New enterovirulent *Escherichia coli* serogroup 64474 showing antigenic and genotypic relationships to *Shigella boydii* 16

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Studies based on the analysis of housekeeping genes indicate that *Escherichia coli* and all *Shigella* species, except for *Shigella boydii* type 13, belong to a single species. This study analysed the phenotypic and genotypic characteristics of 23 *E. coli* strains isolated in different countries from faecal specimens taken from children with diarrhoea. Strains were identified using the VITEK system and typed with rabbit sera obtained against 186 somatic and 53 flagellar *E. coli* antigens and against 45 *Shigella* somatic antigens. Biochemical analysis of these strains showed a typical *E. coli* profile with a defined reaction against both *E. coli* O179 and *S. boydii* 16 somatic antisera. Agglutination assays for flagellar antigens showed a response against H2 in 7 (30 %) strains, H10 in 2 (9 %) strains, H32 in 12 (52 %) strains and H34 in 2 (9 %) strains, demonstrating 4 serotypes associated with this new somatic antigen 64474. A serum against one of these *E. coli* strains (64474) was prepared. Absorption assays of *S. boydii* 16 and *E. coli* 64474 antisera with *E. coli* O179 antigen removed the agglutination response against this O179 antigen completely, while the agglutination titres against both *S. boydii* 16 and *E. coli* 64474 remained the same. Four (17 %) *E. coli* strains showed antimicrobial resistance to piperacillin only, one (4 %) to piperacillin and trimethoprim/sulfamethoxazole, one (4 %) to ciprofloxacin, nitrofurantoin and piperacillin, and two (9 %) strains were resistant to ciprofloxacin, norfloxacin, ofloxacin, piperacillin and trimethoprim/sulfamethoxazole. With regards to PCR assays, one (4 %) of the strains was positive for *Shigella* gene *ipaH*, one (4 %) for *ipaA*, two (9 %) for *ipaB*, one (4 %) for *ipaD*, two (9 %) for *sepA* and three (13 %) for *ospF*. The *rfb* gene cluster in the *E. coli* strains was analysed by RFLP and compared with the gene cluster obtained from *S. boydii* 16. The *rfb*-RFLP patterns for all 23 *E. coli* strains were similar to those obtained for *S. boydii* 16. The results from PCR tests to detect *rfb* genes *wzx* (encoding O unit flippase) and *wzy* (encoding polymerase) belonging to a cluster related to the biosynthesis of the *S. boydii* 16-specific O antigen were positive in 21 (91 %) and 22 (96 %) of the strains, respectively. PCR assays to detect *E. coli* virulence genes were also performed. These assays detected enterotoxigenic *E. coli* genes *ltA1* in 12 of the strains (52 %), *st1a* in 4 (17 %), *cfa1* in 6 (26 %), *cs1* in 1 (4 %), *cs3* in 3 (13 %), *cs13* in 9 (39 %) and *cs14* in 5 (22 %) of the strains. Results from the PFGE analyses confirmed the wide geographical distribution of these strains suggesting that 64474:H2, 64474:H10, 64474:H32 and 64474:H34 are new serotypes of *E. coli* strains with a defined virulence capacity, and share a common O antigen with *S. boydii* 16.

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INTRODUCTION

Escherichia coli is the most important aetiological agent of childhood diarrhoea and is a major public-health problem in developing countries (Davidson *et al.*, 2002). Diarrhoeagenic *E. coli* comprise six major pathotypes: enteropathogenic *E. coli*, enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli*, diffusely adherent *E. coli* and Shiga toxin-producing *E. coli* (Nataro & Kaper, 1998). *Shigella* is another human pathogen that is prevalent in less-developed countries where poor sanitation and lack of personal hygiene increase the incidence of disease (Kotloff *et al.*, 1999; Davidson *et al.*, 2002).

LPS is a key component of the outer membrane of Gram-negative bacteria that comprises three distinct regions: lipid A, an oligosaccharide core and commonly a repeat unit polysaccharide O antigen (Raetz & Whitfield, 2002). *E. coli* serotyping indicates relatedness between clinical isolates and allows preliminary classification into pathotypes in the absence of molecular characterization. The highly variable nature of the O antigen provides the basis for serotyping, and the differentiation into 186 O antigen forms (serogroups) that have been recognized in *E. coli*. Differentiation has led to the categorizing of O174 and O175 to temporary O groups OX3 and OX7, as well as the description of 11 new *E. coli* O groups that include verocytotoxin-producing *E. coli* O176, O177, O178, O179, O180 and O181 (Scheutz *et al.* 2004), and O182 to O186 (T. Cheasty, unpublished data).

Shigella strains are classified into four species *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, also known as subgroups A, B, C and D, respectively (ESNC-IAMS, 1958). Based on antigenic variation in their O antigens, *Shigella* species are also typed into multiple serotypes. Despite some biochemical and antigenic differences, *Shigella* and *E. coli* have always been considered to be very closely related. Studies based on the analysis of housekeeping genes suggested that *E. coli* and *Shigella* belong to a single species (Pupo *et al.*, 2000). This can be supported by studies that show the presence of common somatic antigens between *E. coli* and *Shigella*. The reported structures and sequences for 34 *Shigella* O antigens and for the corresponding *E. coli* O antigens that have significant antigenic cross-reactions with *Shigella* O antigens indicate that 21 of the 34 *Shigella* O antigens and associated gene clusters are identical or closely related to those found in *E. coli*. However, 12 O antigen gene clusters are unique to *Shigella* and are not related to other *E. coli* O antigen gene clusters (Liu *et al.*, 2008; Li *et al.*, 2009); *S. boydii* 16 is one of these.

Over the last 15 years, approximately 10 000 *E. coli* strains isolated from different sources have been typed with rabbit sera in the Laboratory of Enteric Pathogens in the Department of Public Health, National Autonomous University of Mexico (Departamento de Salud Pública, UNAM) utilizing sera obtained against O1 to O181 *E. coli*

antigens and against 45 of the *Shigella* antigenic groups (13 of *S. dysenteriae*, 13 of *S. flexneri*, 18 of *S. boydii* and 1 *S. sonnei* serotype). This typing scheme has been extended to include five new *E. coli* O sera, O182 to O186 (T. Cheasty, unpublished data). Using the expanded serotyping scheme, 23 strains classified biochemically as *E. coli* were identified serologically as having both *E. coli* O179 and *S. boydii* 16 O antigens. The present study was designed to try to explain the significance of these serological cross-reactions.

METHODS

Strains. The 23 *E. coli* strains used in this study were isolated from children with diarrhoea from 3 different geographical areas. Ten strains were isolated from children in Mexico, eleven in Egypt (kindly provided by Dr Stephen Savarino, Naval Medical Research Center, Silver Spring, MD, USA) and two in Bangladesh. *S. boydii* 16 (G1219) and *E. coli* O179:H8 (E43478) were used as reference strains.

Biochemical identification. The identity of the 23 strains was confirmed using both the VITEK automated system (bioMérieux) and standard methods (Barrow & Feltham, 1993).

Serotyping. The strains were serotyped by agglutination assays (Ørskov & Ørskov, 1984) using 96-well microtitre plates and rabbit serum (SERUNAM) obtained against 181 somatic antigens and 53 flagellar antigens for *E. coli*, and against 45 somatic antigens for *Shigella* species. Rabbit serum against *E. coli* strain 64474:H32 was also prepared (Ewing, 1986). In addition, the serotyping of the *E. coli* strains was confirmed with 186 anti-O *E. coli* and 45 anti-O *Shigella* sera from the Laboratory of Gastrointestinal Pathogens, Department of Gastrointestinal, Emerging and Zoonotic Infections, Centre for Infections, Health Protection Agency, London, UK.

Absorption assays. Rabbit sera prepared against *S. boydii* 16, *E. coli* O179 and *E. coli* 64474 strains were absorbed against homologous and heterologous antigens according to the method described by Ewing (1986). In brief, smooth colonies grown overnight at 37 °C on blood agar plates were used to inoculate trypticase soy agar plates, which were incubated overnight at 37 °C. Bacteria were harvested into 10 ml 0.15 M NaCl (saline) solution, heated to 100 °C for 1 h and adjusted to a final concentration of 9×10^8 bacteria ml⁻¹. The antigens were incubated at 50 °C with the homologous and heterologous serum samples for 2 h, the mixture was then centrifuged (6000 g for 10 min, 4 °C) and the serum tested for agglutinating activity.

Antimicrobial susceptibility. Susceptibility to different antimicrobial agents was determined using a VITEK AutoMicrobic system (VITEK-AMS; bioMérieux) with GNS 604 cards containing amikacin (2–32 µg ml⁻¹), amoxicillin/clavulanic acid (4/2–16/8 µg ml⁻¹), cefazolin (4–64 µg ml⁻¹), cefepime (4–16 µg ml⁻¹), ceftazidime (4–64 µg ml⁻¹), ceftriaxone (16–128 µg ml⁻¹), cefuroxime (4–64 µg ml⁻¹), ciprofloxacin (1–4 µg ml⁻¹), gentamicin (0.5–8 µg ml⁻¹), meropenem (2–8 µg ml⁻¹), nitrofurantoin (32 µg ml⁻¹), norfloxacin (4–8 µg ml⁻¹), ofloxacin (1–10 µg ml⁻¹), piperacillin (8–64 µg ml⁻¹), ticarcillin/clavulanic acid (32/2–128/2 µg ml⁻¹) and trimethoprim/sulfamethoxazole (2/38–8/152 µg ml⁻¹). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains.

Virulence genes. PCR assays were used to determine the presence of virulence genes, in a large virulence plasmid, which were directly involved with pathogenic entry into epithelial cells and invasive phenotypes observed in the pathogenesis of *Shigella* strains: *ipaA*–*ipaD* genes, which encode Ipa proteins that induce cytoskeletal

rearrangements required for bacterial entry into epithelial cells (Ménard *et al.*, 1996); the *sepA* gene, which encodes a secreted serine protease of the autotransporter family; *ospB* and *ospF* genes, which encode uncharacterized outer *Shigella* proteins; and the *ipaH* gene, which is also associated with virulence (Venkatesan *et al.*, 2001). The primers used in this study are shown in Table 1. ETEC genes for colonization factor antigens CFA1, CS1, CS3, heat-labile (LTa1) and heat-stable (STa, STb) toxins were amplified using specific primers as reported by Bekal *et al.* (2003) and Schultsz *et al.* (1994). Genes encoding CS13 (PCF O9) and CS14 (PCF O166) were also examined by PCR (Sjöling *et al.*, 2007), as were the O unit flippase (*wzx*) and polymerase (*wzy*) genes for *S. boydii* O16-specific antigen biosynthesis (Liu *et al.*, 2006).

rfb-RFLP. A single colony of each *E. coli* strain was grown in Luria-Bertain broth (Difco) at 37 °C in a shaking incubator for 18 h, and the culture used for DNA isolation and PCR amplification. Chromosomal DNA was isolated using the DNeasy blood and tissue kit (Qiagen). Oligonucleotides described by Wang & Reeves (1998) were used to amplify the O antigen gene cluster (Table 1). Long PCR was carried out with the Expand long template PCR system (Roche). The PCR was carried out as follows: a single denaturation step at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 10 s, annealing at 64 °C for 30 s and extension at 68 °C for 15 min; an additional extension step at 68 °C for 7 min. A 35 µl aliquot of PCR product was digested with 2 U *HincII* (Invitrogen) in the supplied buffer. After overnight incubation at 37 °C, restriction fragments were resolved by electrophoresis on 1.3% agarose gel (agarose 1000;

Invitrogen). Fragment sizes were estimated using lambda ladder (Invitrogen).

PFGE. Genomic DNA for PFGE analysis was prepared using 1.5% low-melting-point agarose (Sea Plaque GTG; FMC Bioproducts) and macro restriction analysis of *XbaI*-digested total DNA was carried out as described by Goering (1993). The digested DNA was analysed by electrophoresis in 1.0% agarose, 0.5×TBE, with a Chef Mapper pulsed-field system (Bio-Rad) for 18 h at 6 V cm⁻¹ with switching times from 2.16 to 54.17 s. *Salmonella enterica* serotype Braenderup DNA was restricted with *XbaI* as standard and used as DNA size markers. Gels were photographed under UV transillumination after staining with ethidium bromide (1 µg ml⁻¹). Identical chromosomal banding patterns were considered to represent the same strain. Isolates that differed by three or fewer restriction fragments were classified as subtypes of the same strain, while organisms differing by more than three restriction fragments were considered sufficiently divergent to be considered as a separate strain (Matushek *et al.*, 1996; Goering, 1993). Similarity relationships were assessed by the UPGMA method.

RESULTS

Identification and serotyping

The *E. coli* strains were isolated from 23 faecal samples from children with diarrhoea in 3 different geographical

Table 1. Oligonucleotide primers used in the PCR assay

Primer	Forward sequence (5'→3')	Reverse sequence (5'→3')	Target gene	Product size (bp)	Reference
CFAI	GGT GCA ATG GCT CTG ACC ACA	GTC ATT ACA AGA GAT ACT ACT	<i>cfaI</i>	479	Bekal <i>et al.</i> (2003)
CS1	GCT CAC ACC ATC AAC ACC GTT	CGT TGA CTT AGT CAG GAT AAT	<i>cs1</i>	321	Bekal <i>et al.</i> (2003)
CS3	GGG CCC ACT CTA ACC AAA GAA	CGG TAA TTA CCT GAA ACT AAA	<i>cs3</i>	401	Bekal <i>et al.</i> (2003)
CS13	GGG ACT GCC ACA ATG AAT TT	CAG CAC CAC CTG CTG ATT TA	<i>cs13</i>	178	Sjöling <i>et al.</i> (2007)
CS14	TTT GCA ACC GAC ATC TAC CA	CCG GAT GTA GTT GCT CCA AT	<i>cs14</i>	162	Sjöling <i>et al.</i> (2007)
LtA1	GGC GAC AGA TTA TAC CGT GC	CCG AAT TCT GTT ATA TAT GTC	<i>ltA1</i>	696	Schultsz <i>et al.</i> (1994)
ST Ia	TCT GTA TTA TCT TTC CCC TC	ATA ACA TCC AGC ACA GGC	<i>stla</i>	186	Schultsz <i>et al.</i> (1994)
ST Ib	CCC TCA GGA TGC TAA ACC AG	TTA ATA GCA CCC GGT ACA AGC	<i>stlb</i>	166	Schultsz <i>et al.</i> (1994)
ipaH	TCT CGG CAC GTT TTA ATA GTC TGG	TAA ATC TGC GGG CCG TGG TAG TC	<i>ipaH</i>	446	This study
ipaA	CCC CGG GGC CAA TGA GAA AAC	AGG CCT GTG TCC CCG AGA AAG AGA	<i>ipaA</i>	400	This study
ipaB	ACT CGC CCC AGA TTC ACC AGA	GCC CCA AGT ATT TTC CCA ACA CA	<i>ipaB</i>	447	This study
ipaC	CTC CGC ACT GCA GAA ACT AAA T	GCA AAG AAA TAT CGG GAG AAC TAA	<i>ipaC</i>	406	This study
ipaD	TTC CCT TAC TAT GCT CAA CGA CAC	AGC CGG CAA GAC TGG AAA GAA C	<i>ipaD</i>	404	This study
ipaJ	TTT GGC GAG GAA GCA GAT G	GTG GAG GCC GAC AGG AAC T	<i>ipaJ</i>	460	This study
sepA	GGA GCG CCG GGA GAC CTA	TGC CGC ATC GAG TTT CAG TTT TT	<i>sepA</i>	423	This study
ospF	CTC AGA GTA AGG GGG ATT TTG TTG	CTC TTG CCT TTC ACT TCC ATC TCG	<i>ospF</i>	401	This study
ospB	AAA GCG AAA ATA AAA ACA GTA AAC	TAG GGA AAA AGC TCT TGA CCA TAG	<i>ospB</i>	404	This study
WL 387–388	TTT CAT TTG TCT TGG GAG	ATC TCC CTT ATT YGC GTA T	<i>wzx</i>	910	Liu <i>et al.</i> (2006)
WL 393–394	TTC ACC GCA TAT CTA ATC	TAT GGC GTG GTG TTT ATG	<i>wzy</i>	777	Liu <i>et al.</i> (2006)
rfb 482–412	CAC TGC CAT ACC GAC GAC GCC GAT CTG TTG CTT GG	ATT GGT AGC TGT AAG CCA AGG GCG GTA GCG T	<i>rfb</i>	11 000	Wang & Reeves (1998)

regions: there were 10 (43 %) strains from Mexico, 9 of which were isolated in 1985 and 1 in 1998; 11 (48 %) *E. coli* strains from Egypt, of which 1 strain was isolated in 1997, 2 in 1999 and 8 in 2006; and 2 (9 %) *E. coli* strains from Bangladesh isolated in 2007.

All 23 strains were identified as *E. coli* by VITEK automated and manual identification procedures. Using automated identification, all *E. coli* strains showed the typical biochemical profile of *E. coli*: glucose, lactose, maltose, raffinose, sorbitol and xylose were fermented; sucrose was not fermented; and lysine was decarboxylated. Using manual identification, both gas production from glucose fermentation in the 23 strains and indole production were confirmed. These biochemical assays were consistent with *E. coli* identification but not with the identification of *Shigella*.

Serotyping of the *E. coli* strains with 186 anti-O *E. coli* and 45 anti-O *Shigella* sera showed a positive agglutination reaction against *E. coli* O179-specific and *S. boydii* 16-specific sera, and against a rabbit serum prepared with *E. coli* strain 64474 (Table 2). A serological reaction was also shown against flagellar antigens H2 in 7 strains (30 %), H10 in 2 (9 %) strains, H32 in 12 (52 %) strains and H34 in 2 (9 %) strains.

Absorption assays

To evaluate the presence of common epitopes between *E. coli* O179, *S. boydii* 16 and the 23 *E. coli* strains from this study, serum samples prepared against *E. coli* O179, *S. boydii* 16 and *E. coli* strain 64474 were absorbed with homologous and heterologous antigens. Absorption of *S. boydii* 16 serum with the heterologous *E. coli* O179 antigen removed only the reaction against the O179 antigen. In contrast, when *S. boydii* 16 antiserum was absorbed with either *S. boydii* 16 or *E. coli* 64474 antigens, the agglutination was removed completely for both *S. boydii* 16 and *E. coli* O179 antigens. Absorption of sera prepared against *E. coli* O179 with either *S. boydii* 16 or *E. coli* 64474 antigens showed a significant reduction in the agglutination titre (1:3200 to 1:1600) when tested against the homologous antigen (O179), and a negative response against both the *S. boydii* 16 and *E. coli* 64474 antigens.

Absorption assays of sera prepared against *E. coli* 64474 with *E. coli* O179 antigen showed a negative response against the *E. coli* O179 and a significant decrease in the agglutination titres against *E. coli* 64474 and *S. boydii* 16 antigens (Table 2).

Antimicrobial susceptibility

Eight (35 %) of the twenty-three *E. coli* 64474 strains showed antimicrobial resistance principally to piperacillin. Four (17 %) *E. coli* strains from Egypt showed antimicrobial resistance to piperacillin only. One (4 %) of the Mexican strains was resistant to both piperacillin and trimethoprim/sulfamethoxazole antimicrobials. One (4 %) strain from Egypt was resistant to ciprofloxacin, nitrofurantoin and piperacillin, and two (9 %) *E. coli* strains from Bangladesh were resistant to five antimicrobial agents (ciprofloxacin, norfloxacin, ofloxacin, piperacillin and trimethoprim/sulfamethoxazole).

Virulence genes

PCR assays to determine the presence of *Shigella* virulence genes gave positive results for *ospF* in three (13 %) strains, *ipaB* and *sepA* in two (9 %) strains, and *ipaH*, *ipaA* and *ipaD* in one (4 %) strain. The 23 *E. coli* strains were negative for *ipaC*, *ipaJ* and *ospB* genes. With regard to ETEC genes, *ltA1* and *st1a* were present in 12 (52 %) and 4 (17 %) of the strains, respectively (Fig. 1). Colonization factor *cfa1*, *cs1* and *cs3* was identified in six (26 %), one (4 %) and three (13 %) strains, respectively, while *cs13* was found in nine (39 %) and *cs14* in five (22 %) strains. The *st1b* gene was not detected. Assays to detect the *wzx* and *wzy* genes for *S. boydii* 16 O antigen biosynthesis were positive in 21 (91 %) and 22 (96 %) strains, respectively. Both *wzx* and *wzy* genes were identified in 10 (43 %) of the Mexican strains, as well in the 2 (9 %) Bangladeshi *E. coli* strains. In terms of the Egyptian strains, 9 (39 %) were positive for the *wzx* gene and 10 (43 %) for the *wzy* gene.

PFGE

PFGE analysis (Fig. 1) of the 23 *E. coli* (64474, *S. boydii* 16) strains showed 18 electrophoretic types. Three main

Table 2. Agglutination titres of unabsorbed and absorbed *E. coli* O179, 64474 and *S. boydii* 16 sera

Antigen	Titres of unabsorbed sera			Titres of sera absorbed with boiled cultures					
	<i>E. coli</i> O179	<i>E. coli</i> 64474	<i>S. boydii</i> 16	<i>E. coli</i> O179 absorbed with:		<i>E. coli</i> 64474 absorbed with:		<i>S. boydii</i> 16 absorbed with:	
				<i>S. boydii</i> 16	<i>E. coli</i> 64474	<i>E. coli</i> O179	<i>S. boydii</i> 16	<i>E. coli</i> O179	<i>E. coli</i> 64474
<i>E. coli</i> O179	1:3200	1:800	1:800	1:1600	1:800	–	–	–	–
<i>S. boydii</i> 16	1:1600	1:3200	1:6400	–	–	1:800	–	1:3200	–
<i>E. coli</i> 64474	1:1600	1:1600	1:3200	–	–	1:1600	–	1:1600	–

clusters (I, II, III) were identified within the dendrogram. Cluster I was divided into three subgroups (A, B, C). Subgroup A included the six strains isolated in Egypt in 2006. Five of these Egyptian strains harboured the *ltA1*, *wzx* and *wzy* genes. The majority of the ten strains in subgroup B were isolated in Mexico in 1985. Six strains of subgroup B harboured the *ltA1* gene; eight the *wzx* gene and nine the *wzy* gene. Finally, subgroup C included the two strains isolated in Bangladesh and Egypt in 2007 and 2006, respectively. These strains also harboured genes *wzx* and *wzy*, but only the strain isolated from Egypt was positive for the *ltA1* gene.

The reference strain of *S. boydii* 16 and three strains isolated in different years, namely two in Egypt 1999 and one in Mexico 1998, formed cluster II. The *wzx* and *wzy* genes were detected in the three *E. coli* strains in this cluster with two Egyptian strains harbouring the *ltA1* also. Finally, cluster III included only one of the strains isolated in Egypt in 1997 and the *E. coli* O179:H8 reference strain. The strain isolated in Mexico harboured genes *wzx* and *wzy*,

which were not detected in the *E. coli* O179:H8 type strain. Although these results show a high degree of genetic diversity among the different *E. coli* strains, serological characterization suggests four new possible serotypes in strains isolated from children with diarrhoea (64474:H32/64474:H2/64474:H10/64474:H34).

RFLP

The *rfb*-RFLP *Hinc*II restriction pattern of the 23 *E. coli* strains used in the study was the same as that obtained for *S. boydii* 16 (Fig. 2). This pattern had six bands (2300, 2000, 1400, 1000, 500 and 360 bp) and is clearly different from that obtained for *E. coli* O179 with five bands (3800, 2700, 1700, 1250 and 500 bp).

DISCUSSION

In this study we have characterized the genetic and phenotypic profiles of 23 *E. coli* enterotoxigenic strains

Fig. 1. Dendrogram showing the genetic relationship of the PFGE profiles of *E. coli*. The dendrogram was constructed using Nei's minimum distance by the UPGMA method. The country of origin, year of isolation, serotype and ETEC virulence factors identified in *E. coli* 64474 are shown. +, Gene detected; *, numbers in parentheses are the percentages of positive samples detected; ET, electrophoretic type.

Fig. 2. Agarose gel (1.3%) electrophoresis showing the comparison of the *rfb*-RFLP pattern with *HincII* for *E. coli* O179, *S. boydii* 16 and *E. coli* 64474:H2, 64474:H10, 64474:H32 and 64474:H34. Lane 1, molecular size marker (1 kb ladder; Invitrogen); lane 2, *E. coli* O179; lane 3, *S. boydii* 16; lanes 4 to 26, *E. coli* 38026, 39359, 46529, 47582, 64643, 64474, 64827, 64887, 65164, 97465, 96101, 100282, 100292, 112187, 112189, 112190, 112191, 112192, 112196, 112197, 112198, 114203 and 114615.

isolated at different times from patients suffering from diarrhoea in Egypt, Mexico and Bangladesh. All of the strains presented typical biochemical characteristics of *E. coli*, such as gas production from glucose fermentation, lactose, maltose, raffinose, sorbitol and xylose fermentation, indole production and decarboxylation of lysine, and all shared identity with the *S. boydii* 16 somatic antigen as shown by serological tests and by specific absorption assays. However, in contrast to *S. boydii* 16, which like all *Shigella* strains is non-motile due to a deletion of the *flhDC* flagellar master operon (Tominaga *et al.*, 2005), the *E. coli* 64774 strains were motile and had specific flagellar antigens as detected by serological assays. The majority of the strains isolated in Egypt had an H2 antigen, the strains isolated in Mexico an H32 antigen and those isolated in Bangladesh an H10 antigen. The somatic antigen of strain *E. coli* 64474 was found to be identical to that of *S. boydii* 16 and to be closely related to that of *E. coli* O179. This is not a rare event since numerous reports have documented the presence of common or closely related somatic antigens between different serotypes of *E. coli* and *Shigella*, especially among strains with invasive capacity in both groups (Liu *et al.*, 2008; Li *et al.*, 2009). These include: *S. dysenteriae* 3 and *E. coli* O124 (Jonsson *et al.*, 2006); *S. boydii* 15 and *E. coli* O112ab, which have a similar O antigen structure (Perepelov *et al.*, 2008a); *S. dysenteriae* 2 and *E. coli* O112ac, which are identical except the *E. coli* polysaccharide lacks an O acetyl group (Perepelov *et al.*, 2008b); *S. boydii* 1 and *E. coli* O149 (Liu *et al.*, 2008); *S. boydii* 3 and *E. coli* O167 (Linnerborg *et al.*, 1997); *S. boydii* 8 and *E. coli* O143 (Landersjö *et al.*, 1996); and *S. dysenteriae* 11 and *E. coli* O29, which have the same O structure and antigenic cross-reactivity (Perepelov *et al.*, 2006). In order to confirm whether the antigenic cross-reactivity observed between the studied *E. coli* strains and *S. boydii* 16 using specific sera was related to the biosynthesis of the O antigen genes of *S. boydii* 16, an analysis was carried out to determine the presence of the *wzx* (flippase) and *wzy*

(polymerase) genes that are associated with this characteristic. The presence of the *wzx* and *wzy* genes in the *E. coli* strains used in the study confirmed the existence of a common epitope similar to that found in the *S. boydii* 16 pentasaccharide O unit antigen (Liu *et al.*, 2006).

The 23 *E. coli* strains showed an RFLP pattern of the *rfb* locus similar to that of *S. boydii* 16, thus confirming the similarity of the somatic antigen in the bacteria studied. Some studies have reported RFLP patterns similar to the cluster of the *rfb* gene in *Shigella* and EIEC, such as the pattern between *S. dysenteriae* 2 and *E. coli* O112, *S. dysenteriae* 3 and *E. coli* O124, *S. dysenteriae* 8 and *E. coli* O38, *S. boydii* 12 and *E. coli* O7, and *S. boydii* 4 and *E. coli* O53 (Coimbra *et al.*, 1999, 2000).

The PCR assays used in this study to determine the pathogenic type of the *E. coli* strains (Nataro & Kaper, 1998) showed that they belonged to the enterotoxigenic group since they harboured both genes for the production of heat-labile and heat-stable enterotoxins, and for the production of specific colonization factors. The presence of both colonization factors CS13 and CS14 in some strains has been reported in Latin America (Paniagua *et al.*, 1997), South-East Asia (Qadri *et al.*, 2000) and Australia (Heuzenroeder *et al.*, 1990).

E. coli strains with *S. boydii* 16 antigens have also been found in faecal samples from healthy birds and dairy cattle, which are part of ongoing studies to determine the burden of zoonotic transmission of pathogens in humans associated with diarrhoeal disease. This mechanism could be similar to that described for the transmission of *E. coli* O157 from cattle and sheep to humans (Chapman *et al.*, 1997; Hancock *et al.*, 1997). The lack of sanitation and personal hygiene, together with close contact between cattle and birds could be a viable transmission route for these bacteria in developing areas of the world.

The PFGE analysis of the strains in this study indicates a clustering by geographical region, with the majority of ETEC strains isolated in Egypt in 2006 clustering in subgroup A, the strains isolated in Mexico clustering in subgroup B and those isolated in Bangladesh clustering in subgroup C. The strains from Egypt contained the *Shigella* plasmid virulence genes (*ospF*, *sepA*, *ipaH*, *ipaA*), as well as the characteristic ETEC genes *st1a*, *cs1*, *cfa1*, *cs13*, *sc14* and *ltA1*. This is suggestive that the acquisition of the *Shigella* plasmid genes by the ETEC strains via a mechanism of horizontal gene transfer (Gogarten *et al.*, 2002; Reid *et al.*, 2000). In addition, the transfer of the O antigen gene cluster from *S. boydii* 16 to the ETEC strains, thereby generating new strains with genetic and phenotypic characteristics, was also shown. Plasmids are known to cross species barriers enabling them to be highly effective vehicles for horizontal gene transfer between bacteria that coexist in a specific environment over a determined period of time such as the intestine (Escobar-Páramo *et al.*, 2003; Schubert *et al.*, 2009). Acquisition and loss of functions by bacteria could yield better-adapted pathogenic variants, such as EIEC and *Shigella* (Peng *et al.*, 2009), and increase the ability to adapt to a human host making them successful human pathogens through convergent evolution from diverse genomic backgrounds.

The categorization of these ETEC strains into three distinct clusters suggests that the emergence of these new serotypes is a phenomenon that has taken place more than once in the evolution of *E. coli*. Similar events to those reported in this study have been shown to occur in *Shigella* species, which suggests that their involvement with the evolution of *E. coli* has not been an isolated incident (Pupo *et al.*, 2000; Rolland *et al.*, 1998). The lack of drug resistance, and in particular multidrug resistance, amongst the majority of the studied strains was interesting. All were clinical isolates and the majority were sensitive to the various antimicrobials tested. In contrast, however, two *E. coli* 64474 Bangladesh strains (included in the cluster II subgroup C) showed a resistance pattern similar to that reported by Qadri *et al.* (2005) in *E. coli* strains isolated from clinical cases from the same country. On the basis of this the persistence of *E. coli* resistant clones in that region is suggested. With respect to the Mexican *E. coli* strains (cluster II subgroup B), all except one were sensitive to the antimicrobials tested in the study. This observation differs from those described by Estrada-García *et al.* (2005) and Amábile-Cuevas *et al.* (2010) in studies performed in Mexico, who reported resistance to ciprofloxacin, ampicillin, trimethoprim/sulfamethoxazole and tetracycline by *E. coli* strains isolated from clinical cases and environmental samples. The Mexican *E. coli* 64474 strains in this study were isolated in the mid-1980s in a rural region (Cravioto *et al.*, 1990). It is believed that the changes in antimicrobial resistance observed in the Mexican *E. coli* strains in this study are associated with the indiscriminate use of these antimicrobial agents resulting in the selec-

tion of resistant strains, especially those resistant to the fluoroquinolones.

The overall findings of this study indicate that *E. coli* 64474:H2, *E. coli* 64474:H10, *E. coli* 64474:H32 and *E. coli* 64474:H34 belong to a new serogroup with at least four different pathogenic serotypes showing a somatic antigen identical to that of *S. boydii* 16 and having the pathogenic characteristics of ETEC strains, with a wide geographical distribution. The existence of this type of *E. coli* strain opens up a new discussion as to whether these strains are an antigenic variant of *S. boydii* 16 or whether these strains are primarily composed of ETEC with an *S. boydii* 16 O antigen. Studies are under way to answer these questions.

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REFERENCES

- Amábile-Cuevas, C. F., Arredondo-García, J. L., Cruz, A. & Rosas, I. (2010). Fluoroquinolone resistance in clinical and environmental isolates of *Escherichia coli* in Mexico City. *J Appl Microbiol* **108**, 158–162.
- Bekal, S., Brousseau, R., Masson, L., Prefontaine, G., Fairbrother, J. & Harel, J. (2003). Rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays. *J Clin Microbiol* **41**, 2113–2125.
- Barrow, G. I. & Feltham, R. K. A. (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.
- Chapman, P. A., Siddons, C. A., Gerdan Malo, A. T. & Harkin, M. A. (1997). A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiol Infect* **119**, 245–250.
- Coimbra, R. S., Grimont, F. & Grimont, P. A. D. (1999). Identification of *Shigella* serotypes by restriction of amplified O-antigen gene cluster. *Res Microbiol* **150**, 543–553.
- Coimbra, R. S., Grimont, F., Lenormand, P., Burguière, P., Beutin, L. & Grimont, P. A. D. (2000). Identification of *Escherichia coli* O-serogroups by restriction of the amplified O-antigen gene cluster (*rfb*-RFLP). *Res Microbiol* **151**, 639–654.
- Cravioto, A., Reyes, R. E., Trujillo, F., Uribe, F., Navarro, A., De La Roca, J. M., Hernández, J. M., Pérez, G. & Vázquez, V. (1990). Risk of diarrhea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. *Am J Epidemiol* **131**, 886–904.
- Davidson, G., Barnes, G., Bass, D., Cohen, M., Fasano, A., Fontaine, O. & Guandalini, S. (2002). Infectious diarrhea in children: working group report of the First World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition. *J Pediatr Gastroenterol Nutr* **35**, S143–S150.

- ESNC-IAMS (1958). Supplement to the third report on the *Shigella* group. *Int Bull Bacteriol Nomencl Taxon* **8**, 93–95.
- Escobar-Páramo, P., Giudicelli, C., Parsot, C. & Denamur, E. (2003). The evolutionary history of *Shigella* and enteroinvasive *Escherichia coli* revised. *J Mol Evol* **57**, 140–148.
- Estrada-García, T., Cerna, J. F., Pacheco-Gil, L., Velázquez, R. F., Ochoa, T. J., Torres, J. & DuPont, H. L. (2005). Drug-resistant diarrheogenic *Escherichia coli*, Mexico. *Emerg Infect Dis* **11**, 1306–1308.
- Ewing, W. H. (1986). *Edwards and Ewing's Identification of Enterobacteriaceae*. New York: Elsevier.
- Goering, R. V. (1993). Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. *Infect Control Hosp Epidemiol* **14**, 595–600.
- Gogarten, J. P., Doolittle, W. F. & Lawrence, J. G. (2002). Prokaryotic evolution in light of gene transfer. *Mol Biol Evol* **19**, 2226–2238.
- Hancock, D. D., Besser, T. E., Rice, D. H., Herriott, D. E. & Tarr, P. I. (1997). A longitudinal study of *Escherichia coli* O157 in fourteen cattle herds. *Epidemiol Infect* **118**, 193–195.
- Heuzenroeder, M. W., Elliot, T. R., Thomas, C. J., Halter, R. & Manning, P. A. (1990). A new fimbrial type (PCFO9) on enterotoxigenic *Escherichia coli* O9:H⁻LT⁺ isolated from a case of infant diarrhea in central Australia. *FEMS Microbiol Lett* **66**, 55–60.
- Jonsson, K. H. M., Weintraub, A. & Widmalm, G. (2006). Structural studies of the O-antigenic polysaccharides from *Shigella dysenteriae* type 3 and *Escherichia coli* O124, a reinvestigation. *Carbohydr Res* **341**, 2986–2989.
- Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Sberdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999). Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ* **77**, 651–666.
- Landersjö, C., Weintraub, A. & Widmalm, G. (1996). Structure determination of the O-antigen polysaccharide from the enteroinvasive *Escherichia coli* (EIEC) O143 by component analysis and NMR spectroscopy. *Carbohydr Res* **291**, 209–216.
- Li, Y., Cao, B., Liu, B., Liu, D., Gao, Q., Peng, X., Wu, J., Bastin, D. A., Feng, L. & Wang, L. (2009). Molecular detection of all 34 distinct O-antigen forms of *Shigella*. *J Med Microbiol* **58**, 69–81.
- Linnerborg, M., Wollin, R. & Widmalm, G. (1997). Structural studies of the O-antigenic polysaccharide from *Escherichia coli* O167. *Eur J Biochem* **246**, 565–573.
- Liu, B., Senchenkova, S. N., Feng, L., Perepelov, A. V., Xu, T., Shevelev, S. D., Zhu, Y., Shashkov, A. S., Zou, M. & other authors (2006). Structural and molecular characterization of *Shigella boydii* type 16 O antigen. *Gene* **380**, 46–53.
- Liu, B., Knirel, Y. A., Feng, L., Perepelov, A. V., Senchenkova, S. N., Wang, Q., Reeves, P. R. & Wang, L. (2008). Structure and genetics of *Shigella* O antigens. *FEMS Microbiol Rev* **32**, 627–653.
- Matushek, M. G., Bonten, M. J. M. & Hayden, M. K. (1996). Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *J Clin Microbiol* **34**, 2598–2600.
- Ménard, R., Prévost, M. C., Gounon, P., Sansonetti, P. & Dehio, C. (1996). The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. *Proc Natl Acad Sci U S A* **93**, 1254–1258.
- Nataro, J. P. & Kaper, J. B. (1998). Diarrheogenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142–201.
- Ørskov, F. & Ørskov, I. (1984). Serotyping of *Escherichia coli*. In *Methods in Microbiology*, vol. 14, pp. 43–112. Edited by T. Bergan. London: Academic Press.
- Paniagua, M., Espinoza, F., Ringman, M., Reizenstein, E., Svennerholm, A. M. & Hallander, H. (1997). Analysis of incidence of infection with enterotoxigenic *Escherichia coli* in a prospective cohort study of infant diarrhea in Nicaragua. *J Clin Microbiol* **35**, 1404–1410.
- Peng, J., Yang, J. & Jin, Q. (2009). The molecular evolutionary history of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Infect Genet Evol* **9**, 147–152.
- Perepelov, A. V., Wang, Q., Senchenkova, S. N., Shevelev, S. D., Zhao, G., Shashkov, A. S., Feng, L., Knirel, Y. A. & Wang, L. (2006). Structure of a teichoic acid-like O-polysaccharide of *Escherichia coli* O29. *Carbohydr Res* **341**, 2176–2180.
- Perepelov, A. V., Liu, B., Senchenkova, S. N., Shashkov, A. S., Feng, L., Knirel, Y. A. & Wang, L. (2008a). Structure of the O-polysaccharide of *Escherichia coli* O112ab containing L-iduronic acid. *Carbohydr Res* **343**, 571–575.
- Perepelov, A. V., Weintraub, A., Liu, B., Senchenkova, S. N., Shashkov, A. S., Feng, L., Wang, L., Widmalm, G. & Knirel, Y. A. (2008b). The O-polysaccharide of *Escherichia coli* O112ac has the same structure as that of *Shigella dysenteriae* type 2 but is devoid of O-acetylation: a revision of the *S. dysenteriae* type 2 O-polysaccharide structure. *Carbohydr Res* **343**, 977–981.
- Pupo, G. M., Lan, R. & Reeves, P. R. (2000). Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. *Proc Natl Acad Sci U S A* **97**, 10567–10572.
- Qadri, F., Das, S. K., Faruque, A. S., Fuchs, G. J., Albert, M. J., Sack, R. B. & Svennerholm, A.-M. (2000). Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. *J Clin Microbiol* **38**, 27–31.
- Qadri, F., Svennerholm, A.-M., Faruque, A. S. G. & Sack, R. B. (2005). Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* **18**, 465–483.
- Raetz, C. R. H. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635–700.
- Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K. & Whittam, T. S. (2000). Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* **406**, 64–67.
- Rolland, K., Lambert-Zechovsky, N., Picard, B. & Denamur, E. (1998). *Shigella* and enteroinvasive *Escherichia coli* strains are derived from distinct ancestral strains of *E. coli*. *Microbiology* **144**, 2667–2672.
- Scheutz, F., Cheasty, T., Woodward, D. & Smith, H. R. (2004). Designation of O174 and O175 to temporary O groups OX3 and OX7, and six new *E. coli* O groups that include verocytotoxin-producing *E. coli* (VTEC): O176, O177, O178, O179, O180 and O181. *APMIS* **112**, 569–584.
- Schubert, S., Darlu, P., Clermont, O., Wieser, A., Magistro, G., Hoffmann, C., Weinert, K., Tenailon, O., Matic, I. & Denamur, E. (2009). Role of intraspecies recombination in the spread of pathogenicity islands within the *Escherichia coli* species. *PLoS Pathog* **5**, e1000257.
- Schultz, C., Pool, G. J., van Ketel, R., de Wever, B., Speelman, P. & Dankert, J. (1994). Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. *J Clin Microbiol* **32**, 2393–2397.
- Sjöling, A., Wiklund, G., Savarino, S. J., Cohen, D. I. & Svennerholm, M.-A. (2007). Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* toxins and colonization factors. *J Clin Microbiol* **45**, 3295–3301.

Tominaga, A., Lan, R. & Reeves, P. R. (2005). Evolutionary changes of the *flhDC* flagellar master operon in *Shigella* strains. *J Bacteriol* **187**, 4295–4302.

Venkatesan, M. M., Goldberg, M. B., Rose, D. J., Grotbeck, E. J., Burland, V. & Blattner, F. R. (2001). Complete DNA sequence and

analysis of the large virulence plasmid of *Shigella flexneri*. *Infect Immun* **69**, 3271–3285.

Wang, L. & Reeves, P. R. (1998). Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infect Immun* **66**, 3545–3551.