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Origin and characteristics of enteroinvasive strains of *Escherichia coli* (EIEC) isolated in Germany

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SUMMARY

Thirty-five *E. coli* strains belonging to O-serogroups associated with enteroinvasive types of *Escherichia coli* (EIEC) isolated in Germany between 1989 and 1995 were investigated for invasivity-associated DNA sequences. Only 11 strains were positive for *ipa*H and thus confirmed as EIEC. All 11 EIEC isolates originated from human infections which were imported to Germany from Eastern Europe. EIEC O124 were most frequent and originated from asymptomatic Romanians arriving at Rostock, Germany in 1992 and 1993. In January 1993, EIEC O124 were isolated from faeces of a laboratory technician with diarrhoea working at the enteric pathogen department of the Institute of Hygiene in Rostock. By comparing her *E. coli* O124 isolate with recently imported O124 strains for *Xba* I restriction fragment length polymorphisms (RFLP) the probable source of infection could be determined. Four major RFLP patterns were found in the group of O124 strains. O124 strains with identical RFLP patterns were found in the group of O124 strains. O124 strains with identical RFLP patterns were isolated from people who were in close contact to each other.

INTRODUCTION

Enteroinvasive strains of *Escherichia coli* (EIEC) cause dysentery in humans and are closely related to shigella in their virulence and other phenotypical properties [1–3]. The known EIEC types are associated with certain *E. coli* O-(lipopolysaccharide) groups such as O28ac, O29, O112ac, O124, O135, O136, O143, O144, O152, O159, O164, and O167 [1, 4, 5]. More recently, additional *E. coli* O-groups such as O121 and O173 were reported as EIEC [6, 7]. The biochemical reactions of EIEC resemble those of shigella, and most of the EIEC-associated O-groups share relationships with O-antigens of *Shigella* species [8]. The colonic cell-invasivity of EIEC and shigella is determined by a number of different genes located on large (140 MD) invasivity (inv)-plasmids and also on the bacterial chromosome [2]. Loss of inv-plasmids and mutations in certain chromosomal sites results in loss of invasive properties in both EIEC and shigella strains [2, 9].

EIEC can be grown from human stool specimens on routine cultivation media [1]. Potential EIEC can be identified by testing invasivity with the Sereny test using laboratory animals [10], but also by identification of bacterial invasivity-associated proteins or genes with specific ELISA tests, DNA-probes or specific PCR assays [11–13]. However, these methods are not easily available for routine diagnosis and are often restricted to a few specialized laboratories. For

this reason, EIEC are often provisionally identified in routine diagnostic laboratories only by O-serotyping with commercially available antisera [1].

The epidemiology of EIEC infections in humans has been investigated in different geographical regions. In Chile, Thailand and Brazil, EIEC were found to be common as diarrhoeagenic agents in humans and in regions where EIEC are endemic, asymptomatic human excretors are frequent [14–18]. In Hungary, EIEC O124 has been isolated frequently from sporadic cases and outbreaks of diarrhoea in humans for many years [19]. In contrast, EIEC are rarely isolated in Western Europe [1, 20, 21]. The epidemiological situation in Europe might have changed since 1989, since tourism and exchange of people between Eastern and Western Europe have increased greatly. The aim of this study was to investigate the epidemiology of EIEC infections in Germany since 1989 and the possible influence of imported infections from regions in Europe where EIEC are endemic.

MATERIALS AND METHODS

Bacteria

The reference strains for enteroinvasive *Escherichia coli* (EIEC) belonging to different O-serogroups were from the collection of the International Escherichia and Klebsiella Centre, Statens Serum Institut, Copenhagen, Danmark (Table 1). These were investigated together with 35 *E. coli* strains belonging to the EIEC-associated O-groups and were isolated in different parts of Germany between 1989 and 1995. The 35 *E. coli* strains were faecal isolates from humans and animals which were sent to the Robert Koch-Institut for analysis of their virulence markers (Tables 2, 3). Representative strains of *Shigella boydii*, *Shigella flexneri* and *Shigella sonnei* were from the collection of the Robert Koch-Institut. One hundred and five faecal *E. coli* isolates from humans with diarrhoea which did not belong to any of the EIEC-associated O-serogroups served as controls for the specificity of the *ipa*H PCR.

Serotyping and biotyping of *E. coli*

Serotyping of O, K and H-antigens of *E. coli* was performed as described previously [22]. Biotyping of *E. coli* was done according to the method of Farmer and Kelly [23].

Sereny test

Invasive properties of *E. coli* strains were examined by the kerato-conjunctivitis test [10].

Preparation of total genomic DNA, *Xba* I digestion and pulsed-field gel electrophoresis (PFGE)

Bacteria were grown at 37 °C overnight from single colonies in 10 ml 1% Bacto-Tryptone (Difco Laboratories, Detroit, MI, USA). Grown cultures were harvested by centrifugation and resuspended in the same volume of SE-buffer (10 mM Tris-Cl, 25 mM EDTA and 75 mM NaCl pH 7·5). After a second centrifugation the bacterial pellet was resuspended in 1 ml SE-buffer. A 300 μl aliquot of concentrated bacteria was mixed rapidly with the same amount of molten 2% Rapid-Agarose (Gibco-BRL, Eggenstein, Germany), followed immediately by casting mixtures in sample inserts in a Bio-Rad sample mould (Bio-Rad Laboratories, Richmond, CA, USA) following the instructions of the supplier. Lysis of bacteria embedded in agarose was performed by 15 h of incubation at 56 °C in lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 1% *N*-laurylsarcosine Na-salt, pH 9·5) containing 0·5 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany). After lysis, agarose plugs were washed five times in sterile TE-buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8·0) followed by 1 h incubation at 37 °C in TE-buffer containing RNase (final concentration 20 μg/ml).

For *Xba* I restriction endonuclease cleavage of genomic DNA the agarose plugs were equilibrated for 1 h at 4 °C in *Xba* I restriction enzyme buffer (Reac 2, Gibco BRL) followed by an overnight incubation in Reac 2 containing 50 units of *Xba* I (Gibco BRL) at 37 °C. After enzymatic digestion, the agarose plugs were washed in 10 mM Tris-Cl, 50 mM EDTA, pH 8·0 and were kept at 4 °C in the same type of buffer for long-term storage.

PFGE was performed with the clamped homogeneous electric field (CHEF-DR II) system from Bio-Rad Laboratories following the instructions of the supplier. A slice of agarose plug was sealed into a well of 1% horizontal agarose-gel as described in the Bio-Rad instruction manual. Lambda concatemers (Bio-Rad) were used as size markers. Gels were run at 200 V for 24 h at 14 °C with a pulse time increasing from 5 to 50 s. After electrophoresis, gels were stained with ethidium bromide for visualization of single bands and photographed under UV light as described previously [24].

Table 1. *Properties of EIEC reference strains*

Strain	Serotype and biotype*	Source (reference)	*ipa*H	*ial* and EIEC-probe	Sereny-test
Kattwijk	O28ac:H$^-$	Human diarrhoea [36]	+	−	−
Guanabara	O112ac:H$^-$	Human diarrhoea [36]	+	−	−
EW227	O124:H30	Human diarrhoea [1, 36]	+	−	−
1111-55	O136:H$^-$	Human diarrhoea [36]	+	+	−
4608-58	O143:H$^-$	Human diarrhoea [36]	+	+	+
1624-56	O144:H$^-$	Human diarrhoea [36]	+	+	+
1184-68	O152:H$^-$	Human diarrhoea [1]	+	+	+
145/46	O164:H$^-$	Human diarrhoea [36]	+	+	−
L119B-10	O173:H$^-$	Human diarrhoea [6]	+	+	−

* All strains were biochemically *E. coli*, inactive [23].

Detection of invasivity-associated DNA-sequences

Chromosomal and inv-plasmid-associated invasivity *ipa*H genes were detected by a specific PCR using primers III 5′-GTT CCT TGA CCG CCT TTC CGA TAC CGT C-3′ and IV 5′-GCC GGT CAG CCA CCC TCT GAG AGT AC 3′ [13]. Inv plasmid-associated DNA sequences were specifically detected with the EIEC gene probe as described [15] and by *ial* PCR using primers I 5′-CTG GTA GGT ATG GTG AGG-3′ and II 5′ CCA GGC CAA CAA TTA TTT-3′ [13].

Detection of *eae*A DNA sequences, shiga-like toxins (SLTs), heat labile (LT) and heat stabile (ST) enterotoxins

*Eae*A-specific DNA sequences were detected by DNA hybridization as described previously [25]. All *E. coli* strains were tested for Vero cell toxicity and vero-toxigenic strains were investigated for the presence of *slt*-I and *slt*-II specific DNA sequences by PCR as described previously [25, 26]. LTI specific DNA sequences were detected by PCR as described by Furrer and colleagues [27]. ST-H (ST Ib) was detected by PCR using primers ST-H Start 5′ TCC CTC AGG ATG CTA AAC-3′ and ST-H End 5′ GCA ACA GGT ACA TAC GTT 3′ [28].

RESULTS

Specificity of the *ipa*H and *ial* PCR for detection of EIEC

The specifity of the *ipa*H-specific PCR for detection of EIEC and shigella strains was tested with nine different EIEC reference strains listed in Table 1 and with single isolates of *Shigella flexneri*, *Shigella sonnei* and *Shigella boydii*. All EIEC and shigella strains reacted positively in the *ipa*H-specific PCR. In contrast, none of 105 human faecal *E. coli* strains which were serologically negative for all of the EIEC-associated O-groups was positive for *ipa*H. Inv plasmid-associated DNA sequences were detected in all shigella strains but in only 6 of the 9 EIEC reference strains indicating that inv plasmids were lost in 3 strains. Only 3 (4608-58, 1624-56 and 1184-68) of the 6 EIEC reference strains which were positive for *ipa*H and inv plasmid-associated DNA sequences were also positive in the Sereny test (Table 1).

None of the strains listed in Table 1 was positive for virulence markers of other types of diarrhoeagenic *E. coli*, such as shiga-like toxins (SLT), *eae*A sequences, heat-stable (ST) or heat-labile (LT) enterotoxin.

Origin and properties of EIEC strains isolated in Germany

Thirty-five *E. coli* strains belonging to the EIEC-associated O-serogroups were investigated for invasivity and other virulence markers (Tables 2, 3). Only 11 of the 35 strains were positive for *ipa*H DNA sequences and were thus identified as EIEC (Table 2). Nine of the 11 *ipa*H positive *E. coli* strains were additionally positive for inv plasmid-associated DNA sequences and were invasive in the Sereny test. The two remaining *ipa*H positive isolates had lost their inv plasmids and were also negative for invasion. Most EIEC isolates were serotyped as O124:H$^-$ and O124:H30. All EIEC isolates were from imported infections of humans arriving from Hungary, Romania and the former Yugoslavia. Indigenous cases from Germany were not found. None of these strains was positive for *eae*A, SLT, LT or ST.

Table 2. *Properties of enteroinvasive* Escherichia coli (*EIEC*) *strains isolated from humans in Germany between 1989 and 1995*

Serotype (no. of strains)*	*ipa*H PCR	*ial* PCR and EIEC probe	Sereny-test	Origin, clinical data and year of isolation
O29:H$^-$ ($n = 1$)	+	−	−	Hungary, enteritis, 1990
O124:H$^-$ ($n = 2$)	+	+	+	Romania, asymptomatic, 1992 and 1993
O124:H30 ($n = 6$)	+	+	+	Romania†, asymptomatic, 1992 and 1993
O143:H$^-$ ($n = 1$)	+	−	−	Hungary, enteritis, 1990
O164:H$^-$ ($n = 1$)	+	+	+	Yugoslavia, asymptomatic, 1994

* All strains were biochemically *E. coli*, inactive [23].
† Including one occupationally acquired infection in Germany causing severe gastroenteritis.

Table 3. *Properties of EIEC O-group related non-enteroinvasive* Escherichia coli *isolated in Germany*

Serotype and biotype* (no. of strains)	Source, origin, year of isolation and clinical data†	Virulence markers detected‡
O28ac:H8 ($n = 1$)	Human, Berlin, 1992	—
O29:H$^-$ ($n = 1$)	Human, Bonn, 1993	—
O29:H10 ($n = 1$)	Human, München, 1990, diarrhoea	—
O29:H10 ($n = 1$)	Human, Stuttgart, 1991	—
O29:H10 ($n = 1$)	Human, Berlin, 1992	—
O29:H10 ($n = 1$)	Human, Berlin, 1995	—
O29:H4 ($n = 1$)	Human, Bonn, 1990 diarrhoea	—
O112ac:H16 ($n = 1$)	Human, Bonn, 1993, diarrhoea	—
O124:H25 ($n = 1$)	Human, Karlsruhe, 1994	—
O124:H40 ($n = 1$)	Human, Herford, 1992, diarrhoea	*eae*A
O136:H12 ($n = 3$)	Bovine, Berlin, 1989, healthy	*slt*-I
O136:H20 ($n = 2$)	Ovine, Berlin, 1989, healthy	*slt*-I
O136:H46 ($n = 1$)	Human, Berlin, 1990, diarrhoea	—
O136:H46 ($n = 1$)	Human, München, 1992, diarrhoea	—
O152:H$^-$ ($n = 2$)	Human, Berlin, 1995, healthy	—
O152:H$^-$ ($n = 1$)	Bovine, Berlin, 1990, healthy	*eae*A
O152:H8 ($n = 1$)	Human, Karlsruhe, 1993	—
O167:H9 ($n = 1$)	Human, Karlsruhe, 1993	*eae*A
O173:F51 ($n = 1$)	Human, Berlin, 1994, AIDS and diarrhoea	—

* *E. coli*, active [23].
† All strains were faecal isolates; if known, clinical data are indicated.
‡ Tested were: Shiga-like toxins SLT-I and SLT-II, heat-labile enterotoxin I (LT), heat-stable enterotoxin (ST), *E. coli* attaching and effacing (*eae*A) specific DNA sequences.

Epidemiological analysis of EIEC O124 strains

Seven EIEC O124:H$^-$ and O124:H30 strains were isolated from Romanian refugees arriving at Rostock, Germany between January 1992 and March 1993 (Table 2). The pathogens were detected by routine examination of stools upon arrival and the excretors did not show symptoms of enteric disease. In January 1993, an EIEC O124:H30 strain (CB2634) was isolated from faeces of a German laboratory technician who had suffered from severe gastroenteritis since December 1992. At that time, she had worked at the Federal Institute of Hygiene in Rostock and had been occupied in routine examination of stool specimens. It was presumed that she could have infected herself while working with these pathogens. The possible epidemiological relationship between the different EIEC O124:[H30] isolates was investigated

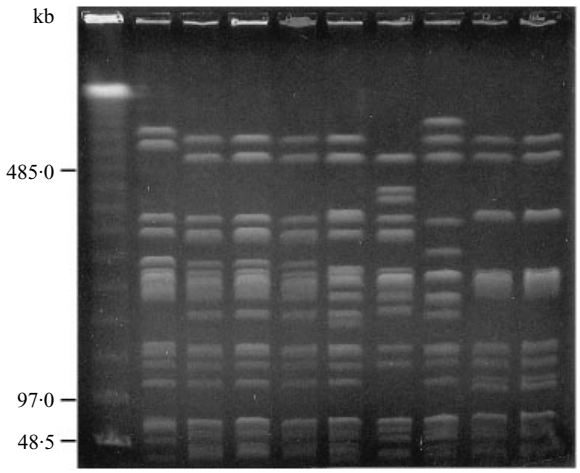

Fig. 1. RFLP of *Xba* I cleaved total DNA of EIEC O124:[H30] strains. Lanes (from left to right): **1**, molecular weight standard (Lambda concatemers), sizes in kb; **2**, EW227 (O124:H30 reference strain); **3**, CB2609 (O124:H30, date of isolation 9.12.92); **4**, CB2610 (O124:H30, 9.12.96); **5**, CB2634 (O124:H30, 6.1.93); **6**, CB2072 (O124:H-, 20.1.92); **7**, CB2638 (O124:H-, 19.1.93); **8**, CB2676 (O124:H30, 3.3.93); **9**, CB2674 (O124:H30, 2.3.93); **10**, CB2675 (O124:H30, 3.3.93).

by comparing strains for their *Xba* I restriction patterns by pulsed-field gel electrophoresis (PFGE) (Fig. 1). The strain CB2634 (lane 5), which was from the stool of the laboratory technician, had an identical *Xba* I restriction pattern as EIEC strains CB2609 and CB2610 (lanes 3 & 4) which were isolated from a Romanian woman and her daughter in December 1992. Thus, it was presented that the three strains were clonally and probably also epidemiologically related to each other. These three O124:H30 strains were also highly similar in their *Xba* I profiles to the O124:H$^-$ strain CB2072 (lane 6) which originated from a 33-year-old Romanian man in January 1992. Identical *Xba* I RFLP-patterns which were different from those described above were also found in two EIEC O124:H30 strains isolated in March 1993 from a pair of young Romanians (lanes 9 & 10). Together, the eight O124:[H30] isolates which originated from Romania could be grouped into four major *Xba* I RFLP profiles which differed in seven bands or more from each other (for representatives see Fig. 1, lanes 2, 7, 8 and 9).

Virulence markers of non-invasive *E. coli* strains belonging to EIEC-associated O-serogroups

Twenty-four *ipa*H and *ial* negative *E. coli* isolates belonging to eight different EIEC-associated O-groups were identified (Table 3). In contrast to many EIEC most of these were motile and all were biochemically active (Table 4). Six strains originated from animals and were positive for slt-I (O136:H12 and O136:H20) or for *eae*A (O152:H$^-$). *Eae*A sequences were also present in both an O124:H40 and O167:H9 strain isolated from humans. All other strains were negative for SLTs, *eae*A, LT or ST.

DISCUSSION

Epidemiological studies performed in different areas have shown that EIEC occur worldwide and are an important cause of diarrhoeae and dysentery in humans [1, 29]. EIEC were found to be more frequent in some countries than in others and similar to shigella. EIEC infections are commonly associated with poor sanitation and low socio-economic status rather than with tropical regions *per se*.

In contrast to shigella, EIEC are more difficult to identify from clinical specimens because of their type variability and because of the lack of established methods suitable for routine detection [1, 29]. Some clinical laboratories use O-serotyping of EIEC-associated O-groups for their detection [1]. Of the 35 *E. coli* strains belonging to EIEC-associated O-groups which were sent to the Robert Koch-Institut for further examination, 11 were confirmed as EIEC by analysis of *ipa*H specific DNA sequences. The remaining 24 strains differed from EIEC not only by the lack of invasivity-associated gene sequences, but also by their biochemical properties (Table 4). We analysed the 24 strains for virulence markers which are typical for human diarrhoeagenic *E. coli* pathogroups such as enteropathogenic (EPEC), enterotoxigenic (ETEC) and enterohaemorrhagic (EHEC) *E. coli*. With the exception of two strains (O124:H40 and O167:H9), all human isolates were negative for any of the tested virulence markers. SLT production was found only in O136 strains from animals. Our data indicate that most of the non-invasive 'EIEC-associated O-group' *E. coli* isolates are not significant as human pathogens.

By comparing Brazilian EIEC and non-EIEC strains sharing common O-antigens, Toledo and Trabulsi [30] observed a high degree of association between absence of lysine decarboxylase and invasivity in laboratory animals. Similar findings were made in this study (Table 4). Routine laboratories using only serological screening as an isolation procedure for EIEC should be recommended in addition to examine their isolates for their biotypes in

Table 4. *Biochemical reactions discriminating between O-group related EIEC and non-EIEC strains*

Group of strains (no. of isolates)	Lysine decarboxylase	Lactose fermentation	Rhamnose fermentation	Gas from glucose	Motility
EIEC ($n = 20$)	0	4	8	12	7*
Non-EIEC ($n = 24$)	24	24	23	20	20

* Only O124:H30 strains were motile.

order to circumvent frequent isolation of EIEC–O group related non-invasive *E. coli* strains.

The relatively low incidence of EIEC infections which we have observed does not necessarily reflect the true incidence. EIEC are probably underdiagnosed in Germany as well as in other countries. In contrast to shigella, EIEC are often omitted from routine laboratory examinations for enteric pathogens. Genetic methods for detection of invasive *E. coli* and shigella are still only infrequently used in most routine clinical laboratories. Moreover, commercially available antisera for detection of EIEC groups may not detect all prevalent EIEC types.

Our results indicate that EIEC are not endemic in Germany. All 11 EIEC strains which were isolated in this study originated from human infections imported from Eastern Europe. The enteroinvasive O124:H^- and O124:H30 (O124:[H30]) isolates which were imported from Romania indicate that, as in Hungary, these EIEC types are prevalent in Romania. Epidemiological data from different parts of the world point to an association between certain EIEC O-types and particular geographical regions. In Europe, EIEC O124:[H30] appear to be the most frequent type [19, 21, 31 and this work].

The first characterized EIEC O124:H30 strain (reference strain EW227, Table 1, Fig. 1) was a faecal isolate from an American soldier with diarrhoea in Italy about 50 years ago [1]. The O124:[H30] strains which were investigated in this work were found to be unrelated to this strain in their *Xba* I restriction profiles. By analysing RFLP in O124:[H30] we found that strains which were isolated from members of families were closely genetically related. However, four major different *Xba* I restriction patterns were found amongst the seven O124:[H30] isolates from Romania, indicating that these strains were heterogeneous [32].

The laboratory-acquired infection with an EIEC O124:H30 strain described here indicates that symptomless carriers of EIEC represent a health risk for exposed people, particularly in regions where EIEC are not endemic. It appears likely that in Germany more sporadic infections due to imported EIEC occur than are detected. Such imported infections are more easily detected if there are large outbreaks, such as that described for EIEC O143:H^- in Houston, Texas, in 1985 [33]. It is also likely, however, that the likelihood of further spread of EIEC in regions with high quality sanitary conditions and high socio-economic status is limited. This may explain why EIEC are not endemic in Germany and in other industrialized countries with high standards of hygiene [34, 35].

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