CHARACTERIZATION OF ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE ISOLATED FROM NEONATES IN A NURSERY WARD USING DNA PROBES, PLASMID PROFILES, AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS

DANIEL MUGANGAI SENERWA, B.V.M.

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Department of Public Health,
Pharmacology and Toxicology,
Faculty of Veterinary Medicine,
College of Agriculture and Veterinary Sciences,
University of Nairobi.

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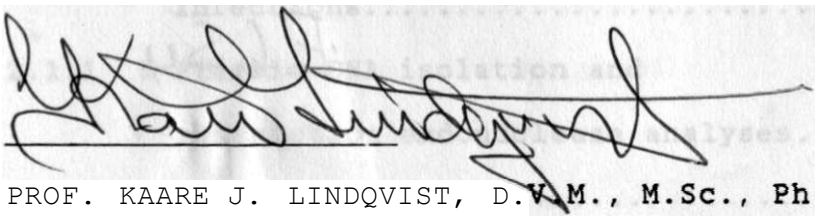
D E C L A R A T I O N

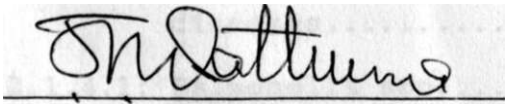
This thesis is my original work and has not been presented for a degree in any other university.

DANIEL MUGANGAI SENERWA

This thesis has been submitted for examination with **our** approval as university supervisors.



PROF. KAARE J. LINDQVIST, D.V.M., M.Sc., Ph D., F.R.C.Pat*



PROF. JOSEPH MWANGI GATHUMA, B.V.Sc., M.Sc., Ph.D.

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ABSTRACT

A laboratory investigation of lactose positive enterobacteria isolated from neonates in the course of several cases of gastroenteritis in a nursery ward is described. During a period of 4 weeks, 30 neonates were involved and 5 of these died. A stool sample was taken from each of the 30 neonates regardless of their clinical status. Ten colonies of lactose positive enterobacteria were obtained from each neonate and identified as Escherichia coli (229 isolates) and Klebsiella pneumoniae (65 isolates). Six strains were lost during laboratory handling.

The pathogenic potential of the strains was examined using an enteropathogenic E. coli adherence factor (EAF) DNA probe. Colony hybridization of DNA from the 294 isolates was performed using the 1 megadalton radiolabelled EAF probe. The EAF probe hybridized with DNA from 78 of the 229 E. coli strains and with none of the DNA from K. pneumoniae strains. The EAF probe positive strains were found in 13 neonates. Colony hybridization tests of DNA from all the E. coli and K. pneumoniae strains using alkaline phosphatase-labelled oligonucleotide probes specific for enterotoxins LT and ST1 of E. coli gave negative results.

There were 28 and 8 different plasmid profile groups of E. coli and K. pneumoniae

respectively. A total of 78 strains of E. coli were shown to belong to one particular plasmid profile group possessing plasmids of molecular weight 65, 1.9, 1.7, and 1.4 megadaltons (MDa). All these strains gave positive results with the EAF probe which was shown to hybridize with the 65 MDa plasmid.

Seven of the EAF probe positive and 6 EAF probe negative E. coli strains were further characterized at Centers for Disease Control, Atlanta, Georgia, USA. The 7"EAF probe positive strains were found to be 0111:HNT (NT=Nontypable). They adhered locally to HeLa cells, and did not produce Vero cell cytotoxins. The 6 EAF probe negative strains all belonged to serotypes other than 0111:HNT, and they neither adhered to HeLa cells nor elaborated Vero cell cytotoxins.

Twenty neonates yielded pure cultures of E. coli, 5 had Klebsiella pneumoniae only, while 5 were positive for both species. The case fatality rates of neonates who harboured EAF probe positive E. coli, EAF probe negative E. coli, and K. pneumoniae were 30.8%, 7.7% and 0% respectively.

Both E. coli and K. pneumoniae strains were resistant to most of the 16 antimicrobial agents tested. In general there was no marked difference in the antimicrobial susceptibility patterns of EAF probe positive and EAF probe negative E. coli strains. However, a lower percentage (10.3%) of the

EAF positive strains were resistant to gentamycin than the EAF negative ones (39.7%). Seventy percent of K. pneumoniae strains were resistant to gentamycin as compared to 30% of E. coli strains. None of the bacterial isolates examined was resistant to cefatoxime, but the majority of the strains (64-99%) were resistant to sulphonamides, trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline, erythromycin, penicillin, and ampicillin. A few strains (1-18.5%) were resistant to cefazolin, cefamandole, amikacin, nalidixan, and nitrofurantoin. No correlation between plasmid profiles and antibiotic resistance pattern was found.

1 INTRODUCTION

Acute infectious enteritis is an important cause of morbidity throughout the world and a major cause of morbidity and mortality of infants in developing nations. The disease is often mild and self-limiting in healthy adults, but in the malnourished, in the aged and in young children, the disease may be severe.

Among the known causes of acute infectious enteritis are: Vibrio cholerae, Shigella spp., Salmonella spp., Escherichia coli, Campylobacter spp., Aeromonas spp., Yersinia enterocolitica, Plesiomonas shigelloides, Giardia lamblia, Entamoeba histolytica, rotavirus and several other viruses (Black et al., 1980). Enterotoxigenic strains of Klebsiella spp., Citrobacter spp., Enterobacter spp., Proteus spp., and Serratia spp. have been isolated from children with diarrhoea in Ethiopia, but the aetiological significance of such organisms awaits confirmation (Gross, 1984).

E. coli cause diarrhoea by different mechanisms (Levine, 1987). Enterotoxigenic E. coli (ETEC) adhere to the mucosa of the small intestine and produce heat-labile (LT) and/or heat-stable (ST) enterotoxin(s) which give rise to diarrhoea. Entero-

invasive E. coli (EIEC) do not produce enterotoxins but are capable of invading and multiplying within colonic epithelial cells. Enterohaemorrhagic E. coli (EHEC) cause haemorrhagic colitis by producing cytotoxins. Enteropathogenic E. coli (EPEC) adhere to epithelial cells of the small intestine and are assumed to produce cytotoxins.

Enteropathogenic E. coli were the first E. coli to be implicated in diarrhoeal disease. Recent reports from developing countries indicate that EPEC is the first or second most important cause of infantile diarrhoea (Levine, 1987). Epidemiological studies of the age-specific and area-specific incidence rates and the elucidation of specific modes of transmission have been hampered by the lack of a simple and reliable test to identify EPEC.

Enteropathogenic E. coli have traditionally been recognized by the presence of certain 0 and H antigens and shown to belong to a relatively small group of distinct serotypes. However, serological identification requires specific reagents frequently limited to central reference laboratories. Motile cultures used for H typing tend to show spontaneous agglutination resulting in inconsistent results between laboratories (0rskov et al., 1987). The classification of a strain as EPEC is further complicated by the fact that, within a serogroup, only certain serotypes have been associated with

diarrhoea. Another, more recently recognized limitation, is the mounting evidence that there is significant genetic diversity within a serogroup and even within the same serotype (Caugant et al, 1985). Identification of EPEC is made even more difficult due to the lack of an ideal animal model for EPEC diarrhoea.

Although serogrouping and serotyping have remained the standard reference methods for the identification of EPEC, several laboratories have encountered serious difficulties in the serotyping of E. coli leading to false diagnosis (Levine and Edelman, 1984; Echeverria et al., 1986; Chatkaemorakot et al., 1987; 0rskov et al., 1987)

Therefore, an obvious need for improved methods for the identification of EPEC has been recognized since their original discovery as causative agents of gastroenteritis. Identification of EPEC by genetic markers offers a potential tool for bypassing problems associated with the definition of gene products. Nataro et al. (1985b) described the use of a highly specific and sensitive EPEC adherence factor (EAF) DNA probe to identify classic serotype EPEC strains isolated from cases of infantile enteritis. Some of the EPEC strains used in their study were given orally to human adult volunteers and caused diarrhoea (Levine et. al., 1978). This probe has been used to successfully identify EPEC

strains isolated from diarrhoeic infants (Chatkaemorakot et al., 1987; Bopp et al., 1988).

In the present study, attempts have been made to characterize by genetic analyses lactose positive enterobacteria. The bacterial strains were isolated from 30 neonates in a nursery ward in the course of an outbreak of gastroenteritis during which 5 neonates died.

The main objectives of the present study were:

1. To evaluate the use of the EAF probe for the identification of EPEC isolates.
2. To determine the location of genes that encode EAF.
3. To analyse the plasmid profiles of the lactose positive enterobacteria isolated from the neonates and assess the use of plasmid profiling for the identification of bacterial clones.
4. To classify the plasmid profile groups in an attempt to determine the lactose positive entero-bacterial colonization pattern and assess the role played by EPEC in this outbreak of diarrhoea.
5. To determine the prevailing antimicrobial resistance pattern and its possible use for therapeutic intervention.

2 REVIEW OF LITERATURE

2.1 Molecular epidemiology of bacterial infections

Bacteria have traditionally been defined by genus, species, biovar, serovar, pathovar, phagovar, and morphovar (Staley and Krieg, 1984). These phenotypic characterizations are based on specific assays for gene products. The identification of epidemic strains by genetic markers offers the potential of bypassing a number of problems inherent in the classical methods of bacterial identification and characterization.

Plasmid profile analysis, plasmid and chromosomal bacterial restriction endonuclease DNA analysis, and DNA hybridization are increasingly being use in clinical microbiology and epidemiology. These DNA techniques have been shown to be useful in unravelling nosocomial and community acquired infections.

2.1.1. Plasmid DNA isolation and restriction endonuclease analyses

Plasmids are extrachromosomal elements that represent a dispensible gene pool found in most bacterial species. Plasmid DNA can be isolated from an overnight bacterial broth (Birnboim and Dolly, 1979; Kado and Liu, 1981). The principle of the methods is based upon the disruption of the bacterial

cell wall by treatment with lysozyme; lysis of internal cell membranes with detergent; and denaturation of chromosomal DNA by alkaline pH. Plasmid DNA is recovered by ethanol precipitation in the cold. Plasmid DNA is electrophoresed through vertical or horizontal agarose slab gel which separate it on the basis of molecular mass during migration towards the anode.

Most of the bacterial plasmid DNA exists in the covalently closed circle (CCC) form (Hardy, 1981). If one of the two polynucleotide strands of CCC plasmid is broken, an open circle (OC) is formed. When both polynucleotide strands are broken, a linear (L) molecule is formed if the two breaks are either exactly opposite, or so close together that the hydrogen bonds between intervening complementary bases are not strong enough to hold the two strands together. Application of shearing forces during plasmid DNA isolation results in the formation of OC form(s). If the shearing forces are excessive, L form(s) may result. Some of the large plasmids are difficult to keep in CCC form during isolation and purification. Different forms of plasmid DNA migrate through agarose gel at different rates during electrophoresis. The rates of migration are in the following decreasing order, CCC, OC, and L (Hardy, 1981).

Different forms of the same plasmid can be

identified by restriction endonuclease analyses. Plasmids may be maintained in the CCC form during isolation by applying minimum shearing force. Recently, some linear plasmids with covalently closed ends have been described (Mayer, 1988). These plasmids encode for outer surface proteins in Borrelia burgdorferi and antibiotic biosynthesis in Streptomyces spp (Mayer, 1988) . During chromosomal DNA isolation, the cells are lysed and proteins are removed by sequential extractions with phenol and chloroform (Brenner el ah, 1969).

The DNA isolated in the above described procedures is of sufficient quality for restriction endonuclease analyses. Restriction endonucleases are enzymes that recognize specific, palindromic, base sequences and cleave those sequences at a defined position. The number of fragments generated should be sufficient for specificity but not so many that coincidental matching of bands occurs (Piatt et al„ 1986).

2.1.2 DNA Hybridization

Hybridization techniques are based on the principle of DNA base pairing. The hydrogen bonds that join complementary bases can be broken with heat or alkali treatment; the double helix will be denatured, and single strands of DNA will be formed (Meinkoth and Wahl,1984). Single strands from one

source can be joined with single strands from a second source under appropriate conditions of pH, temperature, and ionic strength. If the DNA from one source is labelled with a marker such as ^{32}P and is used as a probe, it is possible to detect the hybrids that have been formed (Rigby et. al., 1977).

For bacterial pathogens, the diagnostic probe may be a portion of a virulence gene or a restriction fragment cleaved at random from the organism's chromosome. The fragments of DNA need not be a whole gene (Moseley et al., 1980; 1982), nor need it be from a sequence that encodes a protein (Fitts et al., 1983; Fitts, 1985).

Under conditions of high stringency, only exact matches of DNA will hybridize and stay together. Under conditions of low stringency (low temperature, high salt concentration, or low concentrations of formamide), two strands that are 80-90% homologous may bind together and result in a positive hybridization signal (Tenover, 1988). If the salt concentration or temperature of hybridization is altered, the specificity of the probe will change. The range of conditions that can be tolerated without affecting the specificity of a probe vary depending on the length of the probe and the percentage of guanine and cytosine residues in the probe. The shorter the probe, the more narrow the range of salt concentration and temperature that can be tolerated.

Conventional probes are longer than synthetic (oligonucleotide) probes. The synthetic probes are normally 14-40 basepairs in length and have a high specificity (Tenover, 1988). Under stringent conditions, oligonucleotide probes may detect a change in a single basepair of a DNA or RNA sequence, which is enough to prevent binding to target DNA.

Oligonucleotide probes have several advantages over conventional probes (Tenover, 1988). They are stable for along time and are relatively simple to prepare. Only small amounts are required for hybridization. They hybridize at rapid rates with reaction times of less than 30 minutes. Conventional probes require 4-16 hours for complete hybridization, even with rate enhancers such as dextran sulphate.

Hybridization reactions can be performed on a solid support, in solution, in situ or by using a Southern blot. Nitrocellulose filters, synthetic nylon filters, Whatman no. 541 paper, and microtitre plates are used as solid supports (Gruinstein and Hogness, 1975; Wahl et al., 1979; Maas, 1983; Meinkoth and wahl, 1984).

In solution hybridization, both the target and probe nucleic acid, are free to move, thus increasing the chance of complementary strands aligning. Solution hybridizations go to completion 5-10-fold faster than on solid supports

(Bryan el al. , 1986). After solution hybridization hydroxyapatite is used to selectively bind duplex DNA, leaving single stranded nucleic acids in solution. The duplex DNA is recovered from hydroxyapatite either by increasing salt concentration, healing, or by centrifugalion (Kohne et al. ,1986). A variation of the solution hybridization involves the attachment of the probe DNA to micromagnetic beads, followed by hybridization and removal of the labelled duplex DNA from solution with a magnet (Van and Klausner, 1987).

In most instances, in situ hybridization is carried out on formalin-fixed paraffin-embedded tissues. This technique has proven particularly useful fo detection of viral pathogens.In this method, cytopathology is indicated by areas demonstrating homology to horseradish peroxidase or alkaline phosphatase-labelled probes (Brigatti et al, 1983).

After agarose gel electrophoresis,the DNA can be alkaline denatured followed by neutralisation and trasfer to nitrocellulose by the Southern blot technique (Southern, 1975). The single strands are then fixed on the nitrocellulose by healing at 80°C. The filler is placed in a solution containing the probe, and hybridization is usually determined by autoradio-graphy.

2.1.3 DNA Labelling

The methods used to detect binding of a

probe to target DNA are radioactive labels, biotin-avidin systems, enzyme conjugation, and antibodies. The most commonly used detection system is isotopic labelling where the isotope is directly incorporated into the probe by nick translation (Rigby et al., 1977). The binding of probe DNA to the target is detected by autoradiography or by liquid scintillation counting. This method has the highest sensitivity of all the known methods to date (Tenover, 1988). The shortcomings of radiolabelling include the availability of X-ray film processing equipment or a scintillation counter, frequent probe preparation, and safety.

In the biotin-avidin system, biotin molecules are introduced into the DNA probe and later reacted with enzyme-labelled avidin or antibody. A reaction between the enzyme and its substrate yields a coloured product (Al-hakin and Hull, 1986). The sensitivities of this method are equivalent to those of radiolabelling **and** commercial kits are currently available.

Enzyme-conjugated probes involve the attaching of an enzyme, such as alkaline phosphatase, directly to the probe DNA by using a 12 atom linker arm (Ruth, 1984; Jablonski et al., 1986). This method is best suited for oligonucleotide probes and has been used with good results to detect enterotoxigenic E. coli (Jablonski et al., 1986). Alkaline

phosphatase-labelled probes were found to be less sensitive than ^{32}P labelled probes (Seriwatana et al., 1987).

Antibodies recognise chemically labelled antigenic moieties covalently linked to nucleotides contained within a probe (Tchen et a^., 1984). The antibodies can be labelled with enzymes or other reporter molecules.

2.1.4 Use of DNA techniques in investigation of outbreaks of diseases

Deoxyribonucleic acid transfer between bacteria occurs by conjugation, transduction, and transformation. Bacterial plasmid DNA can encode conjugation, resistance to noxious agents (antibiotics), pathogenicity factors (colonization fimbriae), and enzymes catalysing metabolic reactions (lysine decarboxylase) (Wachsmuth, 1986).

Plasmid analyses cannot be used in bacterial epidemiology if the strains lack plasmids. The plasmid pattern of bacteria keeps on changing with time. The chance of change in the plasmid pattern increases with time, so the method is best applied to isolates made over a limited period (Hawkey, 1987). Virulence plasmids may serve not only as epidemiological markers but also as an indication of pathogenecity. DNA techniques have been shown to be useful in the identification of bacterial pathogens

involved in disease outbreaks described below.

2.1.4.1 Salmonella spp.

A plasmid profile of Salmonella muenchen remained stable to many potential donors in the human intestine, was stable for over three months in marijuana, and was stable when isolated from individuals from geographically diverse areas of the United States (Taylor et al., 1982). This stable plasmid profile proved to be a specific indicator of a common source outbreak.

In most cases, restriction endonuclease analyses are needed for sufficient strain identification. Riley et al. (1983) investigated outbreaks of Salmonella newport that occurred in the United States of America in June-August 1981. Using the plasmid profiles and restriction endonuclease patterns, they traced the source of human infection to commercial roast beef. Plasmid fingerprinting was used to document the transmission of a resistant Salmonella newport from animals fed subtherapeutic levels of antibiotics to humans (Holmberg et al., 1984a). Using the same techniques Olsvik ejt al. (1985b) showed human infection by both antibiotic sensitive and resistant Salmonella typhimurium acquired from animals not fed antibiotics.

S0rum et al. (1986) used plasmid profiles and restriction endonuclease patterns to map a

nosocomial epidemic caused by Salmonella enteritidis in Oslo, Norway. The findings of their study established the source of infection to two patients who had visited the mediterranean area. The infection later spread to other patients in the same ward and also to the kitchen staff. This finding helped in controlling the outbreak by detecting both the sick and symptomless carriers.

In a retrospective study of S. typhimurium outbreaks Holmberg et al. (1984b) compared the effectiveness of plasmid profile analyses with that of antibiograms and phage typing. Plasmid profiles were found to be at least as helpful as phage typing but both of the former methods were better than antibiogram.

2.1.4.2 K. pneumoniae and E. coli

Plasmid fingerprinting of multiply resistant K. pneumoniae from sequential epidemics revealed that the epidemic strains were quite different and not from a common or continuing exposure (John et a^, . 1983).

In an E. coli diarrhoea epidemic, one toxigenic strain, and one nontoxigenic strain of E. coli 025:NM (NM=Nonmotile) were isolated from the same patient. The plasmid profiles of these two strains were identical but the restriction endo-nuclease patterns revealed that the toxigenic strain

contained two plasmids of molecular weight approximately 60 MDa (Wachsmuth et al., 1979). Hybridization later confirmed that one of the 60 MDa plasmids contained toxin genes. Another epidemic in 1932 included two separate outbreaks of haemorrhagic colitis caused by coli 0157:H7 (Wells et al., 1983). The plasmid profiles of the organisms involved in the outbreaks were identical but the restriction endcnuclease analyses were different. This difference was useful in linking an E. coli 0157:H7 strain isolated from hamburger meat to only one of the outbreaks, and in evaluating previous and subsequent outbreaks.

Use of LT and ST probes has made it possible to screen bacterial clones for the presence of pathogenic, epidemic, or pandemic strains (Olsvik et al.,1985a; Echeverria et al., 1986). In an investigation of a 1985 outbreak, an ST probe was used to detect enterotoxigenic S. coli in contaminated cheese imported into the United states (MacDcnald et al., 1935).

2.1.4.3 Other bacterial spp.

Plasmid fingerprinting was used successfully in the characterization of biochemically atypical strains of Vibrio salmonicida isolated from salmonids with hemorrhagic syndrome (S0rum et. al., 1988). Many studies have shown that plasmid analyses can be

applied to episodes of nosocomial infection by bacteria for which no pre-existing typing scheme exists e.g Enterobacter sakazakii (Muytjens et al., 1983) , Enterobacter cloacae (Markowitz et. al.. , 1983) , Citrobacter diversus (Williams et. al. , 1984), Pseudomonas cepacia (John and Twitty, 1986), and Legionella pneumophila (Brown et al., 1982) .

Plasmids were found less useful in the characterization of Aeromonas salmonicida s s* salmonicida isolated from an outbreak of fish furunculosis (Kvello et al., 1987), epidemiologically defined outbreaks of Campylobacter jejuni (Bopp et al., 1985), and nosocomial strains of Staphylococcus epidermidis (Parisi, 1985). Despite the limitations, plasmid fingerprinting has definite advantages: the same methods are applicable to most bacterial strains, entire analyses of more than 20 samples can be completed in one day, gene expression is not necessary, and microtechniques conserve reagents. (Wachsmuth, 1986).

Bacterial restriction endonuclease DNA analyses (BRENDA) is helpful in typing bacteria that lack plasmids or have serotype specific plasmids (Brown et. a^. , 1982). The large number of bands, many of which are seen in all strains, means that small differences can be missed easily. Plasmids carried by the strains can contribute to the pattern, the effect being unpredictable (Bradburg et al., 1984).

Application of laser scanning of gels and storage of data on microcomputers may enable more to be made of the information provided by this technique (Hawkey, 1987). These difficulties aside, the technique has been successfully used to type Legionella pneumophila serogroup I (Van et al., 1984), Neisseria meningitidis (Bjorvatn et^ al., 1984) and C. jejuni (Bradbury et_ al., 1984). Hybridization in combination with BRENDA have been used with success in several investigations of Vibro cholerae outbreaks (Kaper et al ., 1982; Morris et al., 1984).

2.2 Diarrhoeagenic E. coli

Because E. coli are common inhabitants of the human gastrointestinal tract, the virulent strains were not recognised as intestinal pathogens until the 1940s. At that time, certain strains were identified as the cause of explosive (often fatal) gastrointestinal illness (Levine, 1987). The four main categories of diarrhoeagenic E. coli are enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), enteropathogenic E. coli (EPEC), and enterohaemorrhagic E. coli (EHEC) (Levine, 1987).

E. coli cause diarrhoea by different mechanisms (Levine, 1987). Enterotoxigenic E. coli strains have the ability adhere to the mucosa of the small bowel and produce a heat labile toxin (LT) or a heat stable toxin (ST) or both which give rise to

diarrhoea. Enteroinvasive E.- coli strains do not produce enterotoxins, but, like shigellae, they have the ability to invade and multiply in colonic epithelial cells. Enterohaemorrhagic E. coli strains cause haemorrhagic colitis by producing elevated levels of shiga-like (Vero cell) cytotoxins. Entero-pathogenic E. coli strains adhere to the small intestine mucosa destroying the absorptive micro-villi, and, some strains, in addition produce shiga-like cytotoxins. Enteropathogenic E. coli strains can further be subdivided into two groups. Class I EPEC strains exhibit localised adherence (LA) to Hep-2 cells, whereas class II EPEC strains exhibit either diffuse adherence (DA) or no adherence (Moon et *al.*, 1983).

There is also a fifth category of diarrhoeagenic E. coli, enteroadherent E. coli (EAEC) (Mathewson et al., 1985; 1986). Although little is known about the pathogenesis, epidemiology, and serotypes of the strains in this category, preliminary evidence suggests that they are indeed capable of causing diarrhoea and do not fit into any of the other four categories.

2.2.1. Enteropathogenic E. coli

In the 1940s and 1950s, E. coli was implicated as the cause of epidemics of infantile diarrhoea, but there was evidence that other

organisms were also important causes (Gross, 1984). The classical study which incriminated E. coli as an important cause of diarrhoea was done by Kauffman and DuPont (1950). They showed that E. coli from outbreaks in several countries belonged to the following O-K-H antigen groups:055:K59:H6, 0111: K59:H6, 0111:K58:H2, and 0111:K58:NM. 0rskov and 0rskov (1984) reported that these K antigens are not true acid polysaccharides, and the antigenic designation has been dropped.

Ewing et al. (1957) showed that strains with serogroups 026, 055, 0111, 0125, 0126, 0127, and 0128 were commonly isolated from patients with diarrhoea. Adult humans given any of these organisms orally developed diarrhoea with an incubation period of less than 24 hours. Neter (1959) coined the term "entero-pathogenic E. coli" to refer to the serotypes of E. coli associated with infant diarrhoea.

The list of EPEC serotypes increased until specific pathogenic mechanisms for ETEC and EIEC were discovered. Between 1971 and 1978 many investigators tested classical serotypes of EPEC strains from patients with diarrhoea for the ability to invade cells and for the presence of LT or ST. With a few exceptions EPEC strains were found to lack these virulence properties (Levine et al., 1983). These observations polarised microbiologists into two groups. One group claimed that EPEC had LT and ST

plasmids when they cause diarrhoea but these plasmids are easily lost on subculture and storage. Others argued that EPEC were indeed capable of causing diarrhoea but by a mechanism distinct from shigellae-like invasiveness or production of LT or ST. This controversy was resolved by a series of studies on adult human volunteers carried out by Levine et al. (1978). These studies demonstrated that EPEC strains isolated 7 years earlier in outbreaks of infant diarrhoea caused definite diarrhoea when given orally to adult volunteers. These EPEC strains were shown to be negative for LT, ST, and shigellae-like invasiveness. These results renewed interest in EPEC and searching for mechanisms by which they cause diarrhoea.

2.2.1.1 Clinical manifestations and treatment of EPEC diarrhoea

Enteropathogenic E. coli produce moderate to severe, watery, dehydrating diarrhoea which may be associated with fever (60%), respiratory symptoms (50%), and abdominal distention (10%) (DuPont et al., 1971). Although the average duration of illness is one week, many patients have exacerbations. In addition, EPEC is one of the few known causes of chronic diarrhoea in infants (Levine and Edelman ,1984).

Outbreaks of EPEC diarrhoea in the 1940s and

1950s were associated with case fatality rates as high as 70%. Although the case fatality rate is much lower now, the disease is often more severe than those due to shigellae, ETEC and nonbacterial pathogens (Levine and Edelman, 1984).

Risk factors for death include young age and virulence of the associated bacterial strain. Strains of serogroup 0111 have been associated with more severe clinical illness and a high mortality rate (DuPont et. al., 1971). Babies borne prematurely are immunodeficient and therefore highly susceptible to EPEC diarrhoea (Parker, 1984). About 50% of the children acquire haemagglutinating antibodies at the age of 1 year.It is therefore possible that most babies are infected with EPEC early in life and acquire some immunity (Parker, 1984).

The role of antibiotics in the treatment of EPEC infections is not clear; most studies have failed to show benefit (Gross, 1984). Drug resistance among EPEC is high both in developed and developing areas(Von, 1977). Good antibiotic prescription, though dependent on the clinical skills of the individual physician, can be fostered by collective decisions by the hospital staff to adhere to an "antibiotic policy". This is unlikely to be effective unless good and up-to-date microbiological and pharmacological advice is available to the physician at all times (Parker, 1984).

2.2.1.2 Epidemiology of EPEC diarrhoea

Nearly all episodes of EPEC diarrhoea occur in children under 2 years of age. Illness is uncommon in older children and adults even though they carry the organism (DuPont et al., 1971). However, a few outbreaks have been described in adults. A water-borne outbreak due to E. coli 0111 which affected adults attending a conference in the United States. Two food-borne infections have been reported in Britain (Gross, 1984). The sources of infection for infants are other persons, occasionally from the mother or wet inanimate objects (Parker, 1984).

During the 1950s EPEC outbreaks in industrialised countries occurred in summer months, but by the 1960s the seasonality appeared to have shifted to cooler months. In developing countries, diarrhoea due to EPEC is found most frequently in the warm season (Levine and Edelman, 1984).

Recent studies from several countries in ^r South America and Africa have shown EPEC to be either the first or second most important bacterial cause of diarrhoea in infants (Freiman et: al., 1977; Toledo et al., 1983; Antai and Anozie, 1987; Cravioto et. al., 1988). The frequency with which EPEC occur in industrialised countries is generally not known (Levine and Edelman, 1984; Levine, 1987). The findings of a study by Karch et *al.* (1987) indicate that EPEC disease continues to occur among infants in

West Germany. Enteropathogenic E. coli has recently been shown to cause sporadic outbreaks of diarrhoea in children at day care centers in the United States of America (Rothbaum et al., 1982; Paulozzi et al., 1986; Bopp et al., 1988).

2.2.1.3 Virulence, pathogenesis, and diagnosis of EPEC

Enteropathogenic E. coli strains adhere to human small intestinal epithelial cells causing destruction of microvilli without evidence of further invasion (Polosky et al., 1977; Ulshen and Rollo, 1980; Rothbaum et al., 1982; Tzipori et al., 1985). This lesion causes decreased absorption leading to diarrhoea. In vitro, EPEC strains adhere to Hep-2 cells or HeLa cells in two distinct patterns, localised adherence (LA) or diffuse adherence (DA) (Scaletsky et. al., 1984; Nataro et al., 1985a). Localised adherence is encoded by plasmids with molecular weight ranging from 55 to 65 MDa (Cravioto et al., 1979). The name EPEC adherence factor (EAF) was given to these plasmid encoded adherence.

In a study by Levine et a^L. (1985) it was found that the presence of EAF plasmid in EPEC strain 0127:H6 was responsible for diarrhoea in adult human volunteers. This plasmid encoded a 94 kilodalton (KDa) protein in the outer membrane. The 94 KDa

protein was found to be immunogenic. By using antiserum specific for the 94 KDa protein, this protein has been found in all important EPEC sero-groups (055, 0111, 0119, 0127 and 0142) and not in ETEC, EIEC, EHEC or in strains of E. coli that cause meningitis or pyelonephritis. An attempt to purify this protein and prepare plenty and potent antibodies that can be used in a simple diagnostic test, such as based on agglutination is under investigation (Levine et al., 1985).

A one kilobase segment from the EAF plasmid has been shown to be highly sensitive and specific DNA probe for detection of EPEC that carry the EAF plasmid (Nataro et al., 1985b). Enteropathogenic adherence factor DNA probe positive E. coli of non-classical serotypes have been isolated from infected patients. However, preliminary epidemiological evidence shows that only EAF positive of classical EPEC 0 serogroups appear to be able to cause diarrhoea (Levine, 1987).

At present, EAF negative strains of classical EPEC serogroups are considered class II within EPEC category (Nataro et al , 1985b) . Such a strain of classical serotype 0114:H2 had two plasmids of about 60 Mdal and showed no adherence to Hep-2 cells but caused diarrhoea in adult human volunteers (Moon et al., 1983). This strain caused typical attaching and effacing lesions in enterocytes that

were indistinguishable from those caused by EAF positive EPEC. It has recently been found out that some Hep-2 nonadherent EPEC can adhere to Henle 408 intestinal cells (Karch et al., 1987).

Some EPEC strains produce shiga-like toxin type I (SLT-I) and/or shiga-like toxin type II (SLT-II) (Newland et aJL., 1987). Shiga-like toxin I is neutralised by antibody to shiga toxin. Shiga-like toxin II is not neutralised by anti-SLT-I but apparently shares DNA homology and functional activity with SLT-I (Cleary et al., 1985). Shiga-like toxin II has the same biological activity as SLT-I, but is more lethal for mice and less cytotoxic than SLT-I (Strockbine et. al., 1986). The Vero cytotoxins produced by strains of E. coli isolated from pigs with edema disease or from neonatal pigs with diarrhoea are not neutralised by antisera that are active against SLT-I or SLT-II (Konowalchuk et al., 1977; Smith et *al.*, 1983).

Southern blot hybridization studies revealed that the DNA fragment carrying the SLT-I structural genes has 50% to 60% homology with the DNA of SLT-II structural genes (Newland et al., 1987). This DNA homology supports the conclusion that SLT-I and SLT-II are related toxins that have undergone considerable divergence. Shiga-like toxins production is controlled by phage conversion (Smith et. al., 1983; O'Brien et al., 1984). Susceptible bacteria

that become lysogenic for such toxigenic phages acquire the ability to produce the corresponding *A* toxin.

The mechanism(s) by which SLT-I and SLT-II cause diarrhoea have not been determined (O'Brien and Holmes, 1987). The production of either toxin has been associated with EPEC diarrhoea. The receptor for SLT-I has been shown to be a glycoprotein and is the same as that of the shiga toxin. Shiga-like toxin I inhibits protein synthesis in intestinal epithelial cells. In this model diarrhoea would result from inhibition of absorption rather than secretion (O'Brien and Holmes, 1987).

It is clear that there is considerable diversity among EPEC. It is not yet known how frequent the production of shiga-like toxins and the ability to adhere to enterocytes or Hep-2 cells may be found together (Gross, 1984). Whereas some EPEC strains produce toxin(s) and are able to adhere to cells, others exhibit only one of these properties. The distribution of these two properties in strains that cause infantile enteritis has yet to be determined, and other toxic substances may be discovered in the future.

Diagnosis of EPEC diarrhoea is based on the clinical picture, biotyping, serotyping, cell adherence tests, genetic probing using EAF probe and cytotoxin production bioassays (O'Brien and Holmes,

1987; Levine, 1987). The bioassays of toxin production include HeLa or vero cell cytotoxity, rabbit ileal loops, lethality to mice, and neutralisation of the toxins by their correponding antisera (O'Brien and Holmes, 1987).

The primary limitations of serotyping as a marker are the availability of the necessary reagents, the small number of laboratories that perform typing, and the significant genetic diversity within a serogroup and even within the same 0-H group (Caugant ejt al., 1985). One recent report describing the use of commercial diagnostic antisera was rather discouraging. None of 118 E .coli that agglutinated with the commercial antisera were identified as EPEC serotypes by the reference laboratory (approximately 30% were confirmed as EPEC serogroups). The investigators also tested 105 of these strains with EAF probe and found that none hybridized (Echeverria et al., 1986; Levine and Edelman, 1984). It is therefore important to use caution when interpreting serotype results and to use these reagents only when there are well-documented and compelling clinical reasons.

A study by Chatkaemorakot et al. (1987) compared the effectiveness of serotyping, mannose-resistant adherence to HeLa cells, and EAF probing in identification of EPEC. Gene probing using the EAF probe was found to be more specific and sensitive

than either serotyping or cell adherence. This indicates that identification of enteropathogenic mechanisms is a more reliable way of identifying diarrhoeic E; coli than is screening for E. coli serogroups.

2.3 Antimicrobial resistance

Bacteria become resistant to antimicrobial agents by one or more of the four mechanisms: altering the target site of the antibiotic, modifying the antibiotic so that it is no longer active, preventing the antibiotic from entering the cell, and specifying an enzyme which provides a substitute for the bacteria-specified enzyme which is the target of the antibiotic (Hardy, 1981) .

Genes determining resistance to anti-microbial agents are located on the chromosome, on plasmids, or on both. Acquired resistance in bacteria results from R plasmid transfer by conjugation, transduction, and transformation or by chromosomal DNA mutation. The distinction between plasmid encoded and chromosomally encoded resistance can become blurred because of the dynamic movement of transposons between plasmids and the chromosome (Forster, 1983). Mutation in chromosomal DNA without affecting the fitness of bacteria to survive in the natural environment would be expected to occur over a long period of time (Lyon and Skurray, 1987). In an

evolutionary sense, then, the accumulation of chromosomal mutation would seem to be an unsatisfactory explanation for the rapid emergence of multiresistant bacteria. Most of the clinical antibiotic resistance is mediated by plasmids (Forster, 1983).

Chromosomal beta lactamases are inducible by their substrates in Enterobacter cloacae, Pseudomonas aeruginosa, and Citrobacter freundi but are constitutive in E. coli where the beta lactam gene exists in a repressed state (Sanders, 1987). Induction or derepression of this gene leads to multiple beta lactam resistance which has been clinically observed (Sanders, 1987).

Several factors are known to contribute to the success of plasmid encoded resistance as an evolutionary mechanism. The presence of resistance genes on plasmids, which are normally non-essential for the survival of the organism, provides the bacterial population with a means to reduce the genetic and physiological load on the majority of the cells while, through the carriage of plasmids, a minority are able to maintain the genetic diversity of the population (Clowes, 1972). Plasmids also contribute to chromosomal resistance by transposition and transduction. Insertion elements and transposons make plasmids a major mechanism of gene reassortment.

Hospitals constitute a unique selective

environment because of the multitude of antibiotic drugs in daily use. Hospitals therefore select for plasmids containing multiple determinants to antibiotics and antiseptics. Due to the formation of antibiotic resistance plasmids carrying virulence determinants, clinical and nonclinical antibiotic use will select and favour the dissemination of virulence functions among bacteria and thus select for strains with enhanced virulence (Timmis ejt al, 1986). Termination of gentamycin therapy in a high risk nursery resulted in the disappearance of gentamycin R plasmid responsible for a K. pneumoniae epidemic (John and Twitty, 1986).

Conjugation, the main method of R plasmid dissemination, was until recently thought to be of importance in Gram negative bacteria only. However, conjugal R plasmid transfer has been found to be responsible for the rapid emergence of highly resistant nosocomial strains of Staphylococcus spp. (Hawkey, 1987).

It has been thought for some time that R plasmid mediated antibiotic resistance deterir inants may have evolved before the antibiotic era (Forster, 1983). Bacteria in the genus Streptomyces have mechanisms to protect their potentially susceptible targets from the antibiotics they produce. These genes may have been tranferred to other bacteria as plasmid or transposon encoded drug resistance. The

origin of the enzymes that confer resistance to synthetic drugs trimethoprim and sulphonamides remains a complete mystery. It is possible that mutation in chromosomal genes encoding drug sensitive enzymes could have resulted in drug resistance, although it is difficult to obtain a high level of resistance in the laboratory (Forster, 1983).

3 MATERIALS AND METHODS

3.1 Patients and sampling

In Nov-Dec 1985, an outbreak of diarrhoea occurred in a nursery ward for pre-term babies at Kenyatta National Hospital, Nairobi, Kenya. The ages of the babies varied from 2 to 62 days. A stool sample was taken from each of the 30 neonates in the ward, regardless of whether they had diarrhoea or not. Five neonates that had diarrhoea died later.

Data concerning the ages of neonates, the presence of diarrhoea and its duration, and treatment before stool collection, are listed in Table 1.

3.2 Bacteriological examination

For the purpose of the present study, each stool sample was streaked on a MacConkey agar (Difco Labs, Detroit, Michigan, USA) plate within 6 hours of collection. Ten lactose positive colonies from each MacConkey plate were selected at random and were subcultured onto deep trypticase soy agar (Difco) slants in screw cap test tubes. These slants were stored at room temperature (RT). The bacterial strains on the deep trypticase soy agar slants were identified to species level according to the criteria listed in Bergeys Manual of Systematic Bacteriology Volume 1 (Brenner, 1984).

TABLE 1 Age in days, the presence of diarrhoea and its duration in days, and the antibiotic treatment of the neonates in the nursery ward

NIN	Age	Dia	Dur	Treatment
1	10	Yes	10	Gentamycin + Chloramphenicol
2	21	Yes	3	Ampicillin + Chloramphenicol
3	22	Yes	9	Gentamycin + Chloramphenicol
4	22	No	NA	Ampicillin
5	23	Yes	3	Gentamycin + Chloramphenicol
6	17	Yes	10	Gentamycin
7	21	Yes	15	Gentamycin
8	19	Yes	1	Chloramphenicol
9	29	No	NA	Gentamycin
10	14	No	NA	Gentamycin
11	12	No	NA	None
12	15	No	NA	None
13	35	Yes	5	Chloramphenicol
14	4	No	NA	Gentamycin + Penicillin
15	24	Yes	8	Amikacin
16	9	NI	NI	Gentamycin + Penicillin
17	14	Yes	2	None
18	9	Yes	NI	Gentamycin + Cefazolin
19	5	Yes	NI	None
20	9	No	NA	Gentamycin + Penicillin

(continued)

TABLE 1 (continued)

NIN	Age	Dia	Pur	Treatment
21	5	Yes	1	Gentamycin + Penicillin
22	20	Yes	5	None
23	NI	NI	NI	NI
24	14	No	NA	None
25	5	No	NA	NI
26	28	Yes	1	None
27	7	No	NA	Gentamycin + Penicillin
28	62	No	NA	None
29	3	Yes	NI	None
30	3	No	NA	None

NIN= Neonate identification number.

Dia= Diarrhoea. Dur= duration of diarrhoea.

NI= No information.

NA=Not applicable.

None=No treatment

3.3 Isolation of plasmid DNA

Plasmid DNA was isolated as described by Kado and Liu (1981). Each bacterial strain was inoculated into 3 ml of tryptic soy broth (Difco) and incubated overnight on a roller drum at 37°C. Half of the broth was transferred into new Eppendorf tubes and centrifuged at 14926X g in a Biofuge A centrifuge (Heraeus Christ, Osterode, West Germany). The supernatant was removed and the bacterial cells resuspended in 200 yl of Tris-EDTA-acetate buffer (40 mM Tris-hydroxymethyl-aminomethane (Merck, Darmstard, West Germany) , 2 mM Na_aEDTA (Merck), pH 7.6). To the bacterial suspension, 400 pi of lysis buffer (50 mM Tris, 3% sodium dodecyl sulphate (SDS) (Merck), pH 12.55) was added,.mixed by inversion until the mixture was clear and then incubated at 55° C for 1 hour.

Equal volumes (300 pi) of phenol (Reidel-de Haen Ag, Seelze-Hannover, West Germany) and chloroform (chloroform means a mixture of 24 volumes of chloroform (Merck) and 1 volume of 3-methyl-butanol-(l) (Riedel-de Haen Ag)) were added to the tube, mixed by inversion, and centrifuged for 5 min at 14926X g. Approximately 300 pi of the top aqueous phase that contained plasmids were transferred into new Eppendorf tubes and used for general plasmid profiling.

3.4 Plasmid DNA profiling

3.4.1 Preparation of agarose gels and plasmid DNA electrophoresis

A suspension of 1.5 gm of standard low-endosmotic agarose (3io-Rad Laboratories, Richmond, California, USA) in 150 ml Tris-phosphate-EDTA (TPE) buffer (0.3 M Tris, 0.008 M NazEDTA, pH 8.0) was heated in a microwave oven until the agarose was completely dissolved. The agarose was allowed to cool to 60° C in a water bath. Two "Protean 11™" glass plates (Bio-Rad), one 13.3 by 20.0 cm outer and the other 16.0 by 20.0 cm inner frosted, were separated by two spacers (18.3 by 1.9 by 0.2 cm). The glass plates were put in a P.11 casting stand (Bio-Rad) and held in place by gently tightening the clamps. Agarose was poured into the space between the glass plates, a 20 teeth comb was immediately placed into the agarose, and this was left at room temperature for 30 min to gel. The comb was removed by carefully pulling it upwards. The clamps were loosened and the agarose gel sandwitch was placed in a 16 cm "Protean 11^{T}*" vertical electrophoresis cell (Bio-Rad). Tris-phosphate-EDTA (TPE) buffer was poured into both the upper and lower chambers of the cell. The buffer covered the wells in the upper chamber and 2 cm of the glass plates in the lower chamber.

Plasmid DNA (24 pi) was mixed with 6 pi of loading buffer (0.25% bromophenol blue (Merck), 30%

(v/v) glycerol (Merck) in water) and loaded into the bottom of each well. Cold water (8°C) was switched on to cool the apparatus during electrophoresis for 3 hours at 120 V, 60 mA.

A 1% standard low-endosmotic agarose (Bio-Rad) minigel was prepared using TPE buffer. A sterilising tape was put on the 7 cm sides of a 10 by 7 cm horizontal mini-gel chamber (Bio-Rad). Agarose was poured, an 8 teeth comb placed in, and allowed to gel. The sterilisation tape was removed and the mini-gel was placed in "MIN SUB™ DNA CELL" (Bio-Rad). Tris-phosphate-EDTA buffer was poured into the cell untill it just covered the wells. These apparatus were then used in the electrophoresis of some plasmids and DNA restriction fragments as will be described later.

3.4.2 Agarose gel staining, photography, and determination of the molecular weight of plasmid DNA

Agarose gels were stained for 10 min in distilled water containing 0.5 pg ethidium bromide (Sigma, St. Louis, Missouri, USA) per ml and destained for 20 min in distilled water. The gel was then placed on an ultra violet transilluminator (UVP, INC.,San Gabriel, California, USA), and pictures taken using a polaroid MP-3 land camera (Polaroid, Cambridge, Massachusets, USA). A Polaroid type 665

positive/negative black & white instant film was used.

E. coli strains that carried plasmid(s) of known molecular weight(s) in megadaltons (MDa) were provided by Dr. K. Wachsmuth (Centers for Disease Control (CDC), Atlanta, Georgia, USA). These strains of E. coli and the molecular weight(s) in MDa of their plasmid(s) are : V157 (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4), S.a (23), RP1 (35), R1 (62), and pDK9 (140, 105 and two light plasmids).

The plasmid DNA of the above bacterial strains were included in all agarose gels used in plasmid profiling. A standard curve was drawn for every gel by plotting the log. to base ten of the molecular weights (MDa) of the known plasmids against their migration distances in mm. The migration distances of the plasmids from the bacterial strains used during this study were measured and their molecular weights read from the standard curve (Maniatis et al., 1981). For electrophoresis of DNA restriction fragments, a molecular weight ladder standard composed of DNA fragments (Toyobo Biochemicals, Osaka, Japan) of known size was used as standard.

The plasmid profile groups were established according to plasmid molecular weights. Isolates which possessed more than five plasmids were placed in the same plasmid profile group if at least 4

plasmids had an identical molecular weights. Isolates which carried 5 or less plasmids were placed in the same plasmid profile group if all plasmids had identical molecular weights. Bacterial strains that carried no plasmid were regarded as one plasmid profile group.

3.5 DNA probing

All the E. coli and pneumoniae strains were probed with oligonucleotide DNA probes for the presence of LT and STI genes. All isolates were also probed with a cloned DNA fragment for genes encoding EPEC adherence factor.

3.5.1 Preparation of EPEC adherence factor (EAF) DNA probe

An E. coli K-12 strain carrying the plasmid pMAR22 was provided by Dr. S. Moseley (University of Washington, Seattle, Washington, USA). The EAF probe is a one kilobase BamHl-Sall fragment of this plasmid. The plasmid pMAR22 was isolated according to Kado and Liu (1981). The plasmid sample was deproteinised and concentrated using a "Gene Clean" kit (BIO 101 Inc., La Jolla, California, USA) following the manufacturers recomendations. The reagents in the kit were "glass milk", "wash solution", and sodium iodide solution.

Plasmid pMAR22 (300 **mD**, sodium iodide

solution (750 |il), and "glass milk" (5 |il) were mixed in a new Rppendorf lube and left to stand at room temperature (RT) for 5 min. The mixture was centrifuged at 14926x g for 5 seconds and the supernatant removed. The pellet was washed 3 times using the wash solution provided by the manufacturer (400 pi) with a centrifugation step of 14926x g for 5 seconds in between the washes. After the last wash, the pellet was resuspended in 10 ƒŸ1 of sterile deionised water, incubated at 50°C for 3 min and centrifuged for 30 seconds at 14926x g. The supernatant containing the plasmid was transferred into a new Eppendorf tube. Plasmid pMAR22 (1 pi) was electrophoresed in parallel with known concentrations lambda DNA (Toyobo Biochemicals) at 100 V, 50 mA for 20 min on a horizontal mini-gel. The concentration of plasmid pMAR22 was visually estimated using known amounts of lambda DNA (Toyobo Biochemicals) from the photograph to be approximately 0.5 ng/ml.

The medium salt buffer and restriction enzymes were supplied by Toyobo Biochemicals. Restriction analysis was done according to Maniatis et al.(198l). The restriction mixture consisted of: 34 pi (17 ng) pMAR22, 4 pi medium salt buffer, 1 pi (10 units) Bamlll (from Bacillus amyloquefaciens H), and 1 pi (10 units) Sail (from Streptomyces albus G). The mixture was incubated at 37°C for 2 hours followed by the addition of 1 pi each of BamMl and Sail and incubated for 2 hours at 37°C.

The plasmid pMAR22 BamHl-Sall restriction fragments were separated electrophorentially using a 1% low melting agarose (Bio-Rad) at 5°C, 50 V, 35 mA, for 2 hours on a horizontal mini-gel. Linear DNA fragments of known weights (Bethesda Research Labs, Middlesex, England) were included in the mini-gel and used to estimate the weight of pMAR22 BamHl-Sall fragments.

The agarose was stained with ethidium bromide, placed on a UV transilluminator, and the sections containing the one kilobase pMAR22 BamHl-Sall fragment were cut out of the gel using a scalpel blade. This one kilobase fragment was separated from agarose using the "Gene Clean" kit (BIO 101 INC). The agarose section (0.4 gm) containing the EAF probe DNA was mixed with 1 ml of sodium iodide solution, incubated for 7 min at 50°C. Five microlitres of "glass milk" was then added and the DNA fragment was isolated as described earlier for plasmid pMAR22. The concentration of the EAF probe in the final preparation was estimated to be 0.35 ng/ml.

3.5.2 Preparation of cultures for colony hybridization

This was done according to Maniatis et al. (1981). E. coli and K. pneumoniae strains were inoculated onto nylon NEF-978X 82 mm membranes

(Biotechnology Systems, Boston, Massachusets, USA), placed on MacConkey agar (Difco) plates. The bacteria were allowed to grow on the membranes overnight at 37°C. The membranes were pealed off and placed on (colony side up) 3 MM Whatman chromatography papers (Whatman International Ltd, Maidstone, England) moistened with the following solutions: 10% SDS for 3 min; 0.5 M NaOH for 5 min; and 1.5 M NaCl, 0.5 M Tris-HCl, pH 8 twice, 5 min each. The membranes were then tranferred onto dry Whatman papers and later placed on the UV transilluminator for 5 min to fix the DNA to the membranes. The membranes could then be stored for months at room temperature.

3.5.3 Preparation of agarose gels for the Southern blot technique

Samples were processed as described by Southern (1975). The unused parts of the gel were trimmed off after electrophoresis and photography. The gel was soaked 3 times for 15 min in each of the following solutions: depurinating (0.25 M HC1); denaturing (0.5 M NaOH, 1.5 M NaCl); and neutralising (1.5 M NaCl, 1.0 M tris-HCl pH 7.5).

A piece of 3 MM Whatman chromatography paper was cut to the same width as the gel, but long enough to form a wick between the buffer reservoir and the gel. A petri dish was placed in a plastic container (reservoir). The transfer buffer used was 20x

standard saline citrate (SSC). Standard saline citrate was made of 150 mM sodium chloride, 15 mM sodium citrate (Merck), pH 7.0. The tranfer buffer was poured into the plastic container to just below the top of the petri dish. A glass plate was placed on the petri dish. The 3 MM Whatman chromatographic paper was placed on the glass plate with the shorter sides dipping into the tranfer buffer. The gel was placed on top of the paper and air bubbles were pushed out. A piece of nylon membrane (Biotechnology Systems) was cut to the same size as the gel. The membrane was placed to exactly cover all the space on top of the gel and air bubbles were pushed out by a glass rod. Three 3 MM Whatman papers were placed on the membrane. A stack of absorbent paper 5 cm high was cut to the same size as the gel and placed on the Whatman papers. A one kg weight was placed on the absorbent paper. The DNA transfer process was allowed to go on for 24 hours at RT. Wet absorbent papers were replaced with dry ones in the course of DNA transfer.

After blotting, the membrane was carefully separated from the gel. The gel was stained and photographed to check whether the DNA transfer had gone to completion. The nylon membrane was soaked in 6x SSC at RT for 5 min to remove any adhering agarose and transferred to Whatman paper to dry. The membrane was then placed on a UV transilluminator for

5 min to fix single stranded DNA to the membrane surface.

3.5.4 Radiolabelling of the EAF DNA probe

The random labelling method of Feinberg and Vogelstein (1983; 1984) was used. The reagents used were contained in the RPN.1601Y/Z multiprime DNA labelling kit (Amersham International, Amersham, England). Radiolabelled deoxycytidine-5 (alpha-^{32}P) triphosphate (dCTP) triethylammonium salt (10 mCi/ml) were supplied by Amersham. The EAF probe DNA fragment containing 24.5 ng (7pl) was sucked into a capillary tube. The tube was sealed at both ends on a bunsen flame and the DNA denatured in boiling water for 5 min. The tube was then transferred immediately into an ice bath to avoid DNA renaturation.

The radiolabelling mixture was made up of the following reagents in an ice bath; 7 pi (24.5 ng) denatured probe DNA, 10 pi labelling buffer, 5 pi primer solution, 21 pi sterile deionised water, 5pl (0.05 mCi) ^{32}P radiolabelled dCTPs, and 2 pi Klenow fragment. The mixture was mixed with a pipette tip, centrifuged at 14926X g for 5 min. The labelling process was allowed to continue for 18 hours at RT.

3.5.5 Measurement of incorporated radioactivity and removal of unincorporated nucleotides

After radiolabelling, 2 pi of the probe solution was mixed with 200 \i\ of stop solution (20 mM Tris, 20 mM Na2EDTA,0.2% SDS) and 5 jj.1 of this mixture was placed on a 2.5 cm GF/C glass microfibre filter (Whatman International Ltd) which was dried in an oven for 10 min at 70°C. The glass filter was placed in a servo plastic container (Packard Instrument, Downers Grove,Illinois, USA) containing 10 ml of scintillatillation fluid. The scintillation fluid was made up of 10 gm of 2,5-Diphenyl-Oxadiazole (referred to as PPO; Packard Instrument), 0.125 gm of l,4-bis(5-phenyl-2-OxazolyDbenzene; 2,2-p-Phenylene-bis(5-phenyloxazole)(referred to as POPOP; Packard Instrument), dissolved in 2500 ml of toluene (Merck). The plastic container was placed in a servo tray and the total radioactivity measured using a liquid scintillation counter model 2405 (Packard Instrument).

The mixture of probe solution and stop solution (5 jil) was mixed with 5 ml of 10% trichloroacetic acid (TCA) (Merck) and 100 |il of carrier DNA (Amersham) in a siliconised glass tube.This reaction mixture was incubated in an ice bath for 15 min. A GF/C glass microfibre filter was fitted into a water operated vacuum pump and the reaction mixture applied. Ice cold 10% TCA was poured onto the microfibre filter 4 times (5 ml each time) and followed by 5 ml of 70% ethanol. The filter was treated as described earlier and the incorporated

radioativity was found to be 48% of the total radioactivity.

The radiolabelling reaction was stopped by adding 175 ul of the stop solution. Unincorporated labelled nucleotides were removed by gel filtration. A 2.5 GF/C glass microfibre filter was fitted into a 2 ml syringe attached to an injection needle. The syringe was filled with Sephadex G50 (Pharmacia, Uppsala, Sweden) in 10 mM Tris-HCl, pH 8. This was centrifuged at 1600X g for 5 min in a Hereaus Christ labofuge (Keraeus Christ Gmbh, Osterode, West Germany). and the eluate was discarded. The probe solution (200 pi) was added to the top of the syringe and centrifuged at 1600X g for 5 min in a Heraeus Christ labofuge. The eluate (probe DNA) was denatured in boiling water for 5 min and immediately placed in an ice bath.

3.5.6 Colony and Southern blot hybridization with the EAF DNA probe

The E. coli strain containing the plasmid pMAR22 was used as the EAF probe positive control. Hybridization was done as described in "Membrane tranfer and detection methods" (Amersham, 1985) The stock solutions used were 20 x standard saline phosphate with EDTA (SSPE) (3.6 M NaCl,200 mM $NaH_2PO«$, 20 mM Na_2EDTA) and 50x Denharts solution (5 gm of each of the following ficoll,

polyvinylpyrrolidone, bovine serum albumin (all from Sigma) and sterile deionised water was added to a final volume of 500 ml). The prehybridization solution was made up of 50 ml 20xSSPE, 20 ml 50x Denharts solution, 10 ml 10% SDS, 4 ml (1 mg/ml) denatured herring sperm DNA (Boehringer Mannheim, GmbH, West Germany), and 116 ml sterile deionised water. An addition of 50 pi of denatured probe DNA to the prehybridization solution made up the hybridization solution. These solutions were both prewarmed to 65°C, the experimental temperature.

Prehybridization and hybridization were carried out in plexi glass containers with tight lids in a shaking water bath at 65°C. The nylon membranes were placed into the plexi glass containers. The prehybridization solution at 65°C was poured into the containers till the mebranes were submerged. The lid was tightly closed and the glass container was incubated at 65°C in the shaking water bath for 2 hours. Probe DNA (50 pi) was added to the glass container and hybridization was allowed to go on for 18 hours. The nylon membranes were washed at 65° C in the following solutions: 15 min in 2x SSC, 30 min in lxSSC with 1% SDS, 10 min in 0.1x SSC. The membranes were placed on 3 MM Whatman chromatography papers and allowed to air dry at RT.

A 42.5 x 34.9 cm piece of hard paper was wrapped in "glad pack" (Union Carbide, Dusseldorf,

West Germany). The nylon membranes were transferred onto the hard paper. "Glad pack" was rolled around the paper to hold the membranes in place. The paper was put in a 45 x 37 cm Kodak X-Omatic casette (Eastern Kodak, Rochester, New York, USA) with the membranes on the top side. A 42.6 x 35.4 cm Kodak X-ray diagnostic film was placed on the nylon filters. A 42.9 x 35.3 Dupont Cronex Hi-Plus intesifying screen (Molecular Biosystems, San Diego, California, USA) was placed on the film with the shiny side on the under side. The casette was closed and incubated at -70°C for 24 hours. The film was developed for 2 min with Kodak LX 24 X-ray developer and fixed for 5 min in Kodak AL 4 X-ray film fixer at RT. The developed film was air dried at RT.

3.5.7 Colony hybridization with LT and STI synthetic enzyme labelled DNA probes

Positive LT and STI control strains were provided by Dr. K.Wachsmuth. The probing kit was obtained from Molecular Biosystems. Hybridization was done according to the Snap^{R} hybridization kit manual (Molecular Biosystems, 1987)

Prehybridization/hybridization buffer was made up of 250 ml 20x SSC, 550 ml deionised water, 5 g bovine serum albumin (Pentex fraction), 5 gm polyvinylpyrrolidone (M.W 40,000), and 10 gm SDS. The nylon membranes were placed in plastic containers

prehybridization solution, prewarmed to 50° C was added followed by an incubation of 10 min at 50°C. The membranes were then transferred into plastic bags which were then sealed using an electric sealer (Audio Electric, Oslo, Norway). The LT or STI enzyme labelled DNA probe (10 pi) in 1 ml hybridization buffer was injected into the plastic bags which were incubated at 50°C for 15 min. The nylon filters were washed twice, 15 min each, in each of the 3 washing solutions 1: (1xSSC,1% SDS) , 2: (1xSSC,1% triton) and 3: (IxSSCfor LT) or (2xSSCfor STI). The washes in the first two solutions were done at 40°C for LT and 50°C for STI. The washes in the third solution were carried out at RT.

The washed filters were placed in plastic bags and sealed- Alkaline phophatase (AP) substrates (7.5 ml AP buffer, 33 gl nitro blue tetrazolium, and 25 pi 5-bromo-4-chloro-3-indoyl-phosphate, all from Molecular Biosystem) were injected into the plastic bags. The bags were sealed and incubated in the dark at RT for 24 hours. Development of a purple colour indicated the presence of LT or STI genes. The membranes were washed in distilled water to remove excess substrate.

3.6 Serotyping, adhesiveness, and Vero cell cytotoxin production

Thirteen representative strains of E. coli were sent to Dr. K.Wachsmuth, Centers for Disease Control (CDC), Atlanta, Georgia, USA, where they were serotyped and assayed for adherence to HeLa cells and production of Vero cell cytotoxin(s).

3.7 Antimicrobial resistance

The bacterial strains were streaked on trypticase soy agar (Difco Labs) plates and incubated at 37°C overnight. The inoculum onto Muller-Hinton agar (Difco Labs) plates was standardised according to the recomendations by an international collaborative study (ICS) on antibiotic sensitivity testing (Ericsson and Sherris, 1971). A single colony was picked using a sterile pasteur pipette and suspended in 5 ml of 0.9% sterile NaCl solution. The emptied pipette was rinsed in a second tube with 5 ml 0.9% sterile NaCl solution. The resulting bacterial suspension from the second tube was flooded onto Muller Hinton agar plates and excess liquid was sucked off. The bacterial concentration in the second tube was found to be 3-4 x 10^6 cells per ml. The Neo-Sensitabs were obtained from Rosco Diagnostica (Taastrup, Denmark).

Each Muller Hinton agar plate was subdivided into 4 quarters. A Neo-Sensitab was placed at the

centre of each quarter and the plates were incubated overnight at 37°C. The zone of inhibition (tablets included) was measured to the nearest 0.1 mm using sliding vernier calipers. The results were interpreted according to Ericsson and Sherris (1971) as listed in Table 2. Nalidixan is a trade name for Nalidixic acid.

Table 2: Interpretation of growth inhibition zones by antimicrobial agents according to Ericsson and Sherris (1971).

Antimicrobial agent	Diameter of inhibition zone in millimetres		
	Resistant	Intermediate	Sensitive
Penicillin	<16	16-33	>34
Ampicillin	<18	18-27	>,28
Ap-s	<18	18-27	>,28
Cefazolin	<18	18-27	>28
Cefamandole	<20	20-27	>28
Cefotaxime	<18	18-26	>/26
Gentamycin	<26	-	>,26
Amikacin	<23	-	>23
Sulfonamides	<23	23-27	>28
Tr-su	<28	-	^28
Oxytetracycline	<23	23-27	>,28
Chloramphenicol	<26	-	>26
Erythromycin	<23	23-29	>30
Colistin	<22	-	>22
Nitrofurantoin	<24	-	>24
Nalidixan	<24	-	V24

Ap-s=Ampicillin-sublactam

Tr-su=Trimethoprim-sulfamethoxazole.

4 RESULTS

4.1 Bacteriological findings

Three hundred lactose positive entero-bacterial strains were isolated from 30 neonates. Out of these, six strains were lost during laboratory handling. Two hundred and twenty nine (77.9%) were found to be E. coli, and 65 (22.1%) K. pneumoniae.

4.2 LT and STI colony hybridization

All 294 strains were found to be negative for both LT and STI genes by using enzyme labelled oligonucleotide probes. The results of colony hybridization using an alkaline phosphatase labelled LT probe are shown in Figure 1.

4.3. Plasmid profiles, EAF colony hybridization, Southern blot hybridization, serotyping, HeLa cell adherence, and Vero cell cytotoxin assay of E. coli strains

The 229 E. coli strains are shown according to their plasmid profile groups in Table 3. The number of neonates and the number of strains from which a particular plasmid profile group was found are shown, as well as the result of the hybridization test using the DNA probe for the EPEC adherence factor (EAF). Twenty eight different plasmid profile groups could be defined, of which plasmid profile 1

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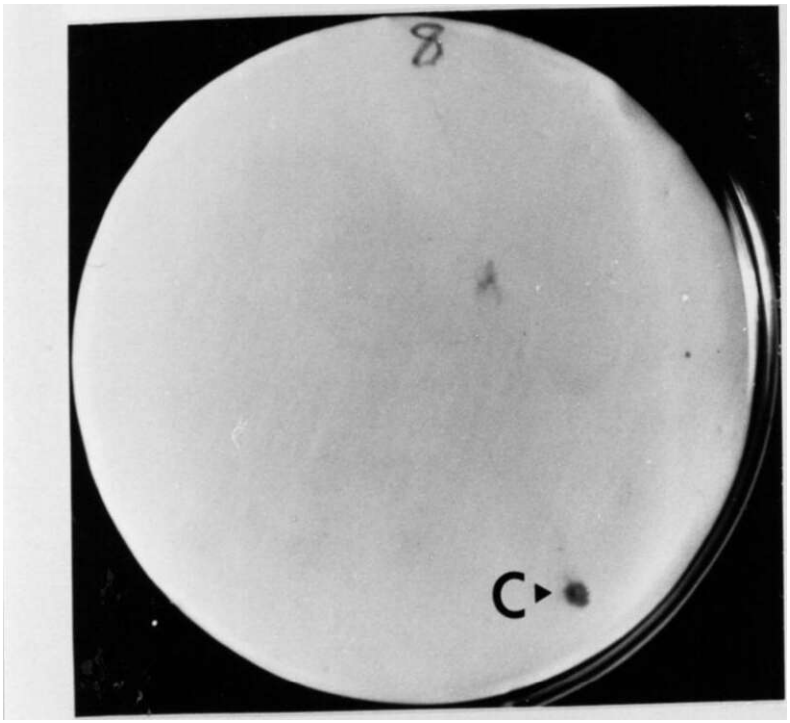

Figure 1. Colony hybridization of DNA from 80 E. coli strains using an alkaline phosphatase labelled oligonucleotide LT probe

Only the DNA from the positive control, marked C, shows a positive reaction.

Table 3: Plasmid profile groups of 229 E. coli strains

pp	NLJ	NS	Molecular weight (Mda) of plasmids
0	2	6	No plasmid
1	12	78	100*, 65, 44*, 42*, 12*, 7.6*, 3.8*, 1.9, 1
2	4	25	1C0*, 68*, 62*, 60*, 4.4*, 4.1*, 3.3, 2.9*,
3	3	21	130, 84, 65, 5.2, 4.4, 2.8, 1.4
4	2	18	160, 140, 90, 67, 56, 6.6, 4.7
5	2	9	70, 67, 5.2, 4.4, 2.8, 1.4
6	1	2	130, 105, 83, 64,
7	1	1	180, 83, 64
8	1	7	130, 97, 90, 64
9	1	7	56
10	1	1	160, 92, 85, 62
11	1	1	140, 95, 80, 67
12	1	4	180, 90, 80, 5.4, 4.6, 4.1, 1.4
13	1	1	140, 90, 5.2, 4.8, 3.8, 1.4
14	1	1	140, 90
15	1	1	90. 80, 5.2, 4.8, 3.8, 1.4
16	1	1	180, 85, 60, 5.2, 4.4, 3.8, 1.4
17	1	1	75, 52, 5.2, 4.4, 1.4
18	1	10	115, 84, 67
19	1	1	180, 100, 7.7, 5.4, 4.1, 3.6, 1.4
20	1	10	95
21	1	9	165, 105, 78, 67, 4.3
22	1	2	155, 92, 85, 62
23	1	1	180, 155, 98, 80, 67

(Continued)

Table 3 (Continued)

PP	NN	NS	Molecular weight (MDa) of the plasmids
24	1	1	112, 95, 82
25	1	2	88, 75
26	1	7	85, 68, 4.4, 4.1, 3.3, 2.9, 1.4
27	1	1	95. 80. 60

PP=Plasmid profile group.

NN=Number of neonates who had E. coli strains belonging to the particular plasrr.id profile group isolated from their stools.

NS=Number of E. coli strains which belonged to the particular plasmid profile group.

*=The plasmid was found in some strains belonging to that particular plasmid profile group.

contained the largest number of strains (78 strains from 12 neonates). These strains all hybridized with the EAF gene probe, indicating that they were capable of producing the EPEC adherence factor, the only virulence factor known so far for EPEC strains. All the other E. coli strains were negative with this probe.

Figure 2 shows representative plasmid profiles of some of the E. coli strains. Figure 3 shows colony hybridization of DNA of E. coli strains using a radiolabelled EAF probe.

As shown in Tables 3 and 4, the combination of 65, 1.9, 1.7, and 1.4 MDa plasmids was present in all the 78 strains which made up E. coli plasmid profile group 1. A 65 MDa plasmid was also present in E. coli strains belonging to plasmid profile group 3. The 1.4 MDa plasmid was present in ten other plasmid profile groups representing 136 strains or close to 60% of the number of strains examined. A 42 MDa plasmid was found in all strains in plasmid profile group 1, except one strain which contained a 44 MDa plasmid. Figure 4 shows plasmid profiles representing E. coli plasmid profile group 1. Figure 5 shows hybridization of a Southern blot of plasmid profile group 1 (EAF probe positive strains) using the EAF radiolabelled probe.

The plasmid profile group, serotype, HeLa cell adherence, and Vero cell cytotoxin assay of the

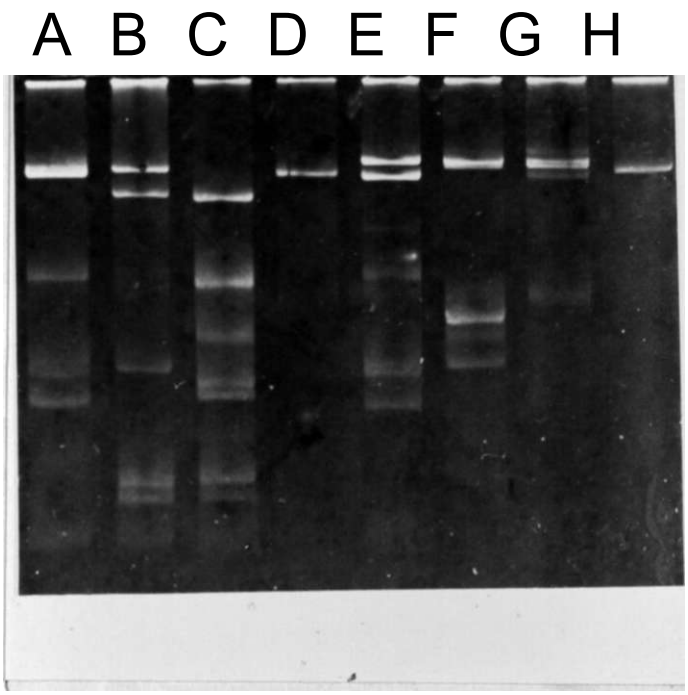

Figure 2. Plasmid profiles representing 5 of the 28 E. coli plasmid profile groups (PP) and plasmid molecular weight standards (m.w.s.)

Lanes A, B, F, G, H show plasmid profiles representing plasmid profile groups (listed in Table 3) *2*, 1, 5, 8, and 9 respectively. Lanes C, D, and E show molecular weight standards (materials and methods page 38) V157, R1, and pDK9 respectively.

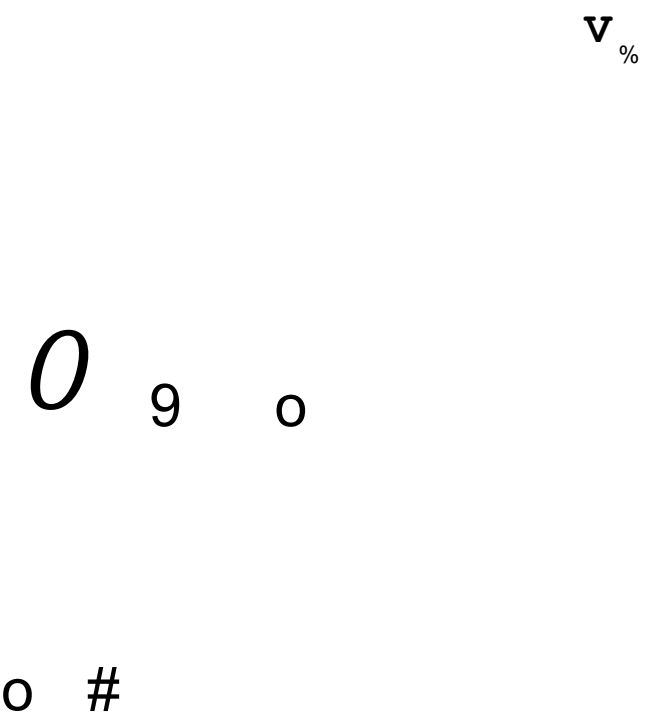

Figure 3. Colony hybridization of DNA from 52 E. coli strains using the 1 megadalton enteropathogenic E. coli adherence factor (EAF) radiolabeled DNA probe

Each of the black spots shows the location of DNA from one of the 22 E. coli strains that hybridized with the EAF probe. The two black spots marked C show the location of positive control strains.

Table 4: Variation in plasmid profile group 1 of E. coli strains (adherence factor positive)

NN	NS	Lane*	Molecular weight (MDa) of plasmids
12	67	A	65, 42, 3.8, 1.9, 1.7, 1.4
3	5	E	65, 42, 1.9, 1.7, 1.4
2	3	C, F	65, 42, 7.6, 3.8, 1.9, 1.7, 1.4
1	1	B	65, 44, 3.8, 1.9, 1.7, 1.4
1	1	G	10Q„ 65, 42, 7.6, 3.8, 1.9, 1.7, 1.4
1	1	D	65, 42, 12, 1.9, 1.7, 1.4

NN=Number of neonates from whom the E. coli strains were isolated.

NS=Number of E. coli strains which carried the listed plasmids.

Lane*=The letters refer to the lanes in which the plasmid profiles are located in Figure 4.

Figure 4. Different plasmid profiles of EAF positive E. coli strains (plasmid profile group 1)

The molecular weights of the plasmids in lanes A, B, C, D, E, F, and G are listed in Table 4. The numbers 1, 2, 3, and 4 show the location of 65, 1.9, 1.7, and 1.4 MDa plasmids respectively.

Figure 5. Hybridization of plasmid DNA obtained from an agarose gel by the Southern blot procedure using the radiolabelled entero-pathogenic E. coli adherence factor (EAF) DNA probe

Lanes A, B, and C contained plasmid DNA belonging to E. coli plasmid profile group 1. Lanes D, E, and F had plasmid DNAs belonging to E. coli plasmid profile groups 2, 3, and 4 respectively. Arrow 1 shows the location of the wells. Arrow 2 shows the location of 65 MDa plasmids. Only the 65 MDa plasmid from plasmid profile group 1 was positive for EAF.

TABLE 5: Plasmid profile groups, serotype, HeLa cell adherence, hybridization results with enteropathogenic E. coli adherence factor probe and Vero cell cytotoxin production of 13 E. coli strains.

PP	NSA	Serotype*	HCA*	EAF	Vero cell cytotoxin*
1	7	0111:HNT	+	+	
2	1	034:H12	-		
3	1	ORough:H18	-		
4	1	NT:NM	-		
17	1	ORough:HI8	-		
18	1	ORough:H4	-		
26	1	0127a :H12			-

*=This was done at Centers for Disease Control, Atlanta, Georgia, USA.

PP=Plasmid profile group.

NSA= Number of strains analysed.

HCA=HeLa cell adherence (+ indicates localised adherence).

EAF=Hybridization with enteropathogenic E. coli adherence factor

NT=Nontypable

NM=Nonmotile

-=Negative

+=Positive

13 E. coli strains analysed at the Centers for Disease control, Atlanta, Georgia, USA are shown in Table 5.

There was complete agreement between the strains with the ability to adhere to Helxi cells and the results of the EAF probing All the HeLa adherence positive strains and the EAF positive strains belonged to plasmid profile group 1 and to serogroup Oil 1 with a nontypable 11 antigen.

4.4 Plasmid profiles and EAF colony hybridization of K. pneumoniae

The plasmid profile groups of the 65 K. pneumoniae strains are listed in Table 6. They were distinct from those of E. coli. Fig 6 shows six of the eight K. pneumoniae plasmid profile groups. Strains belonging to plasmid profile 1 were isolated from 7 neonates. None of the K. pneumoniae hybridized with the EAF probe.

4.5 Gastrointestinal tract colonization patterns with E. coli and K. pneumoniae in the neonates

Plasmid profiles of the strains examined from each of the 30 neonates (including the fatal cases) are shown in table 7. Twenty neonates yielded pure cultures of E. coli, 5 had K. pneumoniae only, while 5 were positive for both species. No correlation was found between the ages (Table 1) and the colonization pattern.

Table 6: Plasmid profile groups of the 65 isolates of K. pneumoniae

PP	NN	NS	Molecular weight (MDa)) of plasmids
O	i	l	N O plasmid
1	7	38	180* , 120, 70*,3.3
2	1	6	140, 120, 90, 46, 10, 5.2, 2.4, 2.0
3	1	6	120, 85
4	1	2	140, 95, 85
5	1	1	52
6	**1**	**1**	**60**
7	1	1	74, 46
8	1	9	100, 62, 56, 5.5, 1.4

PP=Plasmid profile group

NN=Number of neonates who had K. pneumoniae strains belonging to the particular plasmid profile group.

NS=Number of K. pneumoniae strains which were characterized as the particular plasmid profile group.

*= The plasmid was found in some strains belonging to that particular plasmid profile group.

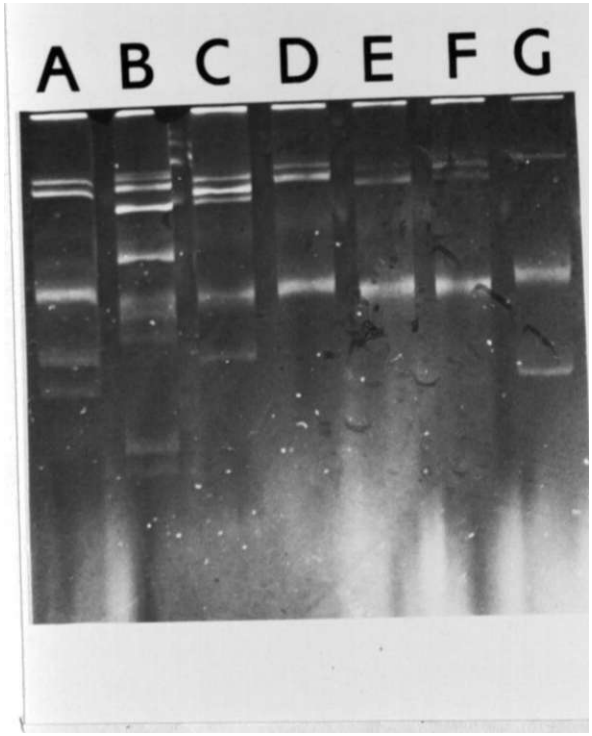

Figure 6. Plasmid profiles representing 5 of the 8 plasmid profile groups (PP) of K. pneumoniae arid the plasmid molecular weight standard (m.w.s)

Lanes B, C, D, E, F, G, show plasmid profiles representing plasmid profile groups (listed in table 6) 2, 8, 4, 3, 3, and 1 respectively. Lane A shows plasmid DNA of molecular weight standard pDK9 (see materials and methods page 38).

Table 7. Plasmid profile groups of bacteria isolated from the 30 neonates in the nursery ward

NIN	NS	PP of 229 bacterial isolates	NK	NE
1	10	9KPP1, 1KPP0	10	0
2(d)	10	10EPP4	0	10
3	10	6KPP3, 2KPP4, 1KPP5, 1KPP6	10	0
4	10	10KPP1	10	0
5*(d)	9	9EPP1	0	9
6	9	9KPP8	9	0
7	10	3KPP1, 7EPP26	3	7
8	10	2EPP6, 1EPP7, 7EPP8	0	10
9	10	1KPP7, 7EPP9, 1EPP10, 1EPP11	1	9
10*	10	1EPP1, 4EPP5, 4EPP12, 1EPP27	0	10
11	»10	1KPP1, 6KPP2, 1EPP13, 1EPP14, 1EPP15	7	3
12*	10	7EPP1, 1EPP2, 1EPP16, 1EPP17	0	10
13	10	10EPP2	0	10
14	10	10EPP18	0	10
15*(d)	10	3EPP1, 7EPP2	0•	10
16*	10	10EPP1	0	10
17*(d)	10	10EPP1	0	10
18	9	4KPP1, 5EPP5	4	5
19*	9	1KPP1, 5EPP1, 2EPP3, 1EPP19	1	8
20	10	10EPP3	0	10
21	10	10EPP20	0	10
22*(d)	10	10EPP1	0	10
23*	10	10EPP1	0	10

(continued)

Table 7 (continued)

NIN	NS	PP of 294 bacterial isolates		
24 *	10	1EPP1, 9EPP3	0	10
25	9	9EPP21	0	10
26	9	5EPP0, 2EPP22, 1EPP23, 1EPP24	0	10
27	10	10PKK1	10	0
28 *	10	1EPP1, 7EPP2, 2EPP25	0	10
29 *	10	1EPP0, -9EPP1	0	10
30 *	10	2EPP1. 8EPP4	0	10

NIN=Neonate identification number.

NS=Number of strains isolated from the stool sample.

PP=Plasmid profile group

EPP=E. coli plasmid profile group.

KPP=K. pneumoniae plasmid profile group.

NE=Number of E. coli strains isolated from the neonate.

NK=Number of K. pneumoniae strains isolated from the neonate.

Neonates who had EAF probe positive E. coli (E .coli plasmid profile group 1)

(d)=Fatal case

Strains belonging to E. coli plasmid profile group 1 (EPP1) were isolated from 13 neonates, in 5 of these neonates all the strains belonged to this plasmid profile group. From 6 other neonates, all the strains isolated belonged to a particular E. coli plasmid profile group (EPP2, EPP3, EPP4, EPP18, EPP20, EPP21).

Out of the 5 fatal cases, 4 had EAF probe positive E. coli (EPP1) isolates. The fifth neonate had all strains belonging to E. coli plasmid profile group 4. E. coli strains with a single plasmid profile group were isolated from 4 of the 5 patients that died.

The case fatality rates for neonates who harboured EAF probe positive E. coli, EAF negative E. coli, and K. pneumoniae were 30.8%, 7.7% and 0% respectively.

4.6 Antimicrobial susceptibility pattern

The enterobacteria isolated from the neonates showed a high resistance to most of the antimicrobial agents tested. Out of the 16 anti-microbials used, the isolates were resistant to at least 7 and at the most 13. Of the 18 neonates who received antibiotic therapy, 13 (72.2%) received gentamycin. A high proportion (69.2%) of the neonates on gentamycin therapy had gentamycin resistant bacterial strains recovered from their stools.

4.6.1 Antimicrobial susceptibility patterns of E. coli and K. pneumoniae

The antimicrobial susceptibility patterns of E. coli and K. pneumoniae are shown in Table 8. The frequency of resistance in E. coli strains and K. pneumoniae strains to sulphonamides, trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline, erythromycin, penicillin (high), and ampicillin varied from 64 to 99% and averaged 85.6% and 81.3% respectively. None of the isolates was resistant to cefatoxime. The frequency of resistance in E. coli strains and K. pneumoniae strains to cefazolin, cefamandole, amikacin, nalidixan, and nitrofurantoin was 2% (1-3.5%), and 10% (4.6-18.5%) respectively. While E. coli strains were more resistant to ampicillin than K. pneumoniae, K. pneumoniae was more resistant to ampicillin-sublactam, gentamycin and colistin. The average frequency of resistance in E. coli and K. pneumoniae to the 16 antimicrobials was 43.2% and 50.3% respectively.

4.6.2 Antimicrobial resistance of EAF probe positive and negative E. coli

Table 9 shows the antimicrobial resistance patterns of EAF probe positive and negative strains. Strains which gave positive reactions with the EAF probe were more resistant to trimethoprim-sulfamethoxazole and chloramphenicol

than those which gave negative reactions. A higher number probe negative strains were more resistant (39.7%) to gentamycin than probe positive strains (10.3%). The average frequency of resistance in both EAF positive and negative E. coli was 43%.

4.6.3 Antimicrobial resistance patterns of E. coli strains in relation to plasmid profile group

The plasmid profile pattern did not correlate with the antibiotic resistance pattern. Some E. coli strains with different plasmid profiles had an identical antimicrobial resistance pattern while others had a different one. There were different antimicrobial resistance patterns even within the same plasmid profile group.

Table 10 shows the relationship between antibiotic resistance of E. coli and plasmid profile groups. The 204 strains in this table represent 89.1% of the E. coli strains characterized in the present study. The frequency of resistance was high (90-100%) with respect to sulphonamides, oxytetracycline$_f$ penicillin, and ampicillin; and variable (0-100%) in case of trimethoprim-sulfamethoxazole, chloramphenicol, gentamycin, ampicillin-sublactam and colistin. The frequency of resistance in E. coli strains belonging to different plasmid profile groups to cefazolin, cefamandole, amikacin, and nalidixan varied from 0 to 30%. The antimicrobial

Table 8: Antimicrobial susceptibility patterns of E. coli and K. pneumoniae

Antimicrobial	E. coli (229)			K. pneumoniae (65)		
	%R	%I	%S	%R	%I	%S
Sp	97.8	0.0	2.2	95.4	0.0	4.6
Tr-su	62.0	6.6	31.4	69.2	20.0	10.8
Cm	74.7	0.0	25.3	76.9	0.0	23.1
Oc	99.0	0.0	1.0	98.5	0.0	1.5
Ec	66.8	29.7	3.5	83.1	16.9	0.0
Pc	99.0	1.0	0.0	80.0	20.0	0.0
Ap	99.0	0.0	1.0	66.2	32.3	1.5
Ap-s	26.6	65.9	5.2	35.3	61.5	3.1
~Cfz	3.5	56.8	39.7	12.3	83.1	4.6
Cfm	1.3	45.9	52.8	18.5	53.9	27.7
Cft	0.0	.1.0	99.0	0.0	4.6	95.4
Gc	29.7	-	70.3	70.8	-	29.2
Ac	2.2	-	97.8	4.6	-	95.4
Nx	1.7	-	98.3	6.1	-	93.9
Nf	1.0	-	99.0	6.1	-	93.9
Ct	26.2	-	73.8	81.5	-	18.5

R=Resistanct I=Intermediate S=Sensitive

Ec=Erythromycin Pc=Penicillin Sp=sulphonamides

Cfz=Cefazolin Ap= Ampicillin Ct=Colistin

Cft=Cefatoxime Gc=Gentamicin Cm=Chloramphenicol

Cft=Cefamandole Oc=Oxytetracycline Nx=Nalidixan

Tr-su=Trimethoprim-sulfamethoxazole Ac=Amikacin

Ap-s=Ampicillin-sublactam Nf=Nitrofurantoin

Table 9: Antimicrobial resistance of E. coli strains with and without the adherence factor(EAF)

Antmicrobial	% of resistant E. coli strains	
	EAF*	EAF⁻
Sulphonamides	97.4	98.7
Timethoprim-sulfamethoxazole	73.1	53.9
Chloramphenicol	94.1	65.6
Oxytetracycline	97.4	100
Erythromycin	64.1	66.9
Penicillin (high)	97.4	100
Ampicillin	97.4	100
Ampicillin-sublactam	19.2	19.2
Cefazolin	5.1	3.3
Cefamandole	2.6	1.3
Cefotaxime	0.0	0.0
Gentamicin	10.3	39.7
Amikacin	2.6	1.3
Nalidixan	0.0	2.6
Nitrofurantoin	0.0	1.3
Colistin	24.4	35.8

EAF* =Enteropathogenic E. coli adherence factor probe positive

EAF-=Enteropathogenic E. coli adherence factor probe negative.

Table 10: Antimicrobial resistance patterns of E. coli related to plasmid profile group.

Anti-microbial	% of resistant E. coli strains belonging to plasmid profile groups:											
	0	1	2	3	4	5	8	9	18	20	21	26
Sp	100	97	89	100	100	100	100	100	100	100	100	100
Tr-su	100	73	89	5	100	0.0	100	0.0	10	100	44	100
Cm	100	95	85	100	100	0.0	100	0.0	10	100	100	100
Oc	100	97	100	100	100	100	100	100	100	100	100	100
Ec	50	64	81	29	83	70	100	71	100	30	100	100
Pc	100	97	100	100	100	100	100	100	100	100	100	100
Ap	100	97	100	100	100	100	100	100	100	100	100	100
Ap-s	33	19	37	5	27	20	50	100	20	20	0.0	43
Cf**z**	0.0	5	0.0	10	11	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cfm	0.0	3	0.0	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cf**t**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gc	100	10	26	33	22	30	100	0.0	90	0.0	55	14
Ac	0.0	3	0.0	0.0	17	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nx	17	0.0	0.0	0.0	17	0.0	0.0	0.0	30	0.0	11	0.0
Nf	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ct	0.0	24	11	14	67	20	64	14	0.0	0.0	0.0	57

Sp=sulphonamides Tr-su= Trimethoprim-sulfamethoxazole

Ec=Erythromycin Ap-s=Ampicillin-sublactam

Cfz=Cefazolin Ap=Ampicicillin Pc=Penicillin

Cfm=Cefamandole Ct=Colistin Nx=nalidixar.

Cft=Cefatoxime Gc=Gentamicin Nf=Nitrofurantoin

Ak=Amikacin Oc=Oxytetracycline.Cm=Chloramphenicol

Table 11: Antimicrobial resistance pattern of K. pneumoniae related to plasmid profile group

Antimicrobial	% of resistant pneumoniae strains belonging to plasmid profile group			
	1	2	3	8
Sulphonamides	97.4	100	100	100
Tr-su	84.2	16.7	16.7	100
Chloramphenicol	100	66.7	0.0	89.9
Oxytetracycline	100	16.7	100	100
Erythromycin	84.2	100	100	55.6
Penicillin (high)	97.4	50.0	0.0	100
Ampicillin	97.4	50.0	16.7	100
Ap-s	60.5	0.0	0.0	33.3
Cefazolin	18.4	0.0	0.0	0.0
Cefamandole	31.6	0.0	0.0	0.0
Cefotaxime	0.0	0.0	0.0	0.0
Gentamicin	63.2	83.3	66.7	100
Amikacin	0.0	0.0	16.7	0.0
Nalidixan	7.9	0.0	0.0	0.0
Nitrofurantoin	2.6	0.0	16.7	0.0
Colistin	73.7	0.0	83.3	44.4

Tr-su=Trimethoprim-sulfamethoxazole.

Ap-s=Ampicillin-sublactam

resistance of E. coli strains carrying no plasmids (plasmid profile group 0) was similar to that of strains possessing plasmids.

4.6.4 Antimicrobial resistance patterns of K. pneumoniae strains in relation to plasmid profile group

The antimicrobial resistance of the different plasmid profile groups of K. pneumoniae which had at least 6 strains are shown in Table 11. The antimicrobial resistance pattern of K. pneumoniae strains varied within and between plasmid profile group(s). The frequency of resistance was high (60-100%) with respect to sulphonamides, erythromycin, and gentamycin; and variable (0-100%) in the case of trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline,penicillin, colistin, and ampicillin.

5 DISCUSSION AND CONCLUSIONS

Enteropathogenic E. coli (EPEC) are defined as E. coli which are epidemiologically associated with infant diarrhoea and do not elaborate heat-labile (LT) or heat-stable (ST) enterotoxins, do not invade the intestinal mucosa and belong to certain classic serotypes (Edelman and Levine, 1983). Enteropathogenic E. coli strains adhere to the small intestinal mucosa destroying the absorbtive microvilli and are assumed to produce cytotoxins.

The term enteropathogenic E. coli adherence factor (EAF) has been applied to the adhesin which is specified by the plasmid pMAR2. This plasmid encodes localised adhesion to both intestinal epithelial cells in vivo and HeLa cells in vitro (Nataro et al., 1985a). Class I EPEC strains exhibit localised adherence (LA) to HeLa or Hep-2 cells, whereas class II EPEC exhibit either diffuse adherence (DA) or no adherence (Moon et *al.*, 1983). Class I EPEC are usually EAF probe positive, while class II EPEC are rarely EAF positive (Levine, 1987).

Enteroadherent E. coli are associated with infantile enteritis. They adhere to Hep-2 cells in a pattern distinct from both LA and DA, and have been shown to cause diarrhoea when given orally to adult human volunteers. Their 0 serogroups have not yet been defined (Mathewson et aJL, 1985; 1986).

Colony hybridization of the 294 lactose positive enterobacteria in the present study using an EAF probe identified 78 positive E. coli. All E. coli strains belonging to plasmid profile group 1 gave positive reactions with the EAF probe. Seven strains belonging to this plasmid profile group were analysed at the CDC and were found to be 0111:HNT, (HNT= H antigens, Non Typable). They adhered locally to HeLa cells and did not produce Vero cell cytotoxin(s). Six EAF probe negative strains examined at CDC were found to belong to various serogroups other than 0111. These strains neither adhered to HeLa cells nor produced Vero cell cytotoxin(s).

It is not yet known how frequent the two potential EPEC virulence factors, enteroadhesiveness and production of Vero cell cytotoxin(s) may be found together (Gross, 1984). Ultrastructural studies by Rothbaum and colleagues (1982) on diarrhoeic infants revealed EPEC adherent to mucosal cells with flattening of microvilli, loss of cellular terminal web, and cupping of the plasma membrane around the individual bacteria. Heavily colonized cells had damaged organelles. These findings indicate that EPEC colonization causes damage to epithelial cells resulting in diarrhoea.

The substance that causes damage of entero-cyte organelles has not been identified. In contrast, ETEC are known to cause diarrhoea by

colonization and production of enterotoxins. Karch et al. (1987) demonstrated that the correlation between Hep-2 cell adherence and elevated production of Vero cell cytotoxins did not always exist. They also found that 76% of the 125 EPEC isolates (belonging to 11 serogroups and 20 serotypes) did not produce cytotoxics). Of the 125 EPEC strains, 13 belonged to 0111 of which only 5 produced low levels of Vero cell cytotoxins. This indicates that EPEC could be capable of causing diarrhoea without producing cytotoxin(s). The production of low levels of cytotoxin(s) is a common trait among EPEC and nonpathogenic E. coli, and may be sufficient to damage the host intestinal cells if the organism adheres avidly to those cells (O-Brien et al., 1982).

Klipstein et al. (1976; 1978) described LT-like and ST-like protein fractions from EPEC strains which were negative in conventional assay systems, but caused fluid secretion in rat jejunal loops when perfused in vivo. At present, it is not possible to ascertain the role played by cytotoxins in the pathogenesis of EPEC diarrhoea. Before this can be done, the cytotoxins must be identified and their mechanism(s) of action elucidated.

The present investigations show that the 7 strains which gave positive results with the EAF probe, adhered locally to HeLa cells. Similar observations have been made by other workers

(Nataro et al.,1985a; 1985b; Chatkaemorakot et al. ,1987; Bopp et. al. , 1988). Hep-2 or HeLa cell adherence has not been an entirely reliable in vitro assay for EPEC pathogenicity. Nataro et al. (1987) found one EPEC strain which did not adhere to Hep-2 cells but was EAF probe positive. This finding may have been due to mutation in the EAF genes or may be an indication that appropriate receptors are not present on the Hep-2 cells. The latter was found to be true by Karch et al. (1987) . They demonstrated that class I EPEC strains which did not adhere to Hep-2 cells adhered to Henle 407 intestinal cells.

Serogrouping, serotyping, genetic probing, ability of cell adherence and cytotoxin production, are at present used to identify EPEC. Chatkaemorakot et al. (1987) compared the effectiveness of serotyping, mannose-resistant adherence to HeLa cells, and colony hybridization using the EAF probe in the identification of class I EPEC. The use of the EAF probe was found to be more specific and sensitive than either serotyping or the cell adherence test. In that case, identification of a virulence gene was found to be a more reliable way of identifying class I EPEC than screening for gene products.

In this study some of the neonates who had only EAF probe negative E. coli strains suffered from diarrhoea. Investigations to establish whether the EAF probe negative E. coli strains were class II

EPEC, enteroinvasive E. coli, enteroadherent E. coli or normal intestinal flora E. coli were not carried out.

Cravioto et al. (1988) showed that LA EPEC strains were more frequently associated with diarrhoea than DA EPEC strains. Twenty five (92%) of 27 children harbouring LA strains were ill, as compared to 10 (24%) of 42 children harbouring DA strains. It has been shown that adult human volunteers given class II DA EPEC strains orally, developed diarrhoea (Levine et al., 1985). In spite of causing diarrhoea in volunteers, it has not yet been established why DA strains are less frequently associated with diarrhoea than LA strains.

In the past, diagnosis of EPEC infections has relied on the detection of E. coli belonging to certain recognized serotypes. Determination of the presence of essential virulence factors using new techniques such as gene probing will allow more precise diagnosis to be made and result in more accurate epidemiological studies.

Based on the detection of EAF genes, 3opp et al. (1988) were able to show that E. coli, 0114:HNM (NM=Nonmotile), possess all the chacteristics of EPEC class I. This serotype, which has previously been considered nonpathogenic, was isolated from diarrhoeic children. The organisms adhered to HeLa cells and gave positive reactions with the EAF probe.

They were negative when assayed for LT and ST enterotoxins, invasiveness, shiga-like toxins I and II and diffuse adhesiveness. It is possible that use of the EAF probe may in the future show that a positive correlation exists between virulence and the serotypes of class I EPEC.

Virulence-specific probes have been shown to be useful in investigations of enterotoxigenic E. coli (Echeverria, et al, 1986), enterohaemorrhagic E. coli (Levine et al., 1987), enteroinvasive E. coli, Shigella bacteria (Venkatesan et al., 1988), and Yersinia enterocolitica (Hill et al., 1983).

The EAF probe in the present study identified EAF positive strains from 13 of the 30 neonates. It is likely that additional EAF positive E. coli would have been detected if more isolates had been obtained from each child or if fecal blots were used.

Southern blot hybridization of the plasmid profiles of EAF probe positive strains showed that the EAF genes were located on a 65 MDa plasmid. This is in agreement with the observations of other workers who showed that EPEC localised adhesion to Hep-2 cell is encoded by plasmids with molecular weights between 55 and 70 MDa (Cravioto et al., 1979; Baldini et al., 1983; Nataro et al., 1985b). The distribution of the 65 MDa plasmid in E. coli strains belonging to plasmid profile group 1, served not only

as a specific epidemiological marker, but also as an indicator of bacterial pathogenicity. A 65 MDa plasmid was also found in E. coli strains that were classified as plasmid profile group 3. However, strains which belonged to this plasmid profile group did not hybridize with the EAF probe. This demonstrated the two 65 MDa plasmids in plasmid profile group 1 and 3 are different. In this case, the presence of a 65 MDa plasmid cannot be used as a characteristic for EAF positive strains.

The EAF plasmid has been shown to be transmissible (Nataro et al., 1985a; 1985b). It is therefore expected that intraspecies or even interspecies transmission may occur. On the other hand, there is a close relationship between serotype and class I EPEC (Levine, 1987).

In this study, the EAF positive strains belonged to the classical EPEC serotype 0111:HNT. It has not yet been established why the EAF virulence plasmids preferentially reside in a limited number of serotypes. A similar restriction of transmissible virulence determinants to certain serotypes has also been documented in ETEC (Levine, 1987). An investigation by Danbara and colleagues (1986) demonstrated that the acquisition and maintenance of stable plasmids encoding colonization factors and enterotoxins is not an attribute of only the restricted serotypes to which ETEC belong. In a recent study it

was showed that enterotoxin plasmids from serotypes of ETEC frequently associated with diarrhoea are nonconjugative, whereas those from serotypes rarely associated with diarrhoea are conjugative (Danbara et al., 1988). These finding suggested that most ETEC strains spread vertically. More information about this is needed also as far as EPEC is concerned.

It is intriguing that despite having identical virulence determinants, only certain serotypes of EPEC and ETEC are frequently associated with diarrhoea. This raises a possible role of the 0 antigen (usually chromosome encoded) in the pathogenesis of diarrhoea. Riley et. al. (1987) described a transmissible EAF plasmid that encoded LA and also a lipopolysaccharide (LPS) which reacted with 0111-specific antiserum. If the genes encoding a group of LPS 0 antigens were always cotransferred with the unique virulence genes of E. coli, an E. coli strain that exhibits a characteristic pathogenic activity would thus belong to a specific serogroup.

The expression of plasmid encoded virulence determinants in the recipient bacteria is usually detected using in vitro models. No attempt has yet been made to administer strains possessing artificially acquired virulence determinants to human volunteers. Such studies would make it possible to determine the ability to cause diarrhoea by bacteria belonging to serotypes other the than classical ones.

Plasmid profile analysis has proved to be a valuable epidemiological tool in the investigations of salmonellosis and E. coli diarrhoeas (Wachsmuth et. al., 1985). Holmberg et al. (1984b) demonstrated that plasmid profile analysis was as helpful as phage typing but superior to antibiogram in the characterization of S. typhimurium.

By using plasmid profiling, the 229 E. coli isolates in the present study were grouped in 28 plasmid profile groups. Only 6 strains had no plasmids. Strains belonging to 7 different E. coli plasmid profile groups were found in several neonates.

All EAF positive E. coli strains belonged to plasmid profile group 1 which was the most commonly represented. All the strains characterized as E. coli plasmid profile group 1 carried plasmids of 65, 42 (except one strain which carried a 44 MDa plasmid), 1.9, 1.7, and 1.4 MDa. The EAF genes were located on the 65 MDa plasmid. A high percentage (89%) of the 78 EAF positive E. coli carried a 3.8 MDa plasmid. The 100, 12, and 7.6 MDa plasmids were found in 5, 1, and 3 EAF positive strains respectively. The 7 strains that were analysed at CDC were all OllliHNT, they adhered locally to HeLa cells and did not elaborate Vero cell cytotoxins. All these findings suggest that EAF probe positive strains may have originated from one bacterial clone. The plasmid

profiles of the EAF positive strains in this study have remained stable in the laboratory for the last two years and were successfully used to characterize these strains.

Gross et al. (1985) used plasmid profiles to show that EPEC 0142:H6 which were the causes of outbreaks of infantile enteritis in British hospitals from 1967 to 1972 had originated from the same clone. All strains isolated from different hospitals over this period possessed two plasmids of 90 and 72 MDa.

The distribution of cryptic plasmids in nearly all genera of medically important bacteria may serve as a means of characterization of strains of the same species (Tompkins, 1985). In the present study, plasmids of 65 and 1.4 MDa were also found in EAF probe negative strains. However, the 1.9 and 1.7 MDa plasmids were only detected in EAF probe positive strains. These two plasmids, though apparently cryptic, and the 65 virulence plasmid, all served as specific epidemiological markers for the 78 EAF positive strains.

Plasmid fingerprinting identified 9 different plasmid profile groups of the 65 K. pneumoniae strains. Strains belonging to plasmid profile group 1 were isolated from 7 neonates indicating that the infection may have been acquired from one source. Plasmid profiling made it possible to differentiate K. pneumoniae strains as there was

no other marker present. Plasmid DNA profiling was found to be a more valuable typing method than klebocin (bacteriocin for K. pneumoniae) typing alone or klebocin typing combined with antibiogram for differentiating epidemiologically related K. pneumoniae from unrelated isolates. Both plasmid DNA profiling and klebocin typing were found to be superior to antibiogram (Walia et al., 1988).

Out of 10 or 9 colonies isolated from each neonates, E. coli and K. pneumoniae strains were isolated in various numbers: from 20 neonates only E. coli strains were isolated, from 5 only K. pneumoniae, while from 5, both species were isolated. Several reasons have been put forward to explain the predominance of a certain coliform species in the neonatal intestine. Tullus et al. (1988) showed that the colonization patterns of neonates differed from ward to ward. There was predominance of Klebsiella spp. in 17 wards, E. coli in 2 wards, Enterobacter spp. in 1 ward, and an equal distribution of the 3 bacterial spp. in 2 wards. They also showed that Klebsiella spp. was prevalent in neonates from 1-2 weeks of age and were gradually replaced with E. coli. However, no correlation between age and colonization pattern was found in the present study.

The factors that stabilize the composition of intestinal microbial communities are many,

complex, and poorly understood. They include various diseases, antimicrobial drugs, starvation, stress, age, immunological factors and dietary changes (Fossum, 1980). Antimicrobial therapy reduces the numbers of susceptible strains in the intestines. Gentamycin was administered to 72.2% of the 18 neonates who received antibiotic therapy. There was no marked difference in the resistance patterns of K. pneumoniae and E. coli to the 16 antimicrobial agents tested except to gentamycin. Of the K. pneumoniae and E. coli strains, 70.8% and 29.7% respectively were resistant to gentamycin. These results show that administration of gentamycin selected for resistant Klebsiella pneumoniae.

Metabolic products of E. coli, Klebsiella spp, and Enterobacter spp. have been shown to inhibit multiplication of Shigella and Salmonella bacteria in the intestines (Hentiges, 1970). The presence of Lactobacillus bacteria inhibits colonization of the vagina by E. coli (Bruce and Reid, 1988). However, no such antagonism has been shown between Klebsiella spp. and E. coli. The factors that control the relative proportions of the lactose positive entero-bacteria in the intestinal microflora have not yet been established.

Three of the five neonates who had only K. pneumoniae isolated from their stools suffered from diarrhoea. These strains were shown not to

produce LT or STI. It is possible that by the method used, EAF positive strains were not detected. Pathogens which were not examined for could have caused diarrhoea. It has also been shown that in 40 to 60% of the diarrhoeal cases, no pathogen is isolated (Freiman et al., 1977).

Goldman (1988) was unable to establish why some strains of K. pneumoniae colonize babies without producing infection, while others are responsible for serious epidemics of pneumonia, sepsis, meningitis, and necrotizing enterocolitis. Finegold (1970) and Gross (1984) described diarrhoea associated with K. pneumoniae. However, no marker has been found to differentiate "pathogenic" from nonpathogenic (normal flora) K. pneumoniae. K. pneumoniae is part of the normal intestinal microflora and until a specific virulence factor is found, it is not possible to assess its role in causation of neonatal enteritis.

In the present study, the case fatality rate of neonates who harboured EAF positive strains was 30.8% as compared to 7.7% for neonates who had EAF negative E. coli, and 0% for those who had K. pneumoniae. Outbreaks of EPEC diarrhoea in the 1940's and 1950's were associated with case fatality rates as high as 70%. Although the case fatality rate is much lower now, the disease is much more severe than those due to shigellae, ETEC, and nonbacterial pathogens (Levine and Edelman 1984).

E. coli 0111:HNT is one of the classic EPEC serotypes (Levine, 1987). This serotype has been incriminated as the cause of infantile diarrhoea by several studies in both developed and developing countries (Kauffman and Dupont, 1950; Komforti et^ al., 1977; Paulozzi et. al., 1986; Karch et al., 1987; Nicolleti et al., 1988; Cravioto et. al., 1988).

All the neonates in the present study were premature babies between 2 and 62 days of age. Risk factors for death include young age and the virulence associated with the bacterial strain (Parker, 1984). Strains of the serogroup 0111 have been associated with severe clinical illness and a high mortality rate (Parker, 1984). Furthermore premature babies are immunodeficient and therefore highly susceptible to EPEC diarrhoea (Parker, 1984) . The results of this study show that the highest mortality rate was among neonates who harboured EAF positive E. coli. Seven EAF positive E. coli which were serotyped all belonged to 0111:HNT.

Sixty to ninety nine percent of the bacterial strains analysed were resistant to sulphonamides, trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline, erythromycin, penicillin, and ampicillin. A similar high frequency of resistance in EPEC isolates was described by Antai and Anozie, 1987. It appears that clinically important resistance to the above antimicrobials is

plasmid mediated (Forster, 1983), except to the beta-lactam antibiotics which is both chromosome and plasmid mediated (Sanders, 1987).

Ninety nine percent of the E. coli and sixty six percent of the K. pneumoniae strains were resistant to ampicillin. The higher resistance in the isolates may be due to production of high levels of beta-lactamase. Elevated beta-lactamase production in E. coli and K. pneumoniae may be due to a high number of copies of the plasmid(s) encoding the enzyme(s) (Forster, 1983). Twenty seven percent of the E. coli and thirty five percent of the K. pneumoniae isolates were resistant to the combination of ampicillin and sublactam. These findings indicate that the resistance to ampicillin was mediated by beta-lactamase(s).

Eight and nine percent of the strains in this study were resistant to cefazolin and cefamandole respectively. None of the strains was resistant to cefatoxime. These findings indicate that the bacteria produced a beta-lactamase which inactivated penicillin and ampicillin but not the 3 cephalosporin beta-lactam antibiotics.

The antimicrobial resistance pattern of six E. coli strains that had no plasmids was similar to that of strains with plasmids. The six strains were resistant to sulphonamides, trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline,

penicillin, ampicillin, and gentamycin. These strains in which no plasmids were found may possess low copy number or high molecular weight resistance plasmids which were not detected by the method used in this study. It is also possible that the resistance to these antibiotics was mediated by chromosomal genes. Chromosomal mechanisms of antibiotic resistance include mutation, tranposition, transduction, and derepression of antibiotic resistance genes (Sanders, 1987).

The importance of plasmids in the emergence and spread of antibiotic resistance cannot be underestimated. However, the detection of antimicrobial resistance genes on plasmids by conjugation does not exclude the location of these genes on the chromosome. Deoxyribonucleic acid probes specific for trimethoprim-, aminoglycoside-, beta-lactam-, tetracycline-, and chloramphenicol resistance genes have been described (Tenover, 1986). These probes could be used to detect the location of resistance genes and thus help in assessing the roles played by the chromosomal and plasmid DNA in the development and dissemination of these genes.

Seventy percent of the K. pneumoniae strains were found to be resistant to gentamycin as compared to thirty percent of the E. coli strains. The resistance of E. coli is much higher than reported by other workers (Sarkar et. al., 1979; Rao

and Naidu, 1982; Antai and Anozie, 1987). The resistance to gentamycin of 236 E. coli strains isolated between 1983 and 1985 at the Kenyatta National Hospital, Nairobi, Kenya was 0.1% (Mutanda et al., 1987). The emergence of gentamycin resistant E. coli at Kenyatta Hospital found in this study emphasizes the role of routine resistance surveillance in effective therapy. Resistance to gentamycin has been shown to be plasmid mediated and transferable (Forster, 1983) . It is therefore possible that the E. coli gentamycin resistance genes may have been acquired from K. pneumoniae.

Ten percent of E. coli strains that were positive with the EAF probe were resistant to gentamycin as compared to thirty nine percent of the probe negative strains. This observation indicates that the EAF positive strains were not selected for by gentamycin therapy. A recent study in the USA, comparing oral gentamycin and placebo, showed gentamycin to be effective in shortening the duration of diarrhoea due to EPEC (WHO., 1988). Based on the results of the present study, it is recommended that treatment of EPEC diarrhoea with antibiotics should be based on the in vitro susceptibility pattern.

Antimicrobial therapy selects for virulent bacterial strains when the antibiotic resistance and virulent determinant genes are located on one plasmid. Genes encoding for LT, ST, and resistance to

several antibiotics have been shown to be located on one plasmid (Timmis et al ., 1986). An average of 43 percent of both EAF probe positive and negative strains in the present study were resistant to the 16 antimicrobials. This indicates that the antimicrobial resistance genes and EAF genes were not located on one plasmid. This finding agrees with that of Nataro et al. (1987) but differs from that of Laporta et. al. (1986) and Riley et al. (1987) who found that the EAF genes and antibiotic resistance genes were locatedon one plasmid. Antibiotic resistance genes can combine reversibly with virulence genes on a plasmid through transposition , transduction or cointegration.

The finding of a high resistance in EAF positive and negative strains indicates that the non-pathogenic strains may provide transmissible resistance genes to pathogenic strains. Gram-negative floras of humans and animals have been shown to serve as a reservoir and source of dissemination for tetracycline resistance determinants (Tenover, 1986).

Between 0% and 6% of the bacterial strains were resistant to amikacin, nalidixan, and nitrofurantoin. This agrees with observations made by other investigators (Antai and Anozie, 1987; Shears et al., 1988).

Transmissible drug resistance to nalidixic acid and its derivatives has not been described until recently. Unfortunately, plasmid-mediated

resistance to nalidixic acid in Shigella dysenteriae type 1 has been reported from Bangladesh, and the number of isolates with this characteristic is rapidly increasing (Munshi et al., 1987).

All bacterial isolates were sensitive to cefatoxime. However, it has been documented that the routine therapeutic use of newer cephalosporins may lead to the emergence of drug-resistant micro-organisms more rapidly than has occurred with the aminoglycosides (Bryan et al., 1985). This may be due to transmissible drug resistance and/or chromosomal cephalosporinases (Forster, 1983; Sanders, 1987).

The incidence of amikacin resistance in several institutions has been reported to remain low, although amikacin was the only aminoglycoside used (Moody et al., 1982; Betts et al., 1984). This is because amikacin is a semisynthetic derivative of kanamycin A which usually cannot be acted upon by kanamycin modifying enzymes (Kawagushi et al., 1972). However, a plasmid (pAZ007) has recently been shown to confer resistance to amikacin. This plasmid has been isolated from several genera of the family Enterobacteriaceae (Tran et al., 1986).

In spite of the wide range of plasmids present, there was no relationship between plasmid profile and antimicrobial resistance pattern. This is not unexpected since the same antimicrobial resistance pattern can be encoded by unrelated

plasmids, transposons , phages, and chromosomal genes. Even identical enzyme mediated antibiotic modification has been shown to be encoded by two different genes (Mayer, 1988).

There was a great variation in the resistance patterns of strains belonging to different plasmid profiles groups. For E. coli strains, the frequency of resistance to chloramphenicol, trimethoprim-sulfamethoxazole, ampicillin-sublactam, colistin and gentamycin varied from 0 to 100%. The frequency of resistance of E. coli strains to ampicillin, penicillin, sulphonamides, and oxytetracycline varied from 97.4% to 100%. The continued uncontrolled use of these antibiotics will select for the resistant strains. Selection of pathogenic, resistant strains will increase the cost of treatment, decrease the number of antibiotics available for effective therapy and eventually lead to serious therapeutic problems. Selection of resistant, non-pathogenic strains leads to the same problem as they might provide a reservoir of transmissible drug resistance genes in pathogenic strains. It has been documented that in patients infected with drug resistant strains, the mortality, the likelihood of hospitalization, and the length of hospital stay were at least twice as great as for patients infected with susceptible strains (Holmberg, et. al., 1987).

Although in most cases other drugs are available for those rendered useless due to resistance, the alternative drug is often less desirable for various reasons. Its efficacy may be lower, it may have greater toxicity, or be more expensive.

The frequency of antimicrobial resistance in the developed countries has remained lower than that in the developing countries (Farrar, 1985). Despite the alarming increase of multiply resistant bacteria in the developing countries, the contributing factors have not been delineated. Availability of antibiotics without prescription, and lack of strict antibiotic policies have been incriminated (Farrar, 1985). There are only limited data available for much of Africa concerning the prevalence and genetic basis of antibiotic resistance.

The effective control of the emergence of antimicrobial resistance depends to a large extent on strict antibiotic policies in both human and veterinary medicine. This is unlikely to be effective unless accurate and up-to-date microbiological and pharmacological advice is available to the physician at all times.

The conclusions made from this study are summarised in the following points:

1. Colony hybridization using the enteropathogenic E. coli adherence factor DNA probe was found

to be a fast, accurate and reliable method for identifying class I enteropathogenic E. coli.

2. Plasmid profiling was shown to be a useful technique in the characterization of E. coli as a 1.7, 1.9 and a 65 MDa plasmid were found to be specific epidemiological markers for strains which gave positive reactions with the enteropathogenic E. coli adherence factor DNA probe.
3. It was shown that the enteropathogenic E. coli adherence genes were located on the 65 MDa plasmid.
4. The highest case fatality rate was among the neonates who harboured E. coli strains which gave positive reactions with the entero-pathogenic E. coli adherence factor DNA probe.
5. A high and similar antimicrobial resistance pattern was found in E. coli strains which gave positive and in those which gave negative reactions with the enteropathogenic E. coli adherence factor DNA probe.
6. No correlation was found between antimicrobial resistance pattern and plasmid profiles.
7. There was an emergence of gentamycin resistant E. coli.
8. K. pneumoniae was more resistant to gentamycin than E. coli.

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