 4, and 6 that may show unique clusters for each serotype (5). PFGE grouped 90% of infant EP isolates collected between 1990 and 1994 in one cluster, indicating minor genetic differences. Meanwhile, NEP and PP isolates formed another cluster, probably in relation to age of the patients.

The obtained data highlight the emergence of *S. flexneri* 1c as an additional serotype in endemic areas. It carries and possibly disseminates drug resistance through variable genetic mechanisms over time and location and should not be overlooked. The spread of multi-resistant strains of this serotype among pediatric and adult populations in endemic areas supports the need for a wiser selection and use of antibiotics. Plans for its proper identification, treatment and control are certainly more urgent than ever.

Consent

Written informed consent was obtained voluntarily from all adult patients and guardians of children before participation or enrollment in the study in keen for publication of this report.

Abbreviations

AMS: Antimicrobial susceptibility; MDR: Multi-drug resistance phenotypes; EP: Egyptian pediatric patients; NEP: Egyptian non-pediatric patients; PP: Pakistani pediatric; AM: Ampicillin; TE: Tetracycline; SXT: Trimethoprim-sulphamethoxazole; C: Chloramphenicol; CIP: Ciprofloxacin; CRO: Ceftriaxone; FEB: Cefepime; CAZ: Ceftazidime; CTX: Cefotaxime; PFGE: Pulsed Field Gel Electrophoresis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SFA carried out the molecular genetic studies, wrote the manuscript. JK participated in the design of the study, help in drafting the manuscript, TH coordinated and helped to draft the manuscript. JM critical revision of manuscript and data content. AN provided final approval for publication. MOW performed data analysis, results interpretation and helped in writing the manuscript. All authors read and approved the final manuscript.

Authors' information

Co-authors: John Klena, Tupur Husain, Jesse Monestersky, Amel Naguib, Momtaz O Wasfy.

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Author details

¹Research Science Directorate, United States Naval Medical Research Unit No. 3, Cairo, Egypt. ²Department of Epidemiology, Ministry of Health and Population, Cairo, Egypt.

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