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Enteroaggregative *Escherichia coli* associated with an outbreak of diarrhoea in a neonatal nursery ward

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SUMMARY

Over a 9-day period in February 1995, 16 newborn babies (age range 2–11 days) and 3 infants (24, 47 and 180 days of age) in a neonatal nursery ward developed diarrhoea accompanied by pyrexia and weight loss. Known enteropathogens were not detected in their stools but *Escherichia coli* displaying aggregative adherence to HEp-2 cells (enteroaggregative *E. coli*) were found in 12 (63%) ill infants and in none of 5 well neonates ($P = 0{\cdot}02$). The illness lasted 3–9 days (mean 5·2) in 16 babies, whereas in 3 neonates it showed a protracted course of 18–20 days. The source of infection and the mode of transmission remained unclear. The outbreak isolates manifested properties common in this new group of diarrhoeagenic *E. coli*: mannose-resistant haemagglutination, haemolysis on blood agar, and clump formation in liquid culture medium. They belonged to the O4 *E. coli* serogroup and expressed multiple antibiotic resistance.

INTRODUCTION

Diarrhoeagenic *Escherichia coli* are divided into four groups on the basis of their virulence properties: enterotoxigenic *E. coli* (ETEC) produce heat-labile (LT) and/or heat-stable (ST) enterotoxins, enteroinvasive *E. coli* (EIEC) invade epithelial cells, verocytotoxin-producing *E. coli* (VTEC) elaborate verocytotoxin (VT), and enteropathogenic *E. coli* (EPEC) attach to enterocytes and cause destruction of brush border microvilli; they also adhere to epithelial tissue culture cells (HEp-2, HeLa) by the formation of bacterial microcolonies referred to as localized adherence (LA). Recently, two new categories of potentially diarrhoeagenic *E. coli* that do not belong to any of the above groups and that adhere to epithelial cells have been described: diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC). DAEC bind uniformly to the entire surface of HEp-2 cells, whereas EAggEC exhibit a third distinct pattern of adherence, so-called aggregative adherence (AA), characterized by 'stacked brick' aggregates of bacteria attached to the surface of cultured cells and, sometimes, to the glass surface between cells [1, 2]. Epidemiological studies performed to date have yielded conflicting results on the role of DAEC and EAggEC as causative agents of diarrhoeal diseases [3–10].

We describe an outbreak of diarrhoeal illness in a neonatal nursery ward with the apparent intestinal colonization of affected neonates with an EAggEC strain.

THE OUTBREAK

The outbreak occurred in the neonatal nursery ward of the Gynaecologic–Obstetrics Clinic (GOC) at the University Clinic Centre (UCC) in Niš, Serbia. The ward consists of four 15–20 baby bed rooms. Cases occurred in only two rooms. The first baby with fever, loss of weight and frequent liquid stools was registered on Tuesday, 7 February 1995. During the next 4 days six new cases with the same signs of illness occurred. The outbreak peaked on 13 February and after 2 days the last new case was registered (Fig. 1). The ill neonates were isolated and on 15 February they were transferred to the Clinic for Infectious Diseases of the UCC for further treatment. Well babies were discharged or displaced to another ward of the GOC, adapted for that purpose. The ward was closed for 2 days for cleaning and disinfection and after that no further cases have been registered among new-borns admitted to the ward. On 14 February the epidemic was reported to the Public Health Centre (PHC), Niš, when the investigation of the outbreak started.

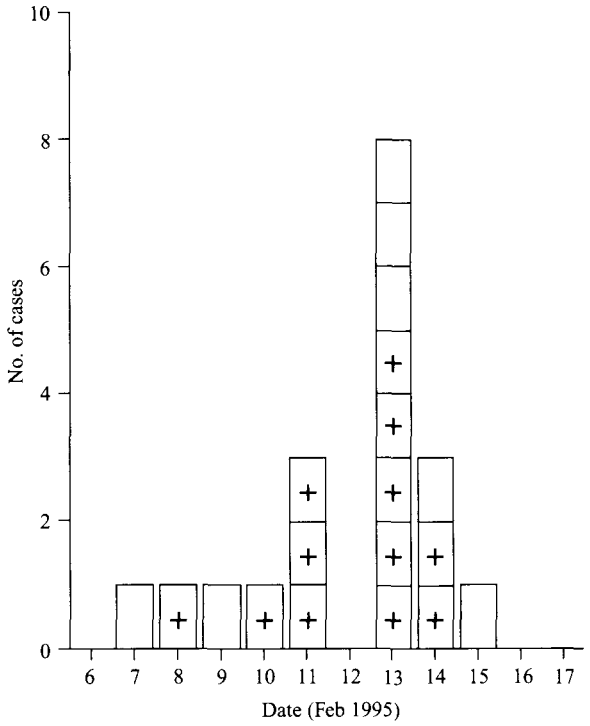

Fig. 1. Diarrhoeal illness by date of onset in the neonatal nursery ward, Niš, Serbia, February 1995. + Enteroaggregative *E. coli* positive case.

METHODS

Specimens

Follow-up stool samples collection from the ill neonates and infants and from 5 babies born on 6 or 7 February who remained well throughout the course of the outbreak started on 14 February. Stool specimens or rectal swabs were also taken from 10 mothers and from 105 staff members of the GOC. Swabs moistened in sterile distilled water were taken from personnel hands and clothes, as well as from items and the environment of the nursery ward, the delivery room, and the operating theatre of the GOC.

Culture

Standard laboratory procedure was used for isolation and identification of salmonella, shigella, yersinia, campylobacter, vibrio, aeromonas, plesiomonas, *E. coli*, klebsiella and *Staphylococcus aureus* [11]. Up to five *E. coli* colonies obtained from any specimen were tested for agglutination [12] with the following antisera (Institute of Immunology, Zagreb, Croatia): O1, O2, O4, O6, O18, O25, O26, O44, O55, O75, O78, O86, O111, O112, O114, O119, O124, O125, O126, O127, O128, O142 and O157. To screen for rotavirus, Rotalex test (Orion Diagnostica, Espoo, Finland) was used. During the processing of specimens it was noticed that the majority of cultures from the ill neonates stools yielded a heavy growth of *E. coli* colonies. Since these isolates agglutinated in the *E. coli* O4 antiserum and exhibited almost the same pattern of resistance to antimicrobials, they were seeded on nutrient agar slants and sent to the Institute of Preventive Medicine, Military Medical Academy, Belgrade, for further investigations.

Test for *E. coli* O4 isolates

Twenty-three *E. coli* O4 isolates were saved. The confirmation of serogroup of these isolates, tests for hydrophobicity and for mannose-resistant haemaglutination of human erythrocytes (MRHA), and for enterotoxin production on CHO K-1 cells (LT) and in the infant mouse (ST) were carried out as previously described [5, 13]. The production of VT was assayed on Vero cells according to Karmali [14]. The invasiveness of these isolates was tested by Sereny's test [15]. The ability of the isolates to adhere to HEp-2 cells in the presence of 1 % D-mannose was investigated using a 6 h incubation period with a wash step after the first 3 h of incubation [16]. The isolates were tested for resistance to ampicillin, cephalosporin, chloramphenicol, kanamycin, streptomycin, tetracycline, ceftri-

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axone, gentamicin, co-trimoxazole and nalidixic acid by the disk diffusion method of Bauer and colleagues [17]. Experiments for drug resistance transfer by conjugation were carried out with *E. coli* K-12, DH-1 (F^-, lac^-, Nal^{-r}) as a recipient strain. The examination of the presence of plasmids in *E. coli* O4 isolates was done by Brinboim and Doly alkaline extraction procedure, modified by Ish-Horowitz [18].

Statistics

Fisher's exact test was used (Epiinfo version 5.0 computer software) to compare the differences in frequencies of isolation of *E. coli* O4 in neonates with and without diarrhoeal disease.

RESULTS

During 4–15 February, 19 of 120 neonates developed diarrhoeal illness (attack rate 16%). All of them were maturely born babies: 11 were male and 8 were female. Sixteen were neonates aged 2–11 days (mean 4·7), and 3 were older infants (24, 47 and 180 days of age) awaiting transfer to an orphanage. One baby had Down's syndrome, but all the others were otherwise healthy. All cases had frequent liquid green, odourless stools. Three cases were noted to have mucus in stools. None had visible blood in stools. Eleven babies had temperatures above 38 °C, and in eight it exceeded 38·5 °C. Only one neonate had vomiting. All suffered loss of weight and all but the three oldest required intravenous rehydration and alimentation. All the ill neonates were treated parenterally with antibiotics (ceftriaxone, 50 mg per kg per day during 3–5 day course). In 16 ill neonates signs of illness lasted 3–9 days (mean 5·2), whereas 3 babies experienced a protracted course of disease for 18–20 days. All fully recovered.

Recognized enteric pathogens were not detected in the cases. *E. coli* O4 was isolated from stools of 12 (63%) out of 19 with diarrhoea and from none of 3–5 stool specimens taken from each of 5 babies who remained well during the course of the outbreak ($P = 0{\cdot}02$). *E. coli* isolates obtained from stools of the remaining seven ill neonates did not agglutinate with the available antisera. A strain of *E. coli* O4 serogroup was also isolated from stool of a mother whose baby developed diarrhoea on 14 February. She was admitted to the GOC on 10 February, and on the same day she underwent caesarean section. She had several loose stools while in the hospital, but she attributed it to a laxative she had received before operation. The outbreak strain was not isolated from the 114 stool specimens or rectal swabs taken from the other mothers and the GOC staff members. No enteropathogens or coliforms were recovered from 111 swabs taken from hands and clothes of the staff, as well as from items and the environment of the nursery ward, the delivery room, and the operating theatre. The duration of excretion of *E. coli* O4 was 2–6 days (mean 4·2) in all except one neonate (her mother's stool was *E. coli* O4 positive) who excreted this strain for 17 days, although signs of illness ceased after 8 days.

Twenty-three saved *E. coli* O4 isolates were obtained from 23 stool specimens. The numbers of these isolates per baby were: 6 from 1, 2 from 5, 1 from 6 babies, and 1 isolate from *E. coli* O4 positive mother. Tube agglutination test confirmed that they were of O4 serogroup. Neither of them produced LT, ST or VT, nor was invasive in the Sereny test. In the adherence test with HEp-2 cells all of them displayed aggregative pattern of adhesion, with the clumps of bacteria attached to the surface of cultured cells (Fig. 2). These isolates also caused MRHA and were haemolytic on blood agar with 5% sheep erythrocytes. They expressed hydrophobicity since they autoagglutinated in 0·1 M solution of ammonium sulphate. EAggEC O4 isolates were multiresistant: of 10 antimicrobials tested, 17 isolates were resistant to ampicillin, cephalosporin, chloramphenicol, kanamycin, tetracycline and gentamicin, whereas 6 isolates were resistant to all mentioned antibiotics except kanamycin. Plasmid profile analysis revealed that all of the investigated EAggEC O4 isolates possessed 7 plasmids of which 3 were large (40–60 Mdal), whereas 4 were small plasmids with the sizes 1·4–3·2 Mdal. In conjugation experiments only resistance to ampicillin was transferred from all isolates to *E. coli* K-12. The transfer frequency was 2×10^{-3} per recipient cell and was associated with the transfer of one of the large plasmids. Of 500 ampicillin-resistant transconjugants no one exhibited AA to HEp-2 cells nor did cause MRHA.

DISCUSSION

This is one of a few reports on epidemic occurrence of EAggEC infections, but the first extensively described outbreak of diarrhoea associated with this agent. Scotland and colleagues [16] and Smith and colleagues [19] from the same laboratory reported several small community and family outbreaks which occurred in

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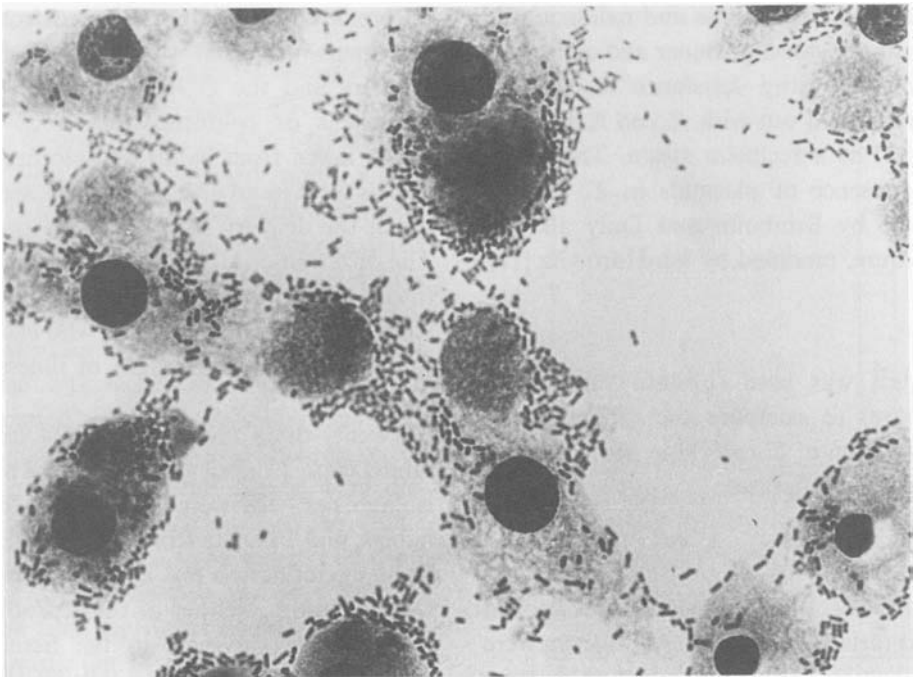

Fig. 2. Aggregative adherence to HEp-2 cells displayed by the outbreak strain of *E. coli*. Magnification × 1000.

Britain, and in which infants, young children, adults and elderly people were affected. In all of these outbreaks strains of *E. coli* belonging to O44:H18 serotype, exhibiting AA to HEp-2 cells, were isolated. Eslava and colleagues [20] reported on an outbreak associated with EAggEC O147:H39 in Mexico, in which 10 infants died. Many more investigations have been devoted to the role of EAggEC in causing sporadic diarrhoea in children in developing countries. The association of these *E. coli* with acute and more prominently with persistent diarrhoea (lasting longer than 14 days) were found in India [4, 9] and Mexico [7], but not in another study in Mexico [6], Thailand [8], Brazil [3] and Chile [10]. Recently, it has been reported that EAggEC may be possible cause of sporadic diarrhoea in adults [21, 22], although not all investigated EAggEC strains elicited diarrhoea in volunteers [23]. Furthermore, the pathogenic potential of these strains was documented in animal models [24, 25]. These data along with the findings of EAggEC-associated outbreaks of diarrhoeal diseases [16, 19, 20] suggest that EAggEC, or at least some clones of these bacteria, could be recognized as a new group of diarrhoeagenic *E. coli*.

Identical plasmid profiles and almost the same phenotypic traits expressed by EAggEC O4 isolates obtained in this study suggest that they originated from a single strain. The outbreak strain isolates exhibited properties common for EAggEC: they caused MRHA [2, 24, 26, 27], were haemolytic [28] and hydrophobic [24], and formed clumps on the surface of liquid culture medium [29]. Knutton and colleagues [27] and Nataro and colleagues [2] described two types of AA: one with bacteria adhering to cultured cells as well as to the glass surface between cells (non-specific AA), and the other when bacteria adhere to cells only (specific AA). The outbreak strain EAggEC O4 could be classified into the later type since in the adhesion assay bacteria were rarely seen between cells. The attachment of bacteria to the surface and to each other has been found to be mediated by various kinds of fimbriae [27] of which aggregative adherence fimbriae I (AAF/I) are best known [2]. These fimbriae also confer MRHA and their genetic determinants are located on 55–65 Mdal plasmid [1, 2, 24].

EAggEC O4 isolates expressed resistance to 5–6 antibiotics, a trait frequently encountered among bacteria causing nosocomial infections. Multiresistant EAggEC have also been isolated from diarrhoeal stools of US soldiers deployed to Egypt [22].

The most common clinical signs in the ill neonates in this outbreak were diarrhoea, fever and loss of weight, but there were no fatal cases, which are frequently registered when other pathogenic *E. coli* are causative agents of diarrhoeal diseases among neonates [30–32]. The presence of blood, described in a proportion of children infected with EAggEC [4, 7],

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was not observed in this outbreak. The mean duration of illness was longer than the duration of EAggEC excretion. The explanation may be that in the late stage of illness the outbreak strain was not the predominant flora in the stools so that microbiological examination failed to detect this agent among other *E. coli* strains.

The source of infection in this outbreak remains unknown. The outbreak strain was not isolated from the GOC staff nor did any of them complain of intestinal disorder before and during the outbreak. The mother from whom EAggEC O4 was isolated is unlikely to be the source since when she was admitted to the GOC four cases of illness had already been registered. Probably she was infected from her baby. Characteristics of the epidemic such as relatively low attack rate and prolonged duration suggest dissemination of infection by direct or indirect contact, which is the most common mode of transmission in nurseries [31, 33].

At the time when the outbreak occurred the potential risk factor was understaffing so that one nurse handled 20–30 babies per day shift. It may be supposed that in that circumstance hygienic practices were not always properly carried out. Although the inspection and the microbiological examination of the GOC environment were correct, they were not a real indicator of hygienic aspects since at that time (14 February) the personnel of the Clinic was aware of the emerging epidemic and strictly implemented hygienic procedures. One feature of the outbreak which is not quite in accordance with contact spread of infection is the cluster of eight new cases of illness, registered on 13 February. The explanation may be that these babies were simultaneously infected by contact with one nurse, but the possibility that contaminated fluid or baby food interfered as a transmission route could not be ruled out.

The data obtained in this study suggest that in searching for causative agent in outbreaks of diarrhoeal diseases EAggEC should also be considered, at least in neonates.

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