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TOXIN PRODUCTION AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* RIVER WATER ISOLATES

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ABSTRACT

Objectives: To establish the types of *E. coli* isolates that are found in river water around Nairobi and to assess the potential risk of use of this water to human health.

Design: Multiple stratified sampling was carried out. Surface sampling was used in the entire study.

Setting: The study was carried out on river waters surrounding Nairobi, Kenya.

Subjects: Forty *Escherichia coli* strains isolated from river water.

Main outcome measures: Serotyping, toxin gene tests and susceptibility to tetracyclines, ampicillin, chloramphenicol and kanamycin were analysed.

Results: None of the isolates could be specifically serotyped using the available antisera. Toxin gene production tests using the colony hybridisation technique revealed that nine (22.5%) of the strains were positive for heat stable (ST) toxin, seven (17.5%) to the heat labile (LT) toxin and two (5%) to both. Using the Agar Disk Diffusion technique, eighty per cent of the strains were susceptible to all four antibiotics, while twenty per cent of the strains showed multiple resistance. None of the strains was resistant to all four antibiotics while no strain showed resistance to kanamycin.

Conclusion: None of the *E. coli* isolates was serotypable and it was therefore not possible to determine whether serologically identical strains of ETEC were haboured by man or animals. Toxin gene tests results showed that there is some risk of infection by diarrhoea causing ETEC to man and animals.

Toxin gene tests results showed that there is some risk of infection by diarrhoea causing ETEC to man and animals if they consume this water untreated and there is evidence to show resistance of bacteria to antibiotics, hence appropriate health measures should be adhered to.

INTRODUCTION

E. coli has been implicated in a variety of infections including gastroenteritis, diarrhoea and urinary tract infections(1,2). Acute diarrhoeal diseases account for the highest infant and childhood morbidity and mortality in tropical developing countries. In recent years, attention has been focused on enterotoxigenic *E.coli* (ETEC) which are among the most commonly encountered entero-pathogens. ETEC strains belong to a heterogenous array of O:K:H serotypes which elaborate a heat-labile enterotoxin (LT) and at least two heat-stable (ST) enterotoxins responsible for intestinal fluid hypersecretion, clinically observed as diarrhoea(4). As a result of their clinical significance, investigation of entero-toxigenic *E. coli* strains has become important as these can be a source of human infection.

Various methods exist in the epidemiological investigations of infections caused by *E. coli*. In the past, diagnosis of E. *coli* relied on the detection of *E. coli* belonging to certain recognised serotypes. There are many known surface antigens in *E. coli* and hence the number of possible serovars is extremely high and even though complete serotyping involving O, K and H antigens has been carried out in few laboratories, it is known that detection of some serovars depends on the antisera available, frequently limited to a few central reference laboratories. Motile cultures used for H typing tend to show spontaneous agglutination resulting in inconsistent results between laboratories(5). The classification of a strain as ETEC is further complicated by the fact that, within a serogroup, only certain serotypes have been associated with diarrhoea. Another more recently recognised limitation, is the mounting evidence that there is significant genetic diversity within a serogroup and even within the same serotype(6). Determination of the presence of essential virulence factors using new techniques such as gene probing allows more precise diagnosis to be made, bypassing problems associated with serotyping and resulting in more accurate epidemiological studies. Virulence specific probes have been shown to be useful in investigations of enterotoxigenic *E. coli*(7), enterohaemorrhagic *E. coli*(8), enteroinvasive *E. coli*, Shigella bacteria(9) and *Yersinia enterocolitica*(10). In

vitro DNA hybridisation assay has been shown to be more sensitive in detecting enterotoxin producing *E. coli* in water and stool than testing isolates for production of enterotoxin(11,12). Probe tests have an advantage of testing quickly a large number of specimens compared to testing *E. coli* for enterotoxin production(7).

The increased prevalence of resistant bacteria is the direct result of antibiotic misuse and overuse in the human and animal environment. There is convincing evidence that both sub-therapeutic and therapeutic doses of antibiotics cause increased antibiotic resistance in the intestinal flora of man and animals(13). 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(14). Laporta *et al*(15) and Riley *et al*(16), found the E. *coli* virulence and antibiotic resistance genes to be located on one plasmid. Transmission of drug resistant micro-organism from farms into the community and subsequently into the hospital has been reported to occur through food(17) and water may play a role in this as well. The present study an investigation on the production of toxins and antmicrobial resistance of *E. coli* isolated from forty water samples collected over a period of five months from April to September 1996, from the Upper Athi River basin, Nairobi, Kenya.

MATERIALS AND METHODS

Bacterial strains: Forty *E. coli* strains isolated from river water were used and analysed at the Kenya Medical Research Institute (KEMRI).

Sero-typing of E. coli isolates: Eight commercial polyvalent test sera (Denka Seiken Co., Japan) as shown in Table 1. A drop of polyvalent test sera was placed on a glass slide and a discrete E. *coli* colony was taken from the Muller Hinton agar (Oxoid, Basingstoke, England) and thoroughly mixed with the test sera. Agglutination was observed by rocking the slide to and from. Each test sera was used against individual *E. coli* isolates.

Test for antibiotic susceptibility: All forty *E.coli* strains were tested for their susceptibility to tetracyclines, ampicillin, chloramphenicol and kanamycin using the Agar Disk Diffusion procedure(18). Bacterial strains were innoculated onto MacConkey agar (Oxoid, Biotec, England) plates and incubated at 37°C overnight. A single colony was picked using a sterile Pasteur pipette and standardised using the McFarland Nephelometer(19). A multipoint inoculator delivering 7 µl volume was used to deliver test strains onto the plates. The bacterial concentration was determined to be 1.5 X 10^8 cells per ml. Antibiotic disks (Becton, Dickinson Microbiology Systems, USA) were then placed on the agar. The plates were incubated overnight at 37°C. *E. coli* strain ATCC 25922 was used as a control for growth. After overnight incubation, the diameter of the zone of inhibition around each disk was measured using sliding vanier callipers to the nearest 0.1 millimetres. The size of the zone was inversely proportional to the Minimum Inhibition Concentration (MIC) of the organisms. Interpretation of the results was done according to the recommendations of the National Committee for Clinical Laboratory Standards(20.21) and a qualitative report of susceptible, moderate susceptibility, intermediate or resistant was obtained.

Test for ability of E. coli isolates to produce heat stable (ST) and heat labile (LT) toxins by use of DNA probes: This was done using the colony hybridisation test as described by Tamatskuri *et al*(22) using synthetic oligonucleotide ST and LT probes. The test organism was inoculated onto Mueller Hinton agar(Oxoid, Basingstoke, England) and was incubated at 37°C for 16-20 hours. A membrane (Gene screen) was cut to fit the number of samples. A colony was picked from the agar with a sterile tooth pick and spotted onto the membrane. One microlitre control plasmid DNA was also spotted onto the membrane. Bacterial cells were lysed and DNA denatured by placing the membrane on a 3 mm Whatman filter paper wetted in pre-warmed 0.5 N NaOH-1% Sodium dodecyl sulphate (SDS) and 5 X Standard saline citrate (SSC) -1% SDS and kept for 10 minutes. The membrane was then transferred onto Whatman 3 MM filter paper wetted with 1M Tris.HCl, pH 7.4 and kept for one minute to neutralise. This procedure was repeated twice for one minute and 10 minutes respectively. The cell debris was gently rubbed out from the membrane by sponge soaked in 5 X SSC-1% SDS and dried at room temperature for one hour to fix DNA to the membrane. The membrane was then placed in the hybridisation buffer (2X SSC, 1% SDS warmed to 50°C) and 5 ul of biotin-labelled probe DNA/ml added and the bag sealed by a heat sealer. The bag was incubated in a water bath maintained at 50°C and hybridisation allowed to take place for 15 minutes. After 15 minutes the hybridisation bag was opened and the membrane transferred using a forceps to about 100 millilitres of 2 X SSC-1% SDS. It was incubated at 50°C for ten minutes with gentle shaking (the first washing). The membrane was again transferred to about 100 millilitres of 1 X SSC- 0.5 % triton X -100, and incubated at room temperature for ten minutes with gentle shaking (the second washing). Hybridisation of probe DNA to E. *coli* DNA was then detected by returning the membrane into the hybridisation bag and 7.5 ml of substrate buffer containing 4.4 microlitres of Nitro blue tetrozolium (NBT) and 3.3 microlitres of 5-Bromo-4-chloro-3-iodolyl phosphate-tuluidine salt (BCIP/ml) alkaline phosphatase buffer added and bag sealed. The hybridisation bag was then placed in a water bath at 37°C, for 30-60 minutes after which the membrane was removed from the bag, washed with deionised water and left to dry. Positive samples and positive control showed a purple colour within thirty minutes while negative samples and negative control showed no colour. The colour intensity was proportional to the homologous DNA fixed to the membrane.

RESULTS

Serotying of E. coli isolates: Using the available anti-sera kindly provided by the Kenya Medical Research Institute (KEMRI), none of the *E.coli* isolates could be specifically serotyped.

Detection of heat labile and heat stable toxin genes: From the forty isolates tested, nine (22.5 %) were positive for heat stable toxin gene while seven (17.5 %) were positive for the heat labile toxin gene. Two of the isolates were positive for both the heat stable and heat labile toxin genes. The results are shown in Table 2.

Table 1

Commercial polyvalent serotypes used and the test sera they represent. (Denka Seikenn Co., Japan)

Polyvalent	Serotypes represented						
1	01	026	086a	0111	0119	0129a	0128
2	044	055	0125	0126	046	0166	
3	018	0114	0142	0151	057	0158	
4	06	027	078	048	0159	0168	
5	020	025	063	0153	0167		
6	08	015	0115	0169			
7	028ac	0112ac	0124	0136	0144		
8	029	0143	0152	0164			

Table 2

Results of antimicrobial sensitivity tests

Antimicrobial agent	% Frequency of resistance
Tetracycline	25
Ampicillin	25
Chloramphenicol	50
Kanamycin	0

A total of 27.5% of the isolates were resistant to one or more antibiotic with 7.5%, 17.5%, and 2.5% being resistant to one, two and three antibiotics respectively. None of the isolates was resistant to all four antibiotics. Twenty per cent of the isolates showed multiple resistance and 80% were susceptible to all antibiotics tested. A Chi-square test for sensitivity independence of tetracycline, ampicillin, tetracycline and chloramphenicol, and ampicillin and chloramphenicol showed no independence between resistance to the three drugs, that is $p = 0.00001$, $p = 0.0443$, and $p = 0.0061$ respectively.

DISCUSSION

The production of enterotoxins by *E. coli* is encoded for by transferable plasmid DNA, with different plasmids governing production of LT alone, LT and ST production or ST alone(23,24). It is also known that the stability of plasmids that encode for enterotoxin production is related to certain O:H types displaying considerable stability(25). The spread of enterotoxin plasmids therefore depends on the availability of serotypes of E. *coli* capable of acquiring these plasmids. In this study, however, none of the *E. coli* isolates was serotypable and it was therefore not possible to determine whether serologically identical strains of ETEC were harboured by man or animals.

From these results, it shows that there is some risk of infection by diarrhoea causing ETEC to man and animals if they consume this water untreated.

Some of the most difficult nosocomial bacterial infections to treat are those caused by organisms which have acquired resistance due to selective pressure of antimicrobial use or have received resistance genes from other organisms. Considering that in Kenya, *E.coli* is an important cause of bacteraemia in nosocomial infections(26) and a significant public health problem, and that antibiotics are widely used in clinical practice, the need to avert the spread of resistance becomes imperative. The findings of this study differed from those of Ombui *et al* (27) who found in forty one *E.coli* isolates from milk samples in Kenya, that 99% were resistant to at least one or more antibiotic, with 29.2% of them showing multiple resistance. Approximately sixty six per cent were resistant to only one antibiotic, with 64.6%, 7.3%, 4.9% and 2.4% of the isolates resistant to 2, 3, 4, 5 antimicrobial respectively. They also varied from those of Bebora *et al* (28) who found from isolates from chicken in Coast Province that 51.4% were resistant to tetracyclines, 62.2% to ampicillin, 13.5% to kanamycin and 100% susceptibility to chloramphenicol. The low level of resistance to antimicrobial agents found in these water isolates could indicate less exposure to antibiotics as compared to isolates from animals where there is direct exposure to the antibiotics. The low resistance to kanamycin by antimicrobial agents can be attributed to its relative low usage in treatment and prophylaxis.

There are adverse implications to the antimicrobial agents-failure due to resistant organisms. Therapeutic failure leads to increased periods of hospitalisation and in some cases to increase in morbidity and mortality. There is higher cost of treatment as the physician may have to resort to newer and more expensive drugs. It is, therefore, extremely important that studies on the epidemiology of antimicrobial resistance are given the priority they deserve and that appropriate measures be taken to curb spread of resistance. From the results, there is evidence that there was some inter-relationship between resistance to the various drugs, that is, resistance to tetracycline was correlated to resistance to ampicillin and chloramphenicol.

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