Epidemiol. Infect. (2012), **140**, 299–310.
doi:10.1017/S0950268811000525

Genetic diversity and antibiotic resistance in *Shigella dysenteriae* and *Shigella boydii* strains isolated from children aged <5 years in Egypt

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(*Accepted 6 March 2011; first published online 7 April 2011*)

SUMMARY

Diversity within *Shigella dysenteriae* ($n=40$) and *Shigella boydii* ($n=30$) isolates from children living in Egypt aged <5 years was investigated. *Shigella*-associated diarrhoea occurred mainly in summer months and in children aged <3 years, it commonly presented with vomiting and fever. Serotypes 7 (30%), 2 (28%), and 3 (23%) accounted for most of *S. dysenteriae* isolates; 50% of *S. boydii* isolates were serotype 2. *S. dysenteriae* and *S. boydii* isolates were often resistant to ampicillin, chloramphenicol and tetracycline (42%, 17%, respectively), although resistance varied among serotypes. Pulsed-field gel electrophoresis separated the isolates into distinct clusters correlating with species and serotype. Genetic differences in trimethoprim/sulfamethoxazole and β-lactam-encoding resistance genes were also evident. *S. dysenteriae* and *S. boydii* are genetically diverse pathogens in Egypt; the high level of multidrug resistance associated with both pathogens and resistance to the most available inexpensive antibiotics underlines the importance of continuing surveillance.

Key words: Antibiotic resistance, bacterial typing, serology, *Shigella*.

INTRODUCTION

Shigellosis remains a significant public health problem in developing countries where it is a major cause of diarrhoea-related morbidity and mortality, especially in children aged <5 years [1]. *Shigella flexneri* and *Shigella sonnei* have been reported as the most common causes of shigellosis in the developing world; in developed countries *S. sonnei* dominates [2, 3]. *Shigella dysenteriae* and *Shigella boydii* are much less commonly reported as causes of shigellosis [1, 2]. However, *S. dysenteriae* can be the cause of large outbreaks of diarrhoeal disease with severe consequences; the increased virulence is primarily due to the secretion of the phage-encoded Shiga toxin [4, 5].

Several epidemiological studies have examined the burden of shigellosis in Egypt [6–10]. Four of these studies examined the *Shigella* serotypes responsible for disease [6–8, 10]. The criteria used to enrol cases in each of these studies were different. Two studies used active surveillance of a birth cohort in which children aged <24 months were enrolled and remained in the study until aged 36 months [6, 7], another study used passive surveillance of paediatric severe diarrhoea where every fifth child (0–59 months) was enrolled

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from two separate outpatient clinics [10], while a fourth study used passive surveillance within all admissions to a Cairo fever hospital (median age 14·1 years; range 4·8–23·4 years [8]. Despite the variable enrolment criteria, *S. flexneri* was the dominant species in all age groups in all four studies. However, in three of the four studies, *S. dysenteriae* was the second most common cause of shigellosis, followed by *S. sonnei*. *S. boydii* was least often associated with gastroenteritis.

Strain characterization of Egyptian isolates of *Shigella* beyond species type is uncommon and where data are available, it pertains almost exclusively to characterization of isolates of *S. flexneri* [11, 12]. Wasfy *et al*. [8] reported that the isolates of *S. dysenteriae* identified in all-age severe cases of diarrhoea were mainly subtypes 1, 2 or 3, but the percentages of isolates were not stated. No information has been reported for *S. boydii*.

Antibiotic treatment of shigellosis is increasingly complicated due to the rising levels of resistance to most widely available and inexpensive antibiotics such as ampicillin (AM), chloramphenicol (C), trimethoprim/sulfamethoxazole (SXT), and tetracycline (TE) [2, 13]. In Egypt only a few studies have examined antimicrobial sensitivities to *Shigella* spp. [6, 8] and one study explored resistance in *S. flexneri* isolates [11]. Almost no data regarding subserotype, mechanism of antimicrobial resistance, and genetic diversity of *S. dysenteriae* and *S. boydii* have been published from Egypt, despite the importance of these pathogens as causes of paediatric diarrhoea.

Our overall goal was to integrate clinical and epidemiological data gathered regarding *Shigella*-associated diarrhoea in Egyptian children with advanced characterization of specific *Shigella* spp. In addition, we wished to document the basis for antibiotic resistance, with a particular emphasis on β-lactam and SXT antibiotics; commonly used to treat diarrhoea in the local paediatric population. In our first report [11] we presented data from children infected with *S. flexneri*. In this report we present data from children infected by *S. dysenteriae* and *S. boydii* exclusively.

METHODS

Bacterial strains and culture conditions

A total of 40 *S. dysenteriae* and 30 *S. boydii* isolates were analysed from stool samples collected from 70 children aged <5 years participating in paediatric cohort and paediatric severe diarrhoea studies conducted during the period 1998–2007, in rural Abu-Homos, Bahira Governorate, the periurban Fayoum district, or the urban district of Moqqatum Hills, Cairo. Briefly, three birth cohort studies centered in the Abu Homos field site were conducted from 1998 to 2007. Cohort study 1, conducted between 1998 and 2000, enrolled 462 children aged <1 month, with follow-up between 6 and 18 months; this study aimed to measure the efficacy of an enterotoxigenic *Escherichia coli* (ETEC) vaccine trial. Cohort study 2 was conducted to determine the age-specific incidence of *Helicobacter pylori* infection in infants and children (1998–2003); 277 children were enrolled within their first month of life and put under active surveillance until achieving their third birthday. Cohort study 3 (2004–2007) was conducted to determine the natural history of ETEC infection; 348 children aged <1 month were enrolled and followed for 2 years. Study subjects in all cohorts were visited twice weekly in their homes to determine if they had loose or liquid stools since the last visit. If such symptoms were present, a rectal swab and stool sample were collected and sent to NAMRU-3 for analysis. For childen enrolled in the paediatric severe diarrhoea studies at Abu Homos Hospital during May 2000 to September 2007, every fifth child aged <5 years who attended the paediatric clinic complaining of diarrhoea was approached for enrolment; a total of 3813 children were screened and enrolled. A total of 3457 children aged <5 years enrolled in outpatient hospital clinics at Moqqatum Hills during October 2002 to September 2007, and in Fayoum district (a hospital paediatric outpatient clinic) during August 2003 to September 2003 a total of 356 children were enrolled. In hospital studies, children aged <5 years who complained of diarrhoea had stool samples collected and rectal swabs performed, after informed consent, which were sent to NAMRU-3 for analysis.

In instances when *Shigella* was isolated more than once from the same child during the same diarrhoeal episode, only one isolate was examined. Rectal swabs were cultured on MacConkey agar (Becton Dickinson, USA) and Salmonella-Shigella agar (Becton Dickinson), and *Shigella* isolates were identified by standard microbiological procedures [14]. *Shigella* isolates were frozen in trypticase soy broth supplemented with 15% glycerol at $-70\,^{\circ}$C until required. Details of these studies have been described elsewhere [10, 15].

Statistical analysis

The values of variables were counted and summarized in tables of frequency. The χ^2 test was used for the analysis of categorical variables. When the frequency in a cell was lower than five, Fisher's exact test was used instead of χ^2. Collected demographic and clinical data of all *S. dysenteriae* and *S. boydii* were analysed using SPSS version 15 (SPSS Inc., USA).

Serotyping

Serogroups were determined using commercial polyvalent group sera and visual inspection of slide agglutination assays as described by the manufacturer (Denka Seiken Co., Japan). Similarly, species serotypes were determined using either 12- or 18-monovalent sera specific for *S. dysenteriae* or *S. boydii*, respectively.

Antimicrobial susceptibility testing (AST)

Isolates of *Shigella* were characterized for antimicrobial susceptibility to seven antimicrobial compounds using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The antibiotic test panel included 10 μg AM; 30 μg C; 30 μg nalidixic acid (NA); 5 μg ciprofloxacin (CIP); 30 μg TE; SXT (1·25 μg trimethoprim/23·75 μg sulfamethoxazole); and 30 μg cephalothin (CR),. After incubation on Mueller–Hinton agar plates at 37 °C for 18 h, zones of inhibition diameters were recorded. Results were interpreted according to CLSI guidelines as susceptible (S), intermediate (I) or resistant (R). For the purpose of reporting, we refer to both intermediate and susceptible isolates as 'non-resistant'. Multidrug resistance (MDR) was defined as resistance to at least three classes of antibiotic as defined previously [6]. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains for AST according to CLSI guidelines [17].

DNA isolation and PCR

Genomic DNA from bacterial isolates was prepared from whole-cell lysates as described previously [17]. Isolates phenotypically resistant to AM and SXT were examined using oligonucleotides specific for bla_{TEM}, bla_{OXA}, class 1 and 2 integrons and sulfonamides genes (*sulI* and *sulII*). PCR amplicons were loaded into 2% agarose (Sigma-Aldrich Inc., USA) gels and DNA subjected to electrophoresis. Molecular mass of DNA fragments was determined by comparison with a commercial DNA standard (100-bp DNA ladder, Promega, USA). *E. coli* ATCC 25922, an antibiotic susceptible strain, was used as negative control in each PCR experiment.

The presence of class I integrons was inferred by amplification of a 280-bp fragment of the integrase gene *intI1* using primers int1-F (5′-cctcccgcacgatgatc-) and intI1-R (5′-tccacgcatcgtcaggc-) as described previously [18]. Class II integrons were inferred by amplification of a 233-bp segment of the integrase gene *intI2* using primers intI2-F (5′-ttattgctgggattaggc-) and intI2-R (5′-acggctaccctctgttatc-) [18]. Detection of resistance to β-lactams was performed amplifying 643- and 599-bp fragments using the following primer sets: $bla_{\mathrm{TEM\text{-}F}}$ (5′-cagcggtaagatccttgaga-) and $bla_{\mathrm{TEM\text{-}R}}$ (5′-actccccgtcgtgtagataa-), and $bla_{\mathrm{OXA1\text{-}F}}$ (5′-aatggcaccagattcaactt-) and $bla_{\mathrm{OXA1\text{-}R}}$ (5′-cttggcttttatgcttgatg-) as described previously [19]. Primers directed towards internal portions of the *sulI* and *sulII* genes amplified fragments of 433 bp (sulI-F, 5′-cggcgtggctacctgaacg-; sulI-R, 5′-gccgatcgcgtgaagttccg-) and 293 bp (sulII-F, 5′-gcgctcaaggcagatggcatt-; sul1II-R, 5′-gcgtttgataccggcacccgt), respectively [20].

*Xba*I pulsed-field gel electrophoresis (*Xba*I–PFGE) and data analysis

*Xba*I digestion and subsequent PFGE of chromosomal DNA was used to detect genetic diversity among *S. dysenteriae* and *S. boydii* isolates. Preparation of intact genomic DNA, digestion and electrophoresis conditions were as described previously [21]. *Xba*I–PFGE profiles were compared by automated analysis using the BioNumerics Software package (version 5.10; Applied Maths, USA) and confirmed by visual inspection. Similarity between PFGE banding patterns was calculated using the Dice coefficient with a 2% tolerance for the band migration distance and clustering was performed using the complete linkage method; this analysis method is considered a more strict interpretation of banding patterns than UPGMA [22].

RESULTS

Epidemiological description of *S. dysenteriae* isolates

S. dysenteriae isolates were recovered from paediatric cohort, paediatric clinic- and hospital-based

https://doi.org/10.1017/S0950268811000525 Published online by Cambridge University Press

Table 1. *Identification and clinical characteristics of paediatric patients with* Shigella dysenteriae (*n* = *40*) *or* Shigella boydii (*n* = *30*) *isolated among predominate serotypes*

	Shigella dysenteriae/serotype, *n* (%)					*Shigella boydii*/serotype, *n* (%)		
Characteristic	2, 11 (28)	3, 9 (22)	7, 12 (30)	Other, 8 (20)	All, 40 (100)	2, 15 (50)	Other, 15 (50)	All, 30 (100)
Study type								
Cohort, *n* (%)	7 (33)	2 (10)	8 (38)	4 (19)	21 (53)	10 (67)	3 (33)	13 (43)
Hospital, *n* (%)	4 (21)	7 (37)	4 (21)	4 (21)	19 (47)	5 (33)	12 (80)	17 (57)
Epidemiological characteristics								
Age, months, median (IQR)	17 (14–24)	15 (9–29)	14 (8–23)	10 (7–11)	15 (9–23)	20 (18–28)	17 (9–22)	20 (13–27)
Male gender, *n* (%)	6 (55)	4 (44)	9 (75)	3 (42)	22 (55)	10 (67)	9 (60)	19 (63)
Warm season, *n* (%)	11 (100)	9 (100)	12 (100)	8 (100)	40 (100)	14 (93)	14 (93)	28 (93)
Clinical characteristics								
Max. no loose/liquid stools, median (IQR)	7 (5–11)	5 (2–5)	8 (4–12)	6 (5–8)	6 (4–8)			7 (4–9)
Duration of illness, days, median (IQR)	3 (2–7)	2 (1–5)	2 (2–5)	6 (3–8)	3 (2–5)	2 (1–3)	3 (2–5)	3 (2–4)
Any vomiting, *n* (%)	3 (27)	3 (33)	2 (17)	1 (13)	9 (23)	4 (27)	7 (47)	11 (37)
Blood in stool, *n* (%)	0	0	2 (17)	0	2 (5)	2 (13)	3 (20)	5 (17)
Febrile (rectal temperature $>38{\cdot}5\ ^{\circ}C$), *n* (%)	2 (18)	5 (56)	7 (59)	2 (25)	16 (40)	5 (33)	6 (40)	11 (37)
Dehydration, *n* (%)	1 (9·0)	0	1 (8·0)	1 (13)	3 (7·5)	0	4 (27)	4 (13)

surveillance studies (Table 1). In total, 40 children with diarrhoea were culture-positive for *Shigella*. Twenty-one of the children were enrolled in the paediatric cohort studies and 19 were enrolled in clinic- and hospital-based studies for paediatric severe diarrhoea. The median age of children with *S. dysenteriae* was 15 [interquartile range (IQR) 9–23] months; 55% (*n* = 22) were males and all infections occurred in the warm months of May to October.

The *S. dysenteriae*-associated children presented with fever (*n* = 16, 40%), vomiting (*n* = 9, 22·5%), dehydration (*n* = 3, 7·5%), and visible blood in stool (*n* = 2, 5%). Three cases reported the use of antibiotics before the stool sample was taken. The median maximum number of loose stool during any diarrhoeal day was 6 (IQR 4–8), while the median duration of a diarrhoeal episode was 3 (IQR 2–5) days. ETEC and rotavirus were isolated as co-pathogens in six and two cases, respectively.

Overall, 43 *S. dysenteriae* strains were isolated from diarrhoeal stool samples from 40 children. Three children had pairs of *S. dysenteriae* isolates, recovered during the same diarrhoeal episode; however, only the first isolate was included in the clinical descriptive and genetic analyses (Table 1).

Epidemiological description of *S. boydii* cases

Similar to *S. dysenteriae*, isolates of *S. boydii* were recovered from all surveillance study sites during the warm season (93%, *n* = 28) (Table 1).

A total of 31 isolates of *S. boydii* were recovered from the 30 diarrhoeal children. Two *S. boydii* isolates were isolated from one child from the same diarrhoeal episode; only the first isolate was analysed. Of the 30 children, 13 (43%) were from the paediatric cohort and 17 (57%) from the paediatric severe diarrhoea study.

The median age for all children with *S. boydii* infection was 20 (IQR 13–27) months and 63% (*n* = 19) were males. ETEC was isolated as a co-pathogen in 8/30 (27%) samples while rotavirus and *Campylobacter* infection were co-pathogens in 14% (3/24) and 3% (1/30) of tested samples, respectively.

Clinical characteristics of diarrhoeal episodes due to *S. boydii* included vomiting (*n* = 11, 37%), fever (*n* = 11, 37%), and visible blood in stool (*n* = 5, 17%). Dehydration was detected in 13% (*n* = 4) of episodes. Antibiotics were used during 7% (*n* = 2) of the episodes. The median duration of diarrhoeal episodes was 3 (IQR 2–4) days and the median maximum

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Table 2. *Antimicrobial resistance results for* S. dysenteriae *and* S. boydii *isolates from Egypt, 1999–2006*

	Number of isolates	
Antibiotic	*S. dysenteriae* (%)*	*S. boydii* (%)
AM	21 (52)	18 (60)
C	17 (42)	5 (17)
CR	5 (12)	12 (40)
NA	2 (5·0)	0 (0)
SXT	10 (25)	19 (63)
TE	23 (58)	23 (77)
Combinations		
AM/C/TE	13 (32)	1 (3·3)
AM/CR/TE/SXT	0 (0)	6 (20)
TE/SXT	1 (2·5)	4 (13)
AM/SXT	2 (5·0)	2 (6·6)
Sensitive	12 (30)	2 (6·6)

AM, Ampicillin; C, chloramphenicol; CR, cephalothin; NA, nalidixic acid; SXT, sulfatrimethoxazole; TE, tetracycline.

* The percentage of resistant isolates is shown within parentheses; for *S. dysenteriae* this percentage was calculated with a denominator of 40 isolates; for *S. boydii*, this percentage was calculated with a denominator of 30 isolates.

number of loose stools passed was 7 (IQR 5–9) times per day. Children with *S. dysenteriae* and/or *S. boydii* diarrhoea were more likely to have vomiting when presenting to hospital for care ($P=0{\cdot}005$).

Diversity of *S. dysenteriae* and *S. boydii* isolate serotypes

Of 40 children infected with *S. dysenteriae* isolates, three serotypes [serotypes 7 (30%), 2 (27·5%), and 3 (22·5%)] accounted for the majority (80%) of all isolates (Table 1). Single isolates of *S. dysenteriae* serotypes 1 and 12 were identified during the 7 years of sample collection. Seventy-five percent of children infected with *S. dysenteriae* serotype 7 were male and their most common symptoms were fever (59%), vomiting (17%) blood in stool (17%) and dehydration (8%). However, 44% of children infected with *S. dysenteriae* serotype 3 were males and presented with fever (56%) and vomiting (33%). All seven *S. dysenteriae* serotypes found in this study were recovered from hospital and clinic study samples while only four serotypes and the untypable isolates (serotypes 2, 3, 4, 7) were present in the cohort studies.

Of the 30 *S. boydii* isolates, nine serotypes were identified; serotype 2 was the most common serotype ($n=15$, 50%) and was isolated in 67% of males. Children infected with *S. boydii* serotype 2 presented with fever (33%), vomiting (27%), blood in stool (13%), although none of the patients were reported as dehydrated. All nine *S. boydii* serotypes recovered were found in hospital samples while only three serotypes (serotypes 2, 4, 14) were found in the cohort studies. However, there was no significant difference in the distribution of serotypes among children with *S. dysenteriae* and *S. boydii* enrolled from hospital or from cohort studies.

Antimicrobial resistance profiles of *S. dysenteriae* and *S. boydii*

S. dysenteriae isolates showed high levels of resistance against TE (58%), AM (52%), C (42%), and SXT (25%, Table 2). Resistance against CR and NA was detected in only 12% and 5% of the isolates, respectively. Only 12 isolates were susceptible to all antibiotics tested, and eight isolates were resistant to either one ($n=4$) or two ($n=4$) antibiotics. The majority of *S. dysenteriae* serotype 2 isolates were susceptible (9/11) to all agents tested. From the 40 *S. dysenteriae* isolates, half ($n=20$, 50%) were MDR. The majority of MDR were resistant to three antibiotics ($n=16$), although three isolates were resistant to four and a single isolate was resistant to six antibiotics. All serotype 3 isolates ($n=9$) were MDR; all were resistant to AM, C and TE. Isolates expressing serotype 7 showed more antimicrobial diversity; three isolates were completely susceptible, a single isolate was resistant to two antibiotics, and eight isolates were MDR. No ESBL-producing isolates were detected in either the *S. dysenteriae* or *S. boydii* collection.

Isolates of *S. boydii* were highly resistant to TE (77%), SXT (63%), AM (60%) and CR (40%); while less resistant to C (17%), and all strains were susceptible to NA (Table 2). The most common MDR phenotypic profile (40%, $n=12$) detected in *S. boydii* were isolates resistant to the combination of AM, CR, TE, and SXT (20%). Over half of the *S. boydii* isolates were MDR ($n=16$, 53%); nine isolates were resistant to a combination of four antibiotics, and six isolates were resistant to three agents. Only one isolate was resistant to five antibiotics. Similarly, only two isolates were sensitive to all the antimicrobials tested in this study. We found six isolates resistant to either one or two antimicrobial agents. Neither *S. boydii* nor *S. dysenteriae* showed resistance to CIP.

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Table 3. *Carriage of antimicrobial resistance genes to β-lactams and sulfonamides among* Shigella*-resistant isolates*

Isolates	Phenotype of resistance	No. of resistant isolates (%)	Detection of integrons, genes (5)		Resistance genes detected	No. of isolates in which resistance genes were detected (%)
			intI1	*intI2*		
S. dysenterae ($n=40$)	AM	21 (53)	19 (90)	5 (24)	bla_{TEM} alone	3 (14)
					bla_{OXA} alone	4 (19)
					OXA with TEM	14 (67)
					Total of bla_{TEM}	17 (81)
					Total of bla_{OXA}	18 (86)
	SXT	10 (25)	5 (50)	5 (50)	*sulI* alone	2 (20)
					sulII alone	1 (10)
					sulI and *sulII*	7 (70)
					Total of *sulI*	9 (90)
					Total of *sulII*	8 (80)
S. boydii ($n=30$)	AM	18 (60)	17 (94)	6 (33)	bla_{TEM} alone	10 (56)
					bla_{OXA} alone	0 (0)
					OXA with TEM	8 (44)
					Total of bla_{TEM}	18 (100)
					Total of bla_{OXA}	8 (44)
	SXT	19 (63)	16 (84)	7 (37)	*sulI* alone	0 (0)
					sulII alone	3 (16)
					sulI and *sulII*	14 (74)
					Total of *sulI*	14 (74)
					Total of *sulII*	17 (89)

AM, ampicillin; SXT, sulfatrimethoxazole.

Genetic basis of AM and SXT resistance

To explore whether there were genetic changes associated in the resistance mechanisms over time, we screened all *Shigella* isolates for genes involved in resistance to AM and SXT (Table 3). Evidence for redundant AM resistance (AM-R) in *S. dysenteriae* was apparent; 18/21 (86%) isolates carried a $bla_{OXA\text{-}7}$-type and 17/21 (81%) harboured a $bla_{TEM\text{-}1}$-type β-lactamase. In contrast, 8/18 (44%) of AM-R *S. boydii* isolates carried $bla_{OXA\text{-}7}$-type and all ($n=18$) harboured a $bla_{TEM\text{-}1}$-type β-lactamase. Only 10 (25%) *S. dysenteriae* isolates were resistant to SXT and the majority of those isolates harboured the *sulI* ($n=9$) and/or *sulII* ($n=8$) genes. Moreover, the majority of the SXT-R *S. boydii* isolates carried *sulI* ($n=14$, 74%) and/or *sulII* ($n=17$, 89%).

In AM-R *S. dysenteriae*, class I integrons (*intI1*) were predominant ($n=19$, 90%); while class II integrons (*intI2*) were detected in only 24% ($n=5$) of these isolates (Table 3). However, the genes *intI1* or *intI2* were detected in only 50% of the *S. dysenteriae* SXT-R isolates. Most of the resistant *S. boydii* carried a marker for *intI1*. For example, *intI1* was detected in the majority of resistant isolates for AM ($n=17$, 94%) and SXT ($n=16$, 84%). In contrast, *intI2* was detected only in 33% ($n=6$) AM-R and 37% SXT-R ($n=7$) isolates.

*Xba*I–PFGE confirms species diversity

*Xba*I–PFGE of *S. dysenteriae* isolates generated 28 unique profiles (Fig. 1). We selected a similarity value of 47% because at this similarity value, isolates separated into the three clusters mainly had the same serotype. At this cut-off value, all of the *S. dysenteriae* serotype 2 isolates ($n=11$) clustered together (cluster 1). Two untypable isolates, the sole serotype 12 isolate, and single serotype 3 isolate were also in this cluster. Cluster 2 consisted of all the serotype 7 ($n=12$) and a single serotype 1 isolates. Cluster 3 consisted of all serotype 4 and all but one of the serotype 3 isolates ($n=8$). Considering a similarity value of 64%, seven subclusters of isolates were evident. Three subclusters fell within cluster 1; one subcluster was strictly populated by serotype 2 isolates; one was composed of only the untypable isolates and serotype 2; the last subcluster was mixed with

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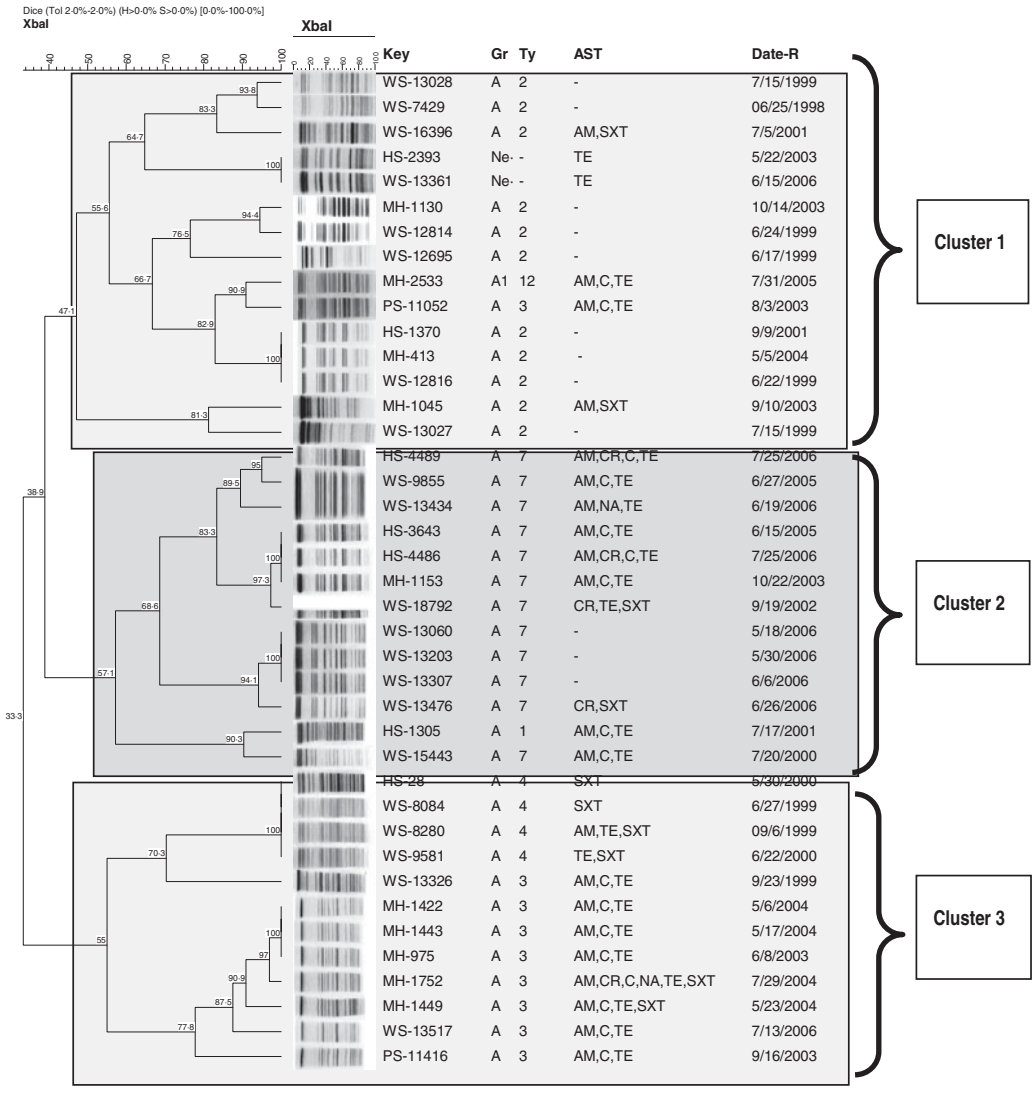

Fig. 1. Dendrogram showing the relationship of *S. dysenteriae* isolates based on *Xba*I–PFGE profiles. Similarity of banding patterns of *Xba*I-restricted whole genomic DNA from isolates of *S. dysenteriae* were compared using Bionumerics software. The bar at the top of the figure indicates decreasing genetic similarity (moving from the right hand to the left hand side of the figure). Numbers located at nodes within the dendrogram indicate percent similarity. Isolates designated as 'WS' were recovered from the paediatric birth cohort; isolates designated as 'HS', 'MH', or 'PS' were recovered from children seeking medical care for diarrhoea-related symptoms. Clusters of isolates determined at a 47% band pattern similarity are shown in the boxes. Other abbreviations: GR, serogroup; TY, serotype; AST, antibiotic sensitivity testing result (indicating resistance to a specific antibiotic); Date-R, specimen receipt date; Ne, did not react with antisera; AM, ampicillin; C, chloramphenicol; CR, cephalothin; NA, nalidixic acid; SXT, sulfatrimethoxazole; TE, tetracycline.

serotype 2, 3 and 12 isolates. Cluster 2 was composed of two subclusters, the first subcluster strictly populated by serotype 7 ($n=11$); the second subcluster was composed of single isolates expressing either serotype 1 or serotype 7. Cluster 3 contained two subclusters, one subcluster composed of serotype 3 exclusively, a second subcluster contained a single serotype 3 isolate and an indistinguishable set of serotype 4 isolates.

The number of *S. dysenteriae* isolates with MDR profiles was significantly different based on the different studies (Fig. 1). For example, 65% of isolates from the hospital studies were MDR compared to 35% from the cohort studies ($P=0{\cdot}028$); however, MDR status was not associated with any specific sign or symptom. MDR status was associated with serotype distribution, ($P=0{\cdot}0001$); 100% of serotype 3 and 67% of serotype 7 were MDR.

Characterization of *S. boydii* using *Xba*I–PFGE (Fig. 2) identified 25 profiles. The *S. boydii* population had a different structure to that observed with

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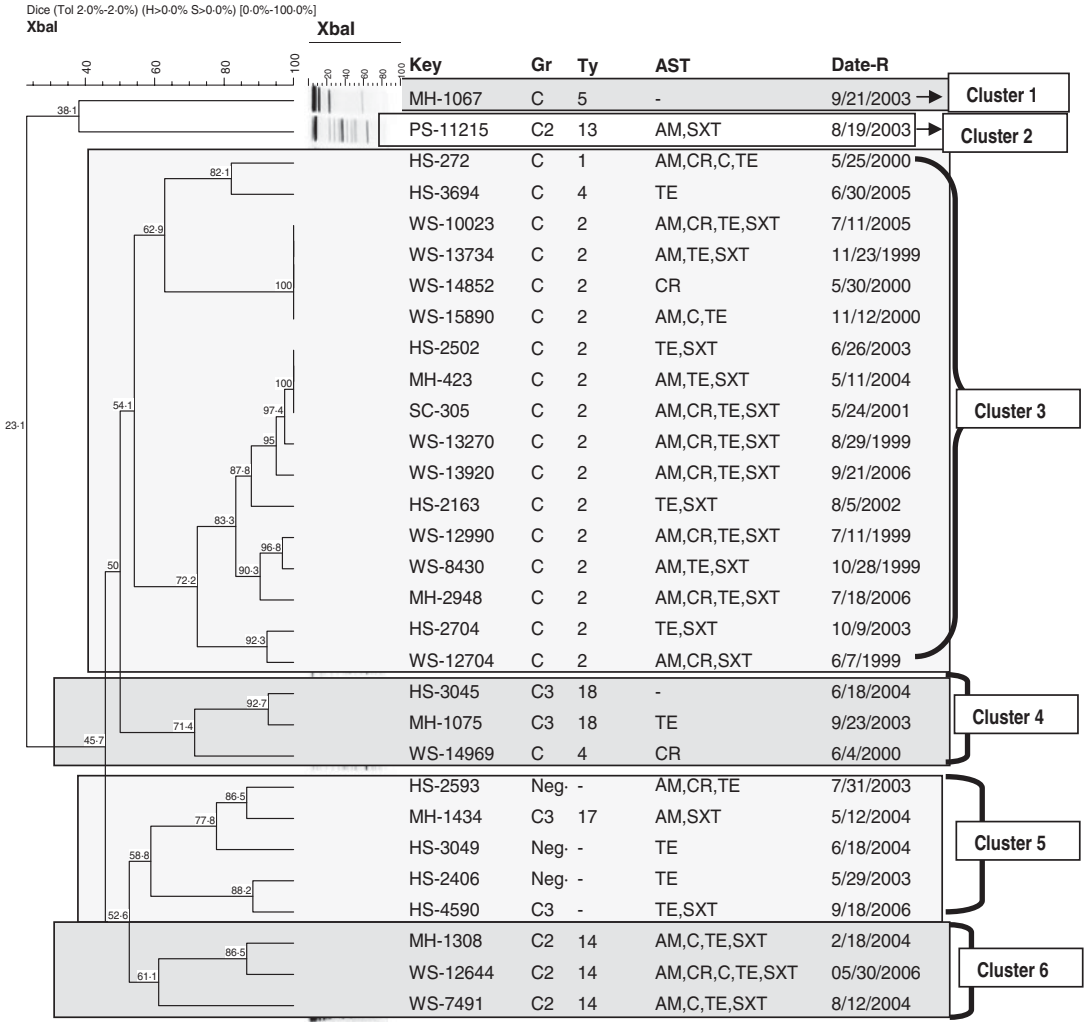

Fig. 2. Dendrogram showing the relationship of *S. boydii* isolates based on *Xba*I–PFGE profiles. Similarity of banding patterns of *Xba*I-restricted whole genomic DNA from isolates of *S. dysenteriae* were compared using Bionumerics software. The bar at the top of the figure indicates decreasing genetic similarity (moving from the right hand to the left hand side of the figure). Numbers located at nodes within the dendrogram indicate percent similarity. Isolates designated as 'WS' were recovered from the paediatric birth cohort; isolates designated as 'HS', 'MH', or 'PS' were recovered from children seeking medical care for diarrhoea-related symptoms. Clusters of isolates determined at a 54% band pattern similarity are shown in the boxes. Other abbreviations: GR, serogroup; TY, serotype; AST, antibiotic sensitivity testing result (indicating resistance to a specific antibiotic); Date-R, specimen receipt date; Ne, did not react with antisera; AM, ampicillin; C, chloramphenicol; CR, cephalothin; NA, nalidixic acid; SXT, sulfatrimethoxazole; TE, tetracycline.

S. dysenteriae and at a 54% similarity level, six clusters of isolates, mainly in agreement with the serotyping results, were evident. Cluster 1 and cluster 2 were single isolates of serotype 5 or serotype 13, respectively. Cluster 3 was the largest cluster, containing all of the serotype 2 isolates ($n=15$), the single serotype 1 isolate and one serotype 4 isolate. Cluster 4 contained both serotype 18 isolates, and one of the two serotype 4 isolates. Cluster 5 consisted of all four untypable isolates and the sole serotype 17 isolate. The final cluster (cluster 6) consisted of all the serotype 14 isolates. Increasing the percentage of similarity to 70% had the greatest impact on isolates in cluster 3. At this similarity level, three subclusters were identified in cluster 3; one subcluster consisted of the serotype 1 and serotype 4 isolates; a second closely related subcluster consisted of a set of four genetically indistinguishable serotype 2 isolates that spanned multiple years; the third subcluster was composed only of highly related serotype 2 isolates ($n=11$) that also spanned multiple years. Cluster 4 contained serotype 18 isolates and the serotype 4 isolate as one group, cluster 5 split into two subgroups; the first group consisted of two untypable strains, and

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the sole serotype 17 isolate; the second subgroup consisted of the remaining two untypable isolates. Cluster 6 also split into two subclusters, representing potential independent lineages of serotype 14.

S. boydii MDR isolates were found more frequently in the paediatric cohort samples (Fig. 2). A total of 10/12 (83%) *S. boydii* isolates from the paediatric cohort were MDR; only 6/18 (33%) hospital samples were MDR. *S. boydii* serotype 2 ($n=15$) were often associated with MDR (11/15, 73%).

DISCUSSION

Despite the potential significance of explosive outbreaks of dysentery [23] and the spread of drug-resistant strains through the consumption or use of contaminated food [24] or water sources [25], there is a paucity of data from Egypt regarding the characterization of non-*flexneri Shigella*. *S. dysenteriae* and *S. boydii* are often the least common serogroups causing shigellosis in developing and developed countries and neither are considered common causes of diarrhoea. Nevertheless, outbreaks of both species have been reported. In a recent report from Sweden, an outbreak of *S. dysenteriae* serotype 2 was linked to sugar snap peas, most probably originating from Kenya, and passing through at least one other European country [24]. Similarly, a cluster of cases of *S. boydii* serotype 2 linked to water transmission was recently reported in Mpumalanga Province in South Africa [25].

In our first report [11] we presented data from children infected with *S. flexneri*. In this report we present data from children infected by *S. dysenteriae* and *S. boydii* exclusively. Data with respect to diarrhoea associated with *S. sonnei* will be reported separately. In this report, we have characterized collections of *S. dysenteriae* and *S. boydii* isolates recovered from children aged <5 years living in Egypt who were enrolled in one of three paediatric birth cohort studies or a paediatric severe diarrhoea study from 1999 to 2006. Wierzba *et al.* [10] reported previously that the dominant bacterial pathogen present in the studies from which our *Shigella* isolates were recovered was ETEC, distantly followed by *Campylobacter* spp. The third most common bacterial pathogen was *Shigella* spp. and *S. flexneri* was the most abundant species isolated from these studies. A study of severe diarrhoeal cases in Dhaka, Bangladesh [26] conducted over 18 months from 2000 to 2001 (all-age diarrhoea) indicated a similar distribution of *S. dysenteriae* ($n=20$) and *S. boydii* ($n=16$) isolates.

As a common concept among physicians in rural clinics, shigellosis is suspected and treated when fever and bloody stool are associated with diarrhoea; however, in our study only 5% and 17% of children with *S. dysenteriae*- and *S. boydii*-associated diarrhoea, respectively, had bloody stools; this finding is consistent with previous cohorts and hospital-based studies from Egypt [8]. Children enrolled in the cohort studies had mainly mild to moderate diarrhoea episodes but those presenting to a medical care facility were more severe as indicated by a significant percentage of vomiting and dehydration. These findings indicate that the clinical presentation of shigellosis due to either *S. boydii* or *S. dysenteriae* can range from mild to severe illness. We also noted greater diversity in isolates of both pathogens presenting to a medical care provider, as determined by serology and *Xba*I–PFGE typing; one possible explanation of this observation is that children presenting to a medical provider were from different geographical areas throughout Egypt while children enrolled in the cohort studies were restricted to a cluster of villages in one governorate in Egypt. A separate observation for which we do not yet have an adequate explanation relates to antibiotic resistance. Perhaps, as expected, the majority of *S. dysenteriae* MDR isolates were present in children presenting to medical providers. This may reflect prior antibiotic use due to the severity of observed disease compared to children in the cohort study. However, the observation of a greater number of *S. boydii* MDR cases in the paediatric cohort, rather than presenting to a medical care provider, is puzzling. Further study is necessary to understand this observation.

Immunity against *Shigella* is serotype specific and there is limited cross-protection between the various serotypes [2]. *S. dysenteriae* isolates were mainly divided into expression of three serotypes (7, 2, 3). Only a single *S. dysenteriae* serotype 1 was isolated. In contrast, we found that the majority of *S. boydii* isolates expressed serotype 2 (50%). The next most prevalent serotype was 14 (10%). This would suggest the use of a multivalent vaccine could effectively control *Shigella* spp. in Egypt.

We also assessed the serotype distribution of *S. dysenteriae* and *S. boydii* from Egypt to determine whether it was unique from that of other countries with endemic shigellosis. Nearly all of the *S. dysenteriae* isolates were able to be serotyped using

commercial antisera (95%); relatively speaking, a much higher percentage of *S. boydii* isolates were untypable (13%). A recent study from Karachi also examined the distribution of *Shigella* serotypes in an endemic setting [27]. This group reported a higher percentage for untypable *S. dysenteriae* isolates (16%) although they had a similar percentage for untypable *S. boydii* isolates (12%) comparing a similar number of *S. dysenteriae* and *S. boydii* isolates to our study. *S. dysenteriae* serotype 7-expressing isolates were most common in Pakistan (24%) in partial agreement with our results (30%). However, our results showed that the distribution of serotypes for both species was different. In Pakistan, *S. dysenteriae* serotype 7-expressing isolates were most common, followed by serotypes 4, 2 and 12. Three *S. boydii* serotypes, 1, 2, and 8, were equally abundant (each 16%) in Karachi.

We observed that AM resistance among *S. dysenteriae* (52%) was slightly lower than *S. boydii* (60%). Different results for AM-resistant isolates were found among *S. dysenteriae* (37%) and S. boydii (25%) in six Asian countries in 2006 [28], while higher resistances were reported in Pakistan in 2009, for *S. dysenteriae* (68%) and *S. boydii* (35%) [27], respectively. In this study, susceptibility to NA in *S. dysenteriae* and *S. boydii* was high (95% and 100%, respectively) and all our isolates were susceptible to CIP. This agrees with reports from Yemen, Iran and Turkey [29–31]. In contrast, the resistance profile in India and Bangladesh is worrisome. NA resistance among *S. dysenteriae* isolates in at least one study in India was alarming (82%), and over half (54·5%) of these isolates were CIP resistant [32]. While not isolated at as great a frequency, *S. boydii* isolates in this study also demonstrated an elevated resistance to NA (57·1%) although CIP resistance was not detected (0%). Although Bangladesh has reported an overall decrease in isolation of *Shigella* over the last 15 years, there has been a rise in the resistance to all WHO-recommended first-line antimicrobial therapies for shigellosis during this time [33]. In Egypt, quinolones are not generally recommended for use in children and this may contribute to the low levels of observed resistance. Other possible factors contributing to the infrequent observation of fluoroquinolone resistance in Egypt may be the relatively high cost and lack of availability. A study measuring the risk factors associated with quinolone resistance in the region could contribute to our understanding of the observed differences.

Occurrence of MDR *Shigella* spp. strains is of great public health concern. In Egypt, Wasfy and colleagues [8] looked at sensitivities to 12 antibiotics; their findings with respect to *S. dysenteriae* indicated that from the 33 isolates analysed, 86% were resistant to erythromycin, 68% to C, 56% to TE, 55% to AM, while only 11% of isolates were resistant to SXT. No isolates were resistant to NA and CIP resistance was not tested. Analysing data from the paediatric cohorts, Putnam *et al.* [6] observed that *S. dysenteriae* isolates cultured from 1995 to 2000 showed little resistance to AM, C, SXT, or TE and only 2/28 isolates were MDR. Our results appear to be more similar to those of Wasfy and colleagues [8]; 48% of our *S. dysenteriae* isolates were MDR (19/40) and 43% of these isolates were resistant to AM, C, and TE. Resistance levels to AM (53%) and TE (58%) remained at nearly 50%, but we observed less resistance to C (43%). In a separate analysis, Wasfy *et al.* [8] suggested that a decrease in the use of C for the treatment of typhoid fever has led to an increased susceptibility of the causative organism; it is possible that a similar change in antimicrobial therapy is having the same effect for isolates of *S. dysenteriae*.

Our findings indicate that the resistance genes of sulfonamides (*sulI* and *sulII*) and β-lactams (bla_{TEM}, and bla_{OXA}) are widely disseminated in *S. dysenteriae* and *S. boydii* isolates in Egypt and many of these isolates carry integrons, potentially providing them with the capability of obtaining antibiotic resistance. Little information on the integron content of *S. boydii* and *S. dysenteriae* isolates is available, but in our study, harbouring a class 1 integron among either *Shigella* spp. was associated with AM resistance, in agreement with results reported previously [34]. Class II integrons (*intI2*) were detected in only 24% of *S. dysenteriae* and 33% of *S. boydii* AM-resistant isolates. In addition, *intI1* or *intI2* was detected almost equally in *S. dysenteriae* SXT-resistant isolates. Among *S. boydii* SXT-resistant isolates, *intI1* detection (84%) was higher than *intI2* (37%). The high prevalence of the OXA-1-type and TEM-1-type β-lactamases in *S. dysenteriae* have been reported previously [34] and is also in agreement with our results (86% and 81%, respectively).

PFGE has been widely used for typing *Shigella* spp. and is considered a molecular typing tool with high discriminatory power for detection of outbreaks [24, 35] and longitudinal observation of bacterial populations [36, 37]. We observed a variety of pulsed-field types present in both *S. dysenteria*e and *S. boydii*.

Comparative analysis of PFGE pulsotypes indicated that most observed clusters also corresponded to the expression of a specific lipopolysaccharide antigen, as determined by their serotyping reaction. This observation was also true in our previous study investigating the diversity in *S. flexneri* isolates [11]. PFGE results in this study showed six sets of *S. dysenteriae* isolates ($n=18$) and two sets of *S. boydii* isolates ($n=7$) that were indistinguishable and with the exception of one *S. dysenteriae* group, the isolates were not epidemiologically linked. These data would appear to indicate that some isolates of *S. dysenteriae* and *S. boydii* are capable of persisting in the environment over time. An intriguing hypothesis that remains to be tested is whether *Shigella* isolates in Egypt might persist in the environment by colonizing *Acanthamoeba* spp., similar to a mechanism proposed for *S. sonnei* [38]. *Acanthamoeba* spp., including the pathogenic T4 genotype, have been documented in the Nile Delta region, and were found associated in areas where cooking, cleaning, washing and fishing took place [39].

In this study, we have shown that there is rich serological and genetic diversity among isolates of *S. dysenteriae* and *S. boydii*. Some limitations of the study are the low numbers of isolated *S. dysenteriae* and *S. boydii* which made determination of statistical significance difficult and our inability to perform follow-up investigations on children seeking medical care in Abu Homos, Fayoum and Moqqatum Hills. Any ongoing vaccine development against shigellosis should take into consideration the amount of variability across multiple geographical locations including Egypt. At least one group of investigators are in advanced clinical trials of a live oral *Shigella* vaccine; it is important to support these trials with additional diarrhoea surveillance studies in other governorates within Egypt and other high-risk countries, to monitor serotype distribution and to determine potential vaccine coverage. In addition, we have observed antibiotic resistance levels and mechanisms of resistance which can be used as a basis for comparison in future studies in Egypt and the Middle East. This study provides useful information for future surveillance of shigellosis and implementation of antibiotic treatment policies in Egypt and elsewhere in the region.

ACKNOWLEDGEMENTS

The opinions or assertions herein are the private views of the authors and should not be construed as official or as reflecting the views of the United States Department of the Navy, Department of Defense, nor the United States Government. This study [DoD no. NAMRU3.2000.0002 (IRB Protocol No. 96), NAMRU3.1997.0002 (IRB No. 72), NAMRU3.1998.0003 (IRB No. 75), NAMRU3.2003.0011 (IRB No. 145)] was reviewed and approved by the Institutional Review Boards of the US Naval Medical Research Unit No. 3 and the Egyptian Ministry of Health in compliance with all Federal regulations governing the protection of human subjects. Informed consents were obtained from all adult participants and from parents or legal guardians of minors.

DECLARATION OF INTEREST

None.

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