ENTEROTOXIGENIC *ESCHERICHIA COLI* OF ANIMAL ORIGIN AS A POSSIBLE SOURCE OF INFECTION FOR MAN.

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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.

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This thesis has been submitted for examination with our approval as University Supervisors.

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THIS WORK IS DEDICATED

TO

MY BELOVED PARENTS.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

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A B S T R A C T

ENTEROTOXIGENIC *ESCHERICHIA COLI* OF DOMESTIC ANIMAL ORIGIN AS A POSSIBLE SOURCE OF INFECTION FOR MAN.

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 *Escherichia coli* (ETEC) which are among the most commonly encountered enteropathogens. The occurrence of ETEC in both animals and man has posed the questions whether animals could serve as sources of infection for man and to what extent transmission between different hosts takes place.

This study deals with these issues. It also aims at determining the prevalance of ETEC in various animal populations, either as a cause of disease, or as normal inhabitants of the intestines of healthy animals. Some samples of human origin have been examined for comparison.

One prerequisite for the causation of disease is the production of enterotoxins, inherent in the term "ETEC" itself. Another is the possession of fimbriae which enable the organism to adhere to tissues.

ETEC elaborate a heat-labile enterotoxin (LT) and at least two heat-stable enterotoxins (ST) responsible for intestinal fluid hypersecretion. Since the discovery of the functional, structural and immunological similarities between LT and the enterotoxin of Vibrio cholerae, assays which were based on these concepts were developed concurrently.

A major problem in the diagnosis of ETEC disease is the inherent difficulty in identifying enterotoxigenic strains among the many non-toxigenic E. coli present in a faecal sample as a part of the normal intestinal flora. There is also no consensus of opinion on how many colonies should be tested for toxin production in order to obtain reliable results.

The bioassays, tissue culture techniques and immunoassays which are available for the detection of enterotoxins are time consuming, expensive and require skilled technical assistance.

The application of the staphylococcal co-agglutination technique (CAG) which allows the examination of a large number of isolated colonies of E. coli from a primary culture of a stool specimen would present a major advantage. An evaluation of this test to determine its suitability for routine laboratory detection of LT was one of the objectives of the present study. The sensitivity and specificity of the coagglutination test were compared with those of the enzyme immunoassay (EIA) for the detection of LT. E. coli isolates were also tested for the heat-stable enterotoxin, STA, by means of the suckling mouse assay.

A total of 10,709 colonies of E. coli from man and animals were examined for LT by means of the EIA and the CAG test. The CAG test had a sensitivity of 93% and a specificity of 100% compared with the EIA. This finding is in conformity with the sensitivity and specificity reported for the previously described tube coagglutination test for the detection of LT.

An average of 20 colonies per stool sample were tested for LT, and in most cases about 50% of these were positive. In a few cases, only 1 to 3 colonies were positive per culture. The CAG test can be performed rapidly and easily for the screening of a

large number of colonies from a primary culture, a feature which may more than compensate for its lower sensitivity compared with EIA.

It was also found necessary to effect the release of LT from cultures of E. coli to achieve uniform, reliable results in the CAG test. Triton X-100 at a dilution of 0.05% was found to serve as an adequate substitute for the more expensive antibiotic polymyxin B.

Enterotoxigenic E. coli, defined as LT positive, ST positive or LT/ST positive, were isolated from 41% (135/329) of children with diarrhoea from Kenyatta National Hospital, and 53% (9/17) of children with diarrhoea from Muhimbili Medical Centre, Dar es Salaam, Tanzania.

ETEC was also isolated from 19% (10/54) of healthy pigs, 33% (18/55) of diarrhoeic pigs 22% (12/55) of healthy cattle, 7% (8/110) of healthy sheep and 1.4% (1/73) of healthy goats. A calf with diarrhoea also harboured ETEC. Most pigs with diarrhoeal disease were less than 12 weeks old. The ages of other domestic animals were unavailable.

ETEC was most prevalent in children within the 0-2 year age group. This is in agreement with findings in Ethiopian children.

ST-producing strains of E. coli were more frequent than LT positive E. coli in both children and animals. This finding is in conformity with results of studies on diarrhoeic patients in Bangladesh, but in contrast to results in Ethiopian children where LT producers were more frequent. Other studies report equal numbers of LT positive and ST positive E. coli isolates.

All ETEC isolates from sheep and goats, which comprised 18% of the ETEC of animal origin, were positive for LT only.

E. coli which produce both LT and ST are known to cause more severe diarrhoea than producers of LT only or ST only. The strains which are positive for both enterotoxins comprised 3% of ETEC from children with diarrhoea at Kenyatta National Hospital and 6% of children with diarrhoea from Muhimbili Medical Centre. These figures are similar to those reported for villagers from the South Nyanza District of Kenya.

LT/ST positive E. coli strains comprised 4% of ETEC from healthy pigs, 16% of diarrhoeic pigs and 5% of healthy cattle. An LT/ST positive strain was also isolated from the calf with diarrhoea.

Adhesion fimbriae of ETEC exhibit considerable host specificity in their attachment to the intestinal mucosal cells of man and animals. Nevertheless, a number of ETEC strains which do not possess the characterised host-specific fimbriae may still cause disease through as yet unknown attachment factors.

F1, F2 and F3 fimbriae were detected only in E. coli isolated from humans, while F4, F5 and F6 were identified only in isolates from domestic animals. F1 was present in ETEC as well as in non-ETEC isolates from children, while F2 and F3 were confined to only ETEC . Strains possessing F2 and F3 comprised 19% and 10% of all ETEC isolates from children.

F4 fimbriae were identified in 29%, F5 in 18% and F6 in 18% of ETEC isolates from pigs. The remaining isolates (36%) were found to be non-fimbriate. The only fimbrial type identified in ETEC isolates from ruminants was F5, which was present in 77% of ETEC from cattle and 38% of ETEC from sheep. The only isolate of ETEC from a goat was also positive for this fimbrial type.

A number of ETEC strains from both children and animals were negative for fimbrial antigens. Due to a lack of sera, these were not serotyped, and the question of serologically identical strains of ETEC being present in both man and animals could therefore not be answered. It is also unclear whether non-fimbriate ETEC may acquire the appropriate

fimbriae through plasmid transfer, which would confer the ability to adhere to the intestinal epithelium of a different species.

Strains of E. coli which colonize an individual at infancy and become established as a part of the normal microflora are non-toxigenic. It is however conceivable that these non-toxigenic E. coli, which may possess appropriate attachment factors, may acquire the ability to produce enterotoxins through plasmid transfer occurring in vivo following the ingestion of ETEC of animal origin. The extent to which this may occur will depend on whether the ingested E. coli of animal origin are capable of transferring these plasmids, as well as the capability of non-toxigenic human strains for acquiring the plasmids which encode for enterotoxin production.

While plasmids which encode for antibiotic resistance are known to be readily transferable within, as well as between species and genera of enteric bacteria, it is largely unknown to what extent transfer of plasmids that encode for the production of enterotoxins and host-specific fimbriae may occur in nature.

Most ETEC strains are restricted to a few O serogroups. Therefore, the frequency of transfer of plasmids encoding for fimbriae and enterotoxin

production may to some extent depend on the presence of E. coli of the appropriate serogroups which can acquire these plasmids.

This study has confirmed that ETEC strains of human origin possess F1 (somatic type 1), F2 (CFA/1) and F3 (CFA/II) fimbriae which are specific for man. ETEC which possess F4 (K88) and F6 (987P) were limited to pigs only, while ETEC strains with F5 (K99) fimbriae were common to pigs, sheep, goats and cattle.

Accordingly, the pattern which has emerged from this study indicates that ETEC strains exhibit strict host specificity, the only exception being F5-fimbriated ETEC which were found in pigs, sheep, goats and cattle. It can therefore be concluded that cross-infections are unlikely to occur between man and animals. Nevertheless, the possibility of interchange of infective plasmids between strains of animal and human origin resulting in interspecies spread of ETEC disease cannot be ignored.

CHAPTER ONE

INTRODUCTION

Diarrhoeal diseases with their wide spectrum of causative agents account for the highest infant and childhood morbidity and mortality in developing countries. Until the early part of the 1970's, the aetiological agents involved in these enteric infections could be identified only in a minority of cases. The introduction of new, improved diagnostic methods has allowed the recognition of rotaviruses, enterotoxigenic Escherichia coli (ETEC), enteropathogenic Escherichia coli (EPEC) and Campylobacter species as the most prevalent causative agents in addition to the traditionally known enteropathogens such as Shigella, Salmonella and Vibrio cholerae (WHO, 1980).

In a study by Puffer and Serano (1973) in Latin America involving 30,000 childhood deaths, at least 30% were directly attributable to infectious diarrhoeal diseases. It is also estimated that 500 million episodes of diarrhoea occurred in children under the age of 5 in 1975. Within the same year it has been estimated that between 5 and 18 million children less than 5 years of age died as a result of infectious diarrhoeal diseases in

Africa, Asia and Latin America (Rohde and Northrup, 1976).

Diarrhoeal diseases are of immense importance also in the domestic animals, in being a leading cause of mortality and morbidity especially in calves, lambs, piglets and kids. Of all the different agents and factors involved in the complex aetiology of enteric diseases of domestic animals, enteropathogens alone serve as the most important cause of disease. Many enteropathogens including viral agents, bacteria, protozoa and helminths have been shown to be involved in enteric diseases of domestic animals. However, in recent years attention has been focused on relatively 'new' infectious agents associated with neonatal diarrhoea in calves, lambs and piglets. Rotaviruses and enterotoxigenic E. coli are among the most frequently encountered pathogens in enteric infections of young domestic animals. In an attempt to identify aetiological agents of diarrhoeal disease in kids, lambs and piglets, Adetosoye (1980) isolated enterotoxigenic E. coli from all animals he examined. Other investigations identified ETEC as the cause of diarrhoeal disease in neonatal piglets (Saunders et al., 1960; Dunne, 1959, Moon et al., 1966). Recent surveys have led to the isolation of ETEC strains from environmental sources as well as from faeces of healthy people and cattle

(Bettelheim et al., 1980; Bettelheim and Wilson, 1982). The carrier rate of ETEC in various domestic animals is largely unknown and the possible role of these animals as reservoirs of ETEC for human infection remains unresolved.

ETEC produce three detectable enterotoxins, a heat-labile toxin (LT) and two heat-stable toxins (STA and STB), which induce the excess fluid secretion in the gut clinically observed as diarrhoea (Smith and Gyles, 1970). It has been shown that LT of E. coli and the enterotoxin elaborated by Vibrio cholerae are closely related in structure and immunologically and both exert a similar action on intestinal mucosal cells through the activation of adenylate cyclase (Evans et al., 1972; Clements et al., 1980). This knowledge made possible the concurrent study of both enterotoxins with the development of various assays over several years. The first generation of tests for the detection of LT were based on animal models and include the infant rabbit test (Dutta and Habbu, 1955), the vascular permeability factor assay (Craig, 1965) and the rabbit ileal loop test (Burrows and Musteikis, 1966). These in vivo tests suffer the drawbacks of being cumbersome to carry out, costly and requiring large numbers of live animals. They are therefore unsuitable for routine diagnostic work in most laboratories. The

Chinese hamster ovary cell culture technique (Guerrant et al., 1974 and the Y-1 adrenal cell culture test (Donta et al., 1974), constitute the next generation of tests which are more sensitive than the previous tests, but suffer from disadvantages of being dependent upon specialized techniques requiring experienced workers and expensive equipment. Their use in developing countries as methods of investigating ETEC disease is therefore limited. Other tests developed for the detection of enterotoxins are the adaptations of the enzyme immunoassays (Yolken et al. 1977; Svennerholm and Holmgren, 1978) based on the original work of Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971), and a recently introduced DNA hybridization technique employing radioactive genetic probes (Kaper et al., 1981; Moseley et al., 1981). These are the most sensitive and specific tests available for the detection of enterotoxins and are obviously of immense importance for research. However, they lack the simplicity required for routine diagnostic work. The absence of an inexpensive, simple method for field investigations is a primary reason for the lack of information on LT producing E. coli in piglets (Guinee et al., 1980). An adaptation of the staphylococcal coagglutination technique of Kronvall (1973) has been

used in detection of LT and represents a simple, rapid screening method for routine laboratory diagnostic work (Brill et al., 1979; Wadstrom and Ronnberg, 1983). No extensive evaluation of this rapid method of detection of LT has been done.

There are at least two classes of E. coli heat-stable enterotoxin, STA and STB. STA is a methanol soluble low molecular weight protein, active in suckling mice through the activation of guanylate cyclase in intestinal mucosal cells, while STB is methanol insoluble, inactive in suckling mice but active in weaned-pig intestinal loops without alteration of guanylate cyclase activity in intestinal mucosal cells (Burgess et al., 1978; Gyles, 1979). The role of STB in causing diarrhoea has been established only in pigs, and its toxigenicity for humans and other species is unknown. Search into the mechanism involved in STB -induced fluid hypersecretion in the intestines of piglets is continuing. STA is detectable by the suckling mouse assay of Dean et al. (1972) whose recent modification by Berry et al. (1983) has greatly improved the ability of this test to detect STA producing E. coli. Recently the applications of radioimmunoassay (Frantz and Robertson, 1981), enzyme immunoassay (Ronnberg et al., in press) and the staphylococcal coagglutination technique (Wadstrom and Ronnberg, 1983)

for the detection of ST have been reported. These techniques await further evaluation in field trials, thus making the suckling mouse assay the preferred test for ST.

A major problem in the diagnosis of ETEC disease is the inherent difficulty in identifying enterotoxigenic strains among the large number of non-toxigenic E. coli present in faecal samples as members of the normal intestinal flora. There is also lack of consensus of opinion with regard to how many colonies in a given routine culture of E coli should be examined for toxin production to justify the conclusion "negative" for ETEC (Merson et al., 1979).

Enteropathogenicity of ETEC not only depends on elaboration of enterotoxins (LT and ST) but also on the ability of ETEC to adhere to the intestinal mucosa, a characteristic conferred onto E. coli by the presence of certain fimbrial antigens which serve as adhesive factors. The best-characterised fimbriae of ETEC of human origin are the F2 and F3, previously referred to as colonization factors I and II (CFA I and CFA II) respectively which enable the organism to adhere to specific receptors on enterocytes of the proximal small intestinal mucosa (Levine, 1981). Fimbrial antigens show specific haemagglutination patterns with erythrocytes from various

species and are also species - specific in the colonization of the small intestines of hosts (Satterwhite et al., 1978). F4 (K88) fimbriae are only encountered in ETEC pathogenic for piglets (Smith and Linggood, 1971). F5 (K99) fimbriae are associated with ETEC pathogenic for calves, lambs and piglets (Ørskov et al., 1975; Moon et al., 1977), while F6 (K987P) are found in only porcine strains of ETEC (Isaacson et al., 1977). Some ETEC strains lacking any of the characterised fimbriae have however been isolated from cases of gastroenteritis in humans and animals suggesting the presence of yet unidentified adhesive fimbriae in some ETEC strains.

Of the many unresolved issues related to diarrhoeal disease the present study has focused on the following aspects of ETEC:-

: Development and evaluation of a simple and rapid method suitable for the screening of large numbers of colonies of E. coli from routine stool cultures for the production of heat-labile toxin (LT).

: Examination of isolates of E. coli from human and animal sources for the production of heat-stable toxin (ST).

: Determination of the prevalence of enterotoxigenic E. coli in diarrhoeic children as well as in healthy and diarrhoeic animals.

: Identification of fimbrial antigens present in enterotoxigenic E. coli of human and animal sources and, based on these attachment factors, attempt to delineate strains which might be pathogenic for both man and animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY OF ESCHERICHIA COLI

In a attempt to identify the causative agent(s) of infantile diarrhoea, the German paediatrician Theodor Escherich isolated Bacterium coli commune (Escherich, 1885, 1886, cited by Sussman, 1985). This microorganism is what is known today as Escherichia coli. The role of a serotype of E. coli in diarrhoeal disease was later established by Bray in 1945, who in an investigation of an outbreak of diarrhoea among infants in a London hospital showed that 42 out of 44 strains of E. coli involved in this outbreak were serologically similar. A few years later other outbreaks of gastro-intestinal disease were reported in which the serotype of E. coli involved was identified (Giles et al.,1949; Taylor et al., 1949).

The earliest indication of a specific virulence factor associated with E. coli was reported by De et al., (1956). They showed that certain strains of E. coli associated with diarrhoeal disease caused secretion of fluid and electrolytes into the lumen of ileal loops of rabbits. Other workers subsequently showed that E. coli

was the cause of neonatal piglet diarrhoea (Saunders et al .,1960; Dunne,1959; Moon et al .,1966). At post-mortem affected piglets often show an oedema of the colon, hence the use of the term "swine oedema disease" to describe this condition. The association of E. coli with enteric disease of both man and animals has been known for nearly a century.

Although E. coli forms a part of the normal intestinal microflora of humans and animals, it has been found to be potentially pathogenic for both man and animals (Dupont et al., 1971; Mundell et al ., 1976; Field, 1979) In recent years however, a lot of emphasis has been placed on the role of E. coli in the aetiology of human diarrhoeal disease especially in tropical developing countries. This new emphasis on infectious diarrhoeal diseases has been outlined by the Regional Diarrhoeal Diseases Study Group of the World Health Organisation (WHO), which described enterotoxigenic and enteropathogenic E. coli together with rotaviruses and Camplylobacter jejuni as being the leading enteropathogens involved in diarrhoeal diseases in Africa (WHO, 1980).

In a study in India, Maiya and co-workers (Maiya et al., 1977) isolated E. coli as the sole enteropathogen from 50 children with symptoms of gastrointestinal disease. Other studies carried out also in children with diarrhoea in Mexico, Kenya and Bangladesh reported isolation of E. coli in 68%, 2.71% and 2.17% respectively. (Donta et al ., 1977, Mutanda, 1980; Black et al., 1980).

2.2 TAXONOMY, MORPHOLOGY AND BIOCHEMICAL CHARACTERISTICS OF ESCHERICHIA COLI

Escherichia coli belongs to the family Enterobacteriaceae and is the only member of the genus Escherichia. Morphologically E. coli is a short Gram-negative, non-spore forming and usually peritrichous and fimbriate bacillus. Some strains possess a capsule or microcapsule with a few strains capable of producing a profuse polysaccharide slime. (Øskov, 1974 cited in Bergey's Manual of Determinative Bacteriology).

E. coli is a facultative anaerobe and most strains frequently ferment lactose although this fermentation may

be delayed or even be abscent in some cases (Ørskov, 1974 cited in Bergey's Manual of Determinative Bacteriology). The major biochemical characteristics ascribed to E. coli are shown in Table 1.

2.3 SEROLOGY

E. coli may be subdivided into stable biotypes based on the fermentation of sugars as well as other biochemical tests, although no correlation exists between these properties and the presence of single antigens. The most useful way of subdividing E. coli is serology based on antigenic properties of various surface structures of the bacterium. Very little was known about the surface characteristics of E. coli until the introduction by Kauffmann (1943, 1944), cited by Danielsson et al. (1979) of the serological classification of E. coli based on different antigens. This serological classification was subsequently reviewed by Kauffmann (1954, 1966) and forms the basis of the typing scheme for E. coli.

Antigens used in serological classification of E. coli include the O or somatic antigen, which denotes the polysaccharide moiety of the cell wall, the K or

Table 1: MAIN BIOCHEMICAL CHARACTERISTICS OF ESCHERICHIA COLI (Ørskov, 1974 in BERGEY'S MANUAL of DETERMINATIVE BACTERIOLOGY)

Optimum growth temperature	37°C
Catalase	+
Oxidase	-
B-Galactosidase	+
Gas from glucose at 37°C	+
KCN (Growth on)	-
Mucate (acid)	+
Nitrate	+
G + c, moles %	50-51
Carbohydrates	
(acid from)	
Adonitol	-
Arabinose	+
Dulcitol	d* (1)
Esculin	D*
Inositol	-
Lactose	+ or X *(2)
Maltose	+
Mannitol	+
Salicin	d*
Sorbitol	+
Sucrose	D*
Trehalose	+
Xylose	D*
Other Carbon Sources	
Citrate	-
Malonate	-
d-Tartrate	d*

Methyl Red Reaction	+
Voges-Proskauer Reaction	+

Protein utilization

Arginine	d*
Gelatin hydrolysis	-
H_2S from Triple Sugar Iron Medium	-
Indole production	+
Lysine decarboxylation	+
Ornithine	d*
Urea	-
Glutanic acid	-
Phenylalanine	-

*(1) d refers to different reactions by different serotypes

*(2) X refers to late and irregularly positive (mutative).

capsular antigen, generally an acidic polysaccharide and the H or flagellar antigens which are protein in nature. Currently 171 different O antigens (designated 01 to 0171), 55H antigens (H1 to H55) and 103K antigens have been identified with the recognition and establishment of new antigens being co-ordinated by the WHO collaborative Centre for Reference and Research on E. coli in Copenhagen (Guinee et al., 1980).

E. coli may also be classified based on the presence of colonization factor antigens or fimbriae on the surface of the organism. These are hair-like structures previously referred to as pili (Duguid, 1955; Brinton, 1959). Fimbriae are non-flagellar filamentous appendages important in bacterial adherence to human and animal epithelial surfaces. These factors are also capable of causing agglutination of erythrocytes of certain species (Duguid et al ., 1979). The different fimbriae of E. coli of human and animal origins have been identified and classified based on the haemagglutination patterns observed with the erythrocytes of different species. The classification of various fimbriae of E. coli

together with the current designations of these fimbriae is presented in Table 2.

Apart from serology, other characteristics of E. coli have been applied in typing schemes for E. coli. Among these are the bacteriophage typing scheme as reviewed by Milch, 1978 and the colicine typing, reviewed by Gillies, 1978, although these have not come into general use.

2.4 ECOLOGY

The primary habitat of E. coli is the gastro-intestinal tract of mammals and birds. This location of E. coli has enabled the extensive use of the organism as an indicator of faecal contamination of water and foods.

E. coli may show opportunistic pathogenicity by causing enteritis, urinary tract infections and neonatal meningitis in man and mastitis in cows (MacDonald et al., 1970; Ørskov, 1974 in Bergey's Manual of Determinative Bacteriology; Sarff et al., 1975). The most important of these diseases appears to be E. coli associated enteric disease in children and young animals. Emphasis in recent years has thus been focused on E. coli induced infant diarrhoeal diseases in less developed countries,

Table 2. CHARACTERIZATION OF E. COLI FIMBRIAL ANTIGENS (ØRSKOV AND ØRSKOV, 1983).

ANTIGENIC DESIGNATION	PREVIOUS DESIGNATION	REFERENCE STRAIN	C O M M O N SOURCE
F1	Somatic type 1	BAM	Pathogenic and non-pathogenic E. coli.
F2	CFA/I	H10407	Human diarrhoea and UTI*
F3	CFA/II	PB176	Human diarrhoea and UTI
F4	K88	E68	Pig diarrhoea
F6	987P	987P	Pigs, calves, lambs diarrhoea
F7	None	C1212	Human UTI*
F8	None	C1254-79	Human UTI*
F9	None	3669	Human UTI*
F10	None	C1960-79	Human UTI*
F11	None	C1976-79	Human UTI*
F12	None	C1979-79	Human UTI*

* UTI refers to urinary tract infections.

travellers' diarrhoea and neonatal colibacillosis in piglets, calves and lambs (Gorbach et al ., 1975).

2.5 COLONIZATION OF THE GUT BY ESCHERICHIA COLI

Colonization of the gastro-intestinal tract by E. coli takes place soon after birth (Escherich, 1885 cited by Sussman 1985; Bettelheim, 1974), with source of the bacteria being the mother and the inanimate environment (O'Farrel et al., 1976). Once introduced, E. coli then becomes a part of the microflora of the intestinal tract. In animals, colonization of the bowel by E. coli also occurs early in life with the faeces of the dam often serving as the source of organisms (Smith, 1965). E. coli population in any individual may consist of a majority serotype with several minority serotypes (Wallick and Stuart, 1943). New serotypes, derived from contaminated food may be introduced into individuals thus increasing the number of serotypes (Cooke et al., 1970; Shooter et al., 1970).

E. coli was thought to be the predominant faecal organism. However, it is now recognized that E. coli forms a small constituent of the total flora present.

2.6 ENTEROPATHOGENICITY

The different pathogenic mechanisms involved in E. coli enteric infections have been classified

by the WHO Scientific Working group on bacterial enteric infections Report (1980) in humans as follows:

1. Enteropathogenic E. coli (EPEC)
2. Enterotoxigenic E. coli (ETEC)
3. Enteroinvasive E. coli (EIEC)
4. E. coli with other pathogenic mechanisms.

2.6.1 EPEC

The history of EPEC began with the early association of certain serotypes of E. coli with outbreak of infantile diarrhoea (Bray, 1945; Giles et al., 1949; Taylor et al., 1949). Other strains of E. coli involved in infantile diarrhoea were soon recognized based on epidemiological and serological considerations. The latter approach was further elaborated by Kauffmann (1954) and was useful for the evaluation of E. coli in infantile diarrhoea.

Enteropathogenic E. coli belong to the "O" serogroups or serotypes O:H, and are capable of causing diarrhoea without producing enterotoxins (heat-labile or heat-stable) and are not invasive (Levine et al., 1983). EPEC were found to be responsible for many cases of infantile gastrointestinal disease with an appreciable mortality even in the developed countries (Rowe, 1979). There has been a drastic reduction in EPEC associated gastrointestinal disease attributable

to the improvement of hygiene in hospitals, institutions and society as a whole, though specific outbreaks are still observed.

About 15 to 20 of the known E. coli serogroups (O, K and H) have been designated EPEC with the most frequently isolated serogroups worldwide being 0111, 055, 026, 0119, 0127, and 0128 (Rowe, 1979). In a study carried out in Sao Paulo, Brazil, Toledo et al., (1983), found serogroups 0111 and 0119 present in 73.4% of EPEC isolates from cases of diarrhoea in children.

The epidemiology of EPEC induced diarrhoeal disease in developing countries is not so well defined as mentioned by the WHO Scientific Working Committee on bacterial enteric infections (WHO, 1980). Available reports on EPEC diarrhoea indicate a continuous presence with occassional and interspersed epidemics. Frieman et al., (1977), reported a clear seasonal occurrence with a peak occurrence of EPEC diarrhoea in the summer months.

There is however, a controversy surrounding the isolation of EPEC in sporadic diarrhoeal disease outbreaks. Some investigators hold the opinion that EPEC should not be searched for in routine investigations in cases of sporadic diarrhoeal outbreaks (Neter,

1976), while others accept and present convincing data on the role of EPEC in endemic infantile diarrhoeal disease (Gurwith et al., 1978).

Colonization of the small intestines is an essential feature of EPEC diarrhoeal disease as indicated by post-mortem studies of children (Thomson, 1955). This colonization is then followed by pathophysiological changes in the intestinal mucosal cells, induced by the EPEC strains (Thomson, 1955; Albert et al., 1978; Banwell and Sherr, 1973). In the pathogenesis of EPEC enteritis, there is no production or release of recognized enterotoxins (Levine et al., 1978).

2.6.2 ETEC

In an attempt to isolate Vibrio cholerae from the faeces of patients with signs and symptoms typical of cholera, De et al. (1956), isolated Bacterium coli. The pathogenicity of these isolates of Bacterium coli, now known as Escherichia coli was demonstrated by inoculation of cultures into ligated rabbit ileal loops leading to dramatic distention of these intestinal loops. The distention was due to excessive fluid accumulation, a finding observed when V. cholerae was used in place of E. coli (De et al., 1956). It was later shown that bacteria-free toxin preparations also caused rapid fluid accumulation in ligated

loops of pig and calf intestine (Smith and Halls, 1967). The enterotoxin responsible for fluid secretion was found to retain its activity after heating to 100°C for 30 minutes and to be non-antigenic. This is now known as the heat-stable enterotoxin (ST). Another enterotoxin produced alone or together with ST was identified by Gyles and Barnum (1969) and the relationship between these enterotoxins was then described by Smith and Gyles (1970). ETEC cause diarrhoea by elaboration of heat-labile toxin (LT) and/or heat-stable toxin (ST) which are classified based on their thermolability (Clements and Finkelstein, 1979).

The production of both LT and ST is encoded for by transferable DNA plasmids, with different plasmids governing production of LT alone, LT and ST production and ST alone (Gyles et al., 1974; Wachsmuth et al., 1976). Since the plasmids encoding for enterotoxin production can be transferred from one E. coli strain to another in vivo, it is therefore not uncommon to find diverse E. coli serotypes being responsible for diarrhoea through production of enterotoxins (Ørskov and Ørskov, 1977; De Boy et al., 1981).

Besides the elaboration of enterotoxins ETEC also possess accessory virulence factors. The best characterised of these factors are the adherence or

colonization factors which permit attachment of ETEC to the mucosa of the small intestines (Smith and Linggood, 1971; Smith and Linggood, 1972; Jones and Rutter, 1972; Nagy et al., 1977), permitting the release of enterotoxin close to the reactive sites. ETEC colonization factors were previously referred to as pili but have been renamed fimbriae (Duguid, 1955, Brinton, 1959). Agglutination of erythrocytes of certain species in the absence and presence of D-mannose has been used as criterion for the identification of fimbriae of ETEC, of animal origin such as F4 (K88) and F5 (K99) Burrows et al 1976). However F6 (type 987P) fimbriae of ETEC pathogenic for pigs does not manifest haemagglutination (Moon et al., 1977).

Colonization factor antigens I and II better known as F2 and F3 of human strains are analogous to F4 and F5 associated with strains of ETEC pathogenic for animals (Evans et al., 1975; Evans and Evans, 1978; Ørskov and Ørskov, 1977). F1 or somatic type 1 fimbriae show ability to attach to epithelial cells (Old, 1972; Salit and Gotschlich, 1977; Isaacson et al., 1978), however, the distribution of these fimbriae in both normal flora as well as pathogenic E. coli makes their role as a virulence factor unclear (Levine et al., 1980). Haemagglutination patterns of some fimbrial antigens is presented in Table 3. Fimbrial types F2, F3 of human ETEC and types F4 and F5 of animal origin are

Table 3. HAEMAGGLUTINATION PATTERNS OF FIMBRIAL ANTIGENS OF E.COLI

PREVIOUS DESIGNATION	PRESENT DESIGNATION	HAEMAGGLUTINATION				SOURCE of STRAIN	REFERENCE
		HUMAN	BOVINE	GUINEA PIG	SHEEP		
Somatic type 1	F1	-	-	MS	-	Common in pathogenic and	Salit and Gotschuld (1977).
CFA I	F2	MR	MR	-	-	H10407 Human diarrhoeal disease and *UTI	Evans et al. (1975).
CFA II	F3	-	MR	-	-	Human diarrhoea and *UTI	Evans and Evans (1978).
K88	F4	MR	-	-	MR	Pig diarrhoea	Jones and Rutter (1972).
K99	F5	MR	-	-	MR	Pig, calf lamb diarrhoea	Burrows et al. (1976).
987P	F6	-	-	-	-	Pig diarrhoea	Moon et al. (1977).

encoded for by transferable plasmids which frequently encode for ST and LT as well (Levine et al., 1983). F4 (K88) fimbriae have been shown to exist in at least three forms (Ørskov et al., 1964; Stirm et al., 1966; Guinee and Jansen, 1979).

The disease caused by ETEC is clinically indistinguishable from clinical cholera (Dupont et al., 1971; Field, 1979; Mundell et al., 1976). Research in various parts of the world especially in the developing countries, using newly developed methods of detecting enterotoxins has shown the importance of ETEC in diarrhoeal diseases. Table 4 shows some recent studies on causative agents of infantile diarrhoea with isolation rates of ETEC from various parts of the world. In a study carried out in Kenya 47% of a population of 782 children with diarrhoea yielded ETEC (Shimotori et al., 1984 personal communication).

2.6.2.1 Heat-labile enterotoxin (LT)

Since the first observation more than 15 years ago that E. coli strains elaborate a cholera-like enterotoxin (Gyles and Barnum, 1969), researchers worldwide have attempted to isolate and characterize the responsible proteins and to consider the enterotoxins in conjuction with choleragen or cholera toxin. Cholera toxin is a protein of molecular weight 84,000 daltons and

Table 4. RECENT STUDIES SHOWING ISOLATION RATES OF ETEC IN CHILDREN WITH DIARRHOEAL DISEASE FROM DEVELOPING COUNTRIES.

	COUNTRY	PERIOD OF STUDY	AGE GROUP	N° OF PATIENTS	ETEC Isolated
1.	Mexico Mexico City	July to Oct. 1974	0-12yr	50	16(32%)
2.	Costa Rica	-a	3-15 s months	62	12(19.35%)
3.	South Africa Johannesburg	Dec. 1976 to Jan 1977	0-2yr	70	19(27.14%)
4.	Kenya Nairobi	1975	-a	36	3(8.33%)
5.	Bangladesh rural area	Feb. 1978 to Jan. 1979	0-2yr	2614	28(1.07%)
		March 1977 to Feb 1978	0-12yr	962	12(1.25%)
		Oct. 1977 to Feb. 1978	0-12yr	86	22(25.58%)
		March to June 1979	0-4yr	175	9(5.14%)

a refers to not specified.

References

1. Donta et al., (1977)
2. Nalin et al., (1979)
3. Robins-Browne et al., (1980)
4. Mutanda (1980)
5. Black et al., (1980)

consisting of 5 B-Subunits (mol. wt. 11,000 each) and a single A subunit. The A subunit is divisible into an A_1 and A_2 (mol. wts 24,000 and 5,000 daltons respectively) (Finkelstein, 1973).

LT like cholera toxin is a protein, molecular weight of 72,000 to 150,000 daltons and consisting of antigenic determinants common to the subunits A and B of cholera toxin (Clements and Finkelstein, 1979). This relationship has been exploited in various serological methods of detection of LT.

The receptors for LT, to which the B subunit binds are located on intestinal mucosal cells. The main receptor is the GM_1- ganglioside, which is also the recognized receptor for cholera toxin (Holmgren, 1973). Holmgren *et al.* (1982) described another receptor, a glycoprotein which lacks affinity for cholera toxin but serves as a receptor for LT.

In the pathogenesis of diarrhoeal disease, the B subunits of LT bind to the receptors on intestinal mucosal cells, such that the A subunit in some way gains entry into these target cells and irreversibly activates adenylate cyclase, with a resultant accumulation of cyclic AMP and hypersecretion of ions and water into the gut or intestinal lumen (Cuatrecasas, 1973; Stavric *et al.*, 1978). The activation of the adenylate cyclase system is usually preceded by a lag phase of approximately 8-10 hours (Evans, 1979).

LT from human ETEC is closely related to, but distinct from that found in porcine ETEC strains (Honda et al., 1981, Geary et al., 1982). LT of human origin also appears to have a higher affinity for the GM_1 - ganglioside receptor than LT of porcine origin (Olsvik et al., 1983).

2.6.2.2 Heat-Stable enterotoxin (ST)

ST from human ETEC as well as animal ETEC strains have recently been purified and characterised (Alderete and Robertson, 1978; Takeda et al., 1979; Staples et al., 1980). Different purified preparations of ST appear to have different molecular weights ranging from 2,000 to 5,000 daltons, corresponding to different numbers of amino acid residues. Burgess et al. (1978) described two E. coli ST activities, STA and STB. STA is methanol - soluble, active in the infant mouse assay and is found in ETEC of human and animal origins. In contrast to STA, STB is methanol-insoluble, inactive in infant mouse assay but active in the ligated pig and rabbit intestinal loop assays. STB has only been identified in ETEC pathogenic for pigs (Burgess et al., 1978; Kapitany et al., 1979). No ETEC of human origin has been shown to produce STB but the search for the infant mouse - negative ST has been limited to date.

The exact process of binding to receptors of target cells by STA is not known (Thomas and Knoop, 1983), although it clearly activates guanylate cyclase activity leading to an intracellular accumulation of cyclic GMP (Field et al., 1978; Guerrant et al., 1980). There is a resulting alteration of the membrane function of the mucosal target cells and a net secretion (Rao et al., 1981).

2.6.3 EIEC

Sakazaki et al. (1967) showed that some strains of E. coli cause a dysentery- like illness in humans. Later Ogawa et al. (1968) isolated E. coli of 0144: K? (B), 043:K(B), 0136:K78, 0124:K72 and 028a, c:K73 O:K types and showed that these organisms possess invasive properties, characteristic of virulent Shigella strains. EIEC do not produce enterotoxins (LT/ST), but invade the colonic mucosal cells in a similar fashion as Shigella. This invasive property is demonstrable by the guinea pig keratoconjuntivitis test (Sereny, 1955). EIEC are also restricted to a small number of serogroups, such as 028 and 0124 (Ørskov, 1974). Serologically some strains of EIEC have been shown to possess somatic antigens (O antigens) related to those of various Shigella serotypes (Edwards and Ewing, 1972), although the significance of this finding is not clearly understood.

Little is known about the prevalence, epidemiology and clinical features of EIEC diarrhoea in developing countries (Rowe, 1979; WHO, 1980). Clinical signs attributed to the few outbreaks of EIEC disease reported, include dizziness, myalgia, headache and the presence of blood in the stool (Dupont et al., 1971, Snyder, et al, 1984). EIEC outbreaks however continue to be reported from different parts of the world (Guerrant et al., 1975; Snyder et al., 1984).

2.6.4 E. coli with other pathogenic mechanisms

A new pathogenic mechanism associated with an E. coli pathogenic for rabbits has been found. The organisms were found adhering to the mucosal surface of the ileum, caecum and colon of the rabbit, without producing enterotoxins or showing any invasive properties (Cantey and Blake, 1977). By means of electron microscopy, this strain of E. coli has been shown to destroy the microvillous border of the host mucosal cells (Takeuchi et al. 1978). These reports form the basis of the possibilities of a fourth mechanism in the enteropathogenicity of E. coli diarrhoeal disease. O'Brien et al. (1977) have suggested the possible involvement of a Shigella -like cytotoxin in the pathogenesis.

The need to investigate the possibilities of involvement of this new mechanisms in E. coli diarrhoeal disease has been stressed by the WHO expert Committee on diarrhoeal diseases (WHO, 1980).

2.7 DETECTION OF ENTEROTOXINS

The development of methods of detecting enterotoxins began with the early use of the rabbit ileal loop model (De *et al.*, 1956). Following this, various methods have been applied to the detection of both ST and LT of *E. coli*. These methods are reviewed here, with emphasis on their advantages and drawbacks.

2.7.1 Infant Rabbit test.

The infant rabbit test of Dutta and Habbu (1955) was probably the first bioassay to be used in detection of cholera toxin and enterotoxins of *E. coli* (Sack, 1975). In this method, seven day old rabbits, starved overnight are hydrated prior to being infected with the test organism by oral administration of 5% dextrose solution. Seven hours post infection, the rabbits are sacrificed and their intestines dissected out. The fluid accumulation is measured and results are expressed as a ratio of intestinal fluid in millilitres over remaining intestinal weight given in grams.

This is a very sensitive test for detection of enterotoxins but suffers the drawbacks of requiring large numbers of rabbits, being cumbersome and as such unsuitable for testing large numbers of *E. coli* strains.

2.7.2 Vascular permeability factor assay

This test detects the presence of cholera toxin or LT of E. coli with the effect being neutralized by antiserum raised against cholera toxin (Craig, 1965). Briefly, cell-free supernates of E. coli to be examined for LT, together with dilutions of anti-cholera serum are injected intradermally into the shaved back of a young adult rabbit, followed 18 hours later by an intravenous injection of Evans blue dye. Capillary permeability indicated by bluing and induration occurs if toxin was present in the supernate.

Sensitivity to cholera is in the range of 0.1 to 3.5 ng/ml. Like the previous method, the bioassay is cumbersome and expensive in terms of large numbers of rabbits required. These drawbacks make this technique of detection of LT inappropriate for routine screening of many E. coli strains.

2.7.3 Rabbit ileal loop test

The rabbit ileal loop test was initially developed for the detection of cholera but was later adapted for most enterotoxin producing organisms including ETEC (Burrows and Musteikis, 1966). Ligated segments or loops of rabbit intestine are injected with cell-free extracts, following which the abdomen of the rabbit

is closed for 6 to 8 hours. The rabbit is sacrificed and the intestine dissected out, measured and weighed to determine the amount of fluid accumulation stimulated by the toxin present in the cell-free extract or supernate. Results are expressed as fluid volume per length of intestinal loop.

False negatives are frequently encountered (Burrows and Musteikis, 1966). The technique is not easy to perform and there is a requirement for large numbers of rabbits, drawbacks which reduce the usefulness of this test as a routine screening technique for identification of ETEC.

2.7.4 Chinese hamster ovary cell test (CHO)

This test is presently the most widely used tissue culture assay for enterotoxin detection. The CHO is very sensitive in being capable of detecting 10pg/ml of purified cholera toxin (Guerrant et al., 1974). Cell-free filtrate of a test culture is mixed with freshly suspended CHO cells and an incubation period of 20 to 24 hours allowed before reading the results. Elongation of more than 10% of the CHO cells is interpreted as a positive test (Guerrant et al., 1974).

Other enteropathogens including Enterobacter spp, Citrobacter, Salmonella spp, Klebsiella spp and

Vibrio fluvialis have been shown to be positive in this test (Sanderfur and Petersen, 1972; Guerrant et al., 1974; Lockwood et al., 1982). This is a specialized test requiring skilled assistance and the use of expensive equipment. These drawbacks make the CHO unsuitable for use in routine screening of E. coli for LT, especially in the developing countries.

2..7.5 Y-1 Adrenal Cell Culture Method

Like the Chinese hamster ovary cell culture technique for detection of LT, this is a tissue culture test with a sensitivity for cholera toxin of the magnitude of CHO (Donta et al., 1974). In this test the amount of steroid produced as a result of the effect of cholera toxin or LT on a monolayer of cells is measured and correlated with the marked cytopathic effect in the same monolayer (Sack and Sack, 1975).

Aeromonas hydrophilla and vibrios produce extracellular products which cause non-specific cell rounding frequently misinterpreted as having been caused by enterotoxins (Kaper et al., 1981). Like other tissue culture techniques for detection of enterotoxins, this test requires skilled assistance and is too expensive for use as a routine diagnostic tool especially in the developing countries where ETEC disease is still a leading cause of morbidity and mortality in children. Another disadvantage of this assay is the

ability of a partial or inactive toxin molecule to give a positive reaction which does not indicate toxigenicity or pathogenicity at all.

2.7.6 Passive immune hemolysis.

One of the serological assays used in the detection of LT of E. coli is the passive immune hemolysis as described by Evans and Evans (1977). This technique involved the use of supernates obtained from polymyxin treated whole culture in conjuction with the spectrophotometric determination of hemoglobin released. A fixed amount of supernate is allowed to react with a suspension of sheep erythrocytes in the presence of antiserum and guinea pig complement with the resulting hemolysis indicating presence of LT in the supernate so tested.

This immunoassay is less sensitive compared with the tissue culture techniques and is not easily adapted for screening of large numbers of colonies of E. coli for LT (Bramucci and Holmes, 1978).

2.7.7 Radial passive immune hemolysis

This method of detecting LT is based on the same principles as the previously described passive immune hemolysis and was described by Bramucci and Holmes (1978). Radial passive immune hemolysis was intended

for the screening of large numbers of bacterial colonies, by using hemolysis produced and observed as clear zones on a blood agar plate. Hemolysis occurs as a result of the reaction of LT or cholera toxin with antiserum against LT in the presence of guinea pig complement.

This method is less sensitive compared with the bioassays and tissue culture tests, although it is reported to be significantly more sensitive than radial immunodiffusion tests for cholera toxin (Bramucci and Holmes, 1978). The radial passive immune hemolysis has not been widely applied to the detection of LT because of its low sensitivity.

2.7.8 Rat intestinal perfusion assay

This *in vivo* method was developed to measure fluid absorption and/or secretion in the rat intestines as a result of the effect of LT or ST. The method monitors the concentration of a non-absorbable marker as a basis of measuring the fluid changes and is capable of detecting both LT and ST (Klipstein *et al.*, 1979). Apart from the drawback of frequent false positives, this method also suffers the disadvantage of other *in vivo* tests of requiring live animals and hence cumbersome to carry out.

2.7.9 Removable intestinal tie-adult rabbit diarrhoea (RITARD)

The removable intestinal tie-adult rabbit diarrhoea technique was recently described by Spira et al., (1981), based on the principle that when the small intestine of an adult rabbit is temporarily ligated so that bacterial cells are not easily cleared, an infection and a fatal diarrhoea were produced by V. cholerae and ETEC.

This test has the disadvantage of giving a number of false positive results (Klipstein et al 1979) being cumbersome and so unsuitable for use in screening large numbers of E. coli colonies.

2.7.10 Radioimmunoassay (RIA)

Radioimmunoassay was first described by Yalow and Berson (1960) and later used with some modifications by Miles and Hales (1968). RIA is among the earliest immunological asays to be applied in the detection of cholera toxin and heat-labile enterotoxin of E. coli within the last 5 years (Greenberg et al., 1977). Briefly this method involved pre-coating polyvinyl microtiter plates with anti-cholera serum, followed by addition of cell-free supernates of E. coli and a subsequent incubation for reaction to occur. This

was followed by a washing of the plates to get rid of any unbound or unreacted material and the addition of ^{125}I-labelled anti-cholera toxin immunoglobulin. Microtiter plates were then washed again and labelled immunoglobulin conjugate added. Detection of radiation was by means of a ratio of residual count or radiation in sample well to the mean residual count of wells without toxin.

The sensitivity and specificity of this test are equivalent to the Y-1 adrenal cell assay of Donta et al. (1974). The method is easily standardized and automated. Among the demerits of this test are the high costs involved in establishing it as well as the highly skilled technical assistance required for its operation. Although the radioimmunoassay is very useful for research on enterotoxin detection, for reasons already mentioned it cannot be used in routine laboratory screening for LT.

2.7.11 Modified Elek Test (Biken test)

Biken test was developed by Honda et al. (1981) based on the principles of Elek (1948) and Ouchterlony (1948). Briefly the method consits of the following: E. coli is inoculated on a special agar containing lincomycin which induces enterotoxin production of ETEC (Levnar

et al., 1977), and after incubation of these cultures for at least 18 hours, polymyxin impregnated paper discs are placed on E. coli colonies. Polymyxin causes the release of periplasmic LT whis is allowed to react with anti-LT antiserum placed in an adjacent well to the test colony of E. coli. The resulting precipitin line indicates the presence of LT produced by the test E. coli colony.

This test is simple and reproducible but requires upto 3 days for results to be obtained (Honda et al., 1981).

2.7.12 Enzyme immunoassay (EIA)

EIA was developed separately by Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971). This was based on the original concept of Miles and Hales (1968) that, enzymes or co-enzymes could replace the radioactive labels in non-competitive radioactive assays.

In an enzyme-linked immunosorbent assay (ELISA) for detection of E. coli LT, the known cross-reactivity between cholera toxin and LT is exploited (Yolken et al., 1977). Briefly, microtiter wells are coated with anti-cholera serum, followed by addition of cell-free supernates of E. coli. A rabbit anti-cholera antibody is then added, followed by an appropriate incubation time and an addition of an alkaline

phosphatase anti-rabbit immunoglobulin conjugate replacing the earlier used radioactive label of Greenberg et al., 1977). Finally the enzyme substrate (p-nitrophenyl phosphate) is added. Excess reagent is washed off between addition of various reagents. Results are read either by visual observation or by means of a spectrophotometer.

2.7.13. GM_1-ELISA

GM_1- ELISA is an approach to the detection of LT of E. coli which utilizes the known affinity of the enterotoxin for the receptor ganglioside GM_1 of cholera toxin (Holmgren, 1973; Pierce, 1973). The principles involved are the same for ELISA, except that the primary coating antibody is replaced by the GM_1 - ganglioside to which LT binds if present in a test supernate. Following addition of test supernates of E. coli, rabbit anti-cholera serum, anti-rabbit immunoglobulin enzyme conjugate and nitrophenylphosphate substrate are added in that order. Excess or unbound reagent is washed off with an appropriate buffer in between the addition of the different reagents mentioned.

Results are highly reproducible and in close agreement with those obtained in the sensitive adrenal cell culture assay. This test requires expensive

reagents and equipment as well as highly trained personnel. The assay also detects partial or inactive toxin molecules thus giving a positive reaction which does not necessarily indicate toxigenicity or pathogenicity.

2.7.14 Staphylococcal coagglutination technique

Kronvall (1973), first exploited the binding of the Fc portion of IgG of some species to protein A of Staphylococcus aureus allowing the antibody binding sites to freely interact with antigens, in a rapid and simple technique of typing pneumococci. Subsequently, this test has been used for serological grouping of Streptococci, serological identification of Salmonella, Shigella directly from a primary isolation agar plate (Christensen et al., 1973; Danielsson and Kronvall, 1974; Edwards and Hilderbrand, 1976). Staphylococcal coagglutination was adapted for use in the detection of E. coli LT, first as a tube coagglutination test (Brill et al., 1979) and eventually as a slide agglutination test (Wadstrom and Ronnberg, 1983; Sen et al., 1984).

In this test,a killed suspension of Staphylococcus aureus Cowan I strain is coated with rabbit anti-cholera

toxin serum to obtain the reagent needed. A suspension of a test colony of E. coli is made on a glass slide and to this is added the staphylococcal reagent. Results are read visually.

The coagglutination test is simple, rapid and easily performed. The test is suitable for screening of large numbers of colonies. These attributes of the test make it useful for research laboratories as well as hospitals in developing countries. Replacement of some of the expensive reagents and simplification of processes involved in this test would make it even better as an assay system for bacterial enterotoxins.

2.7.15 Hybridization using genetic probes

Since the beginning of the 1980's a new diagnostic tool namely the use of radiolabelled genetic probes has been applied in the detection of E. coli enterotoxins, (Moseley and Falkow, 1980; Moseley et al., 1981; Kaper et al., 1981). Probes consisting of small fragments of DNA (157 to 800 base pairs) labelled with ^{32}P are available for LT and ST genes (Moseley et al. 1982; Kaper et al. 1981).

Briefly, test organisms are denatured, so that the subsequent single-stranded fragment of DNA can

associate or hybridize with radiolabelled probes. This is a very sensitive and highly specific test capable of detecting toxin genes even in unpurified cultures grown overnight from a loopful of diarrhoeal stool (Moseley et al., 1981). For research purposes where reagents, equipment and skilled technical staff are available this is probably the best technique to use.

2.7.16 Infant (suckling) mouse assay.

The infant mouse assay is unique in being able to detect STA specifically. This enterotoxin induces intestinal fluid accumulation causing an increase in intestinal weight (IW) to remaining body weight (RBW) ratio (IW/RBW). Ratios greater than 0.083 are widely accepted as positive or indicative of presence of STA in the test supernates (Gianella, 1976). Alternatively, diarrhoea has also been accepted as an indicator of a positive response (Moon et al., 1978).

More recently a modification of the original method of Dean et al., (1972) employing both (IW/RBW) ratios as well as the degree of diarrhoea has been used (Berry et al., 1983). Briefly the method in-

involves feeding 1 to 3 day old mice intragastrically with 0.1 ml of culture supernates. After 3 hours of incubation, the mice are sacrificed, the intestines weighed and the rest of the body weight recorded. Any evidence of diarrhoea is recorded and graded. Taken together with the ratio of intestinal weight to rest of body weight are used to indicate positive or negative STA reaction.

This technique is cumbersome to carry out, requires large numbers of mice of the right age and the intubation of mice requires training and practice for it to be achieved.

CHAPTER THREE

MATERIAL AND METHODS

3.1 STOOL SPECIMENS

Human samples

Eighty-four children reporting at the Kenyatta National Hospital for treatment for diarrhoea were rectally swabbed using sterile charcoal-tipped cotton swabs. Each swab was placed in a screw-capped tube containing Cary-Blair's transport medium (See appendix 2.1) and sent to the laboratory within 24 hours of collection. The children from whom these samples were obtained ranged in age from a week old to 5 years old.

Cattle samples

Rectal swabs were obtained from 55 healthy cattle, from a farm near Nairobi. These animals were of different ages and sex. A rectal swab was obtained from a calf with diarrhoeal disease brought for treatment at the Large Animal Clinic of the Department of Clinical Studies, Faculty of Veterinary Medicine. Rectal swabs were transported to the laboratory within 24 hours of collection, in screw-capped tubes containing Cary-Blair's transport medium.

Sheep and goat samples

Rectal swabs were obtained from 73 healthy goats sent for slaughter at Ongata-Rongai slaughter house and also from 3 flocks on farms around Nairobi.

One hundred and ten rectal swabs were collected from healthy sheep sent for slaughter at Ongata-Rongai slaughterhouse. The sheep and goats came from Kajiado District. Rectal swabs were transported to the laboratory in Cary-Blair's transport medium.

Pig Samples

A total of 109 rectal swabs were obtained from pigs. The pigs ranged in age from one week old to 12 weeks old. These pigs came from a commercial piggery at Uplands, Kiambu District and from another piggery at Kanyariri, also in Kiambu District.

Of these samples, 54 were from healthy pigs while 55 were from pigs with diarrhoea. Rectal swabs were again transported in Cary-Blair's transport medium to the laboratory.

3.2 E. COLI ISOLATES

A total of 298 samples of E. coli on nutrient agar slants were obtained by the courtesy of Dr. L.N. Mutanda of the Kenya Medical Research Institute (KEMRI),

from children suffering from diarrhoeal disease. Of these samples 195 represented pure E. coli strains while 103 were in the form of mixed E. coli strains. These isolates of E. coli were from rectal swabs or stool specimens obtained from children of 5 years or less in age. These rectal swabs or stool specimens had been tested for rotaviruses, enteropathogenic E. coli, Campylobacter species, Shigella species and Salmonella species.

Seventeen E. coli isolates from children with diarrhoea reporting for treatment at the Muhimbili Medical Centre in Tanzania, were obtained with the kind permission of Professor F. Mhalu.

3.3 PROCESSING OF SPECIMENS

3.3.1 Media and reagents.

Media used included MacConkey agar (Oxoid, England), bromothymolblue lactose agar (Brolac, Merck, Federal Republic of Germany), TCBS cholera medium (Oxoid, England), trypticase soy agar (Oxoid, England), MRVP medium (Oxoid, England), triple sugar iron medium (Oxoid, England, citrate agar (Oxoid, England) and urea agar (Oxoid, England). The preparation of these media is presented under Appendix 2.

3.3.2 Microbiological investigations

The scheme followed in microbiological investigations is presented in Figure 1. Rectal swabs or stool specimens were initially streaked onto MacConkey agar and bromothymolblue lactose agar plates. Lactose fermenting colonies with colonial morphology of Escherichia coli were directly tested for heat-labile enterotoxin (LT) by means of an adaptation of the staphylococcal coagglutination technique. Each colony which was tested this way was also inoculated into casamino acid-yeast extract broths (Biken broth) to be tested in EIA for LT and in suckling mouse assay for STA. The same colonies were also inoculated into various identification media including tryptone water, citrate agar and MRVP medium.

ETEC isolates obtained from tests for LT and STA were further characterized on the basis of the fimbriae present using agglutination of erythrocytes in the absence and presence of D-mannoside and by means of agglutination using specific antisera to the fimbriae. The fimbriae examined for were F1 (somatic type 1), F2 (CFA/I), F3 (CFA/II), F4 (K88), F5 (K99) and F6 (987P).

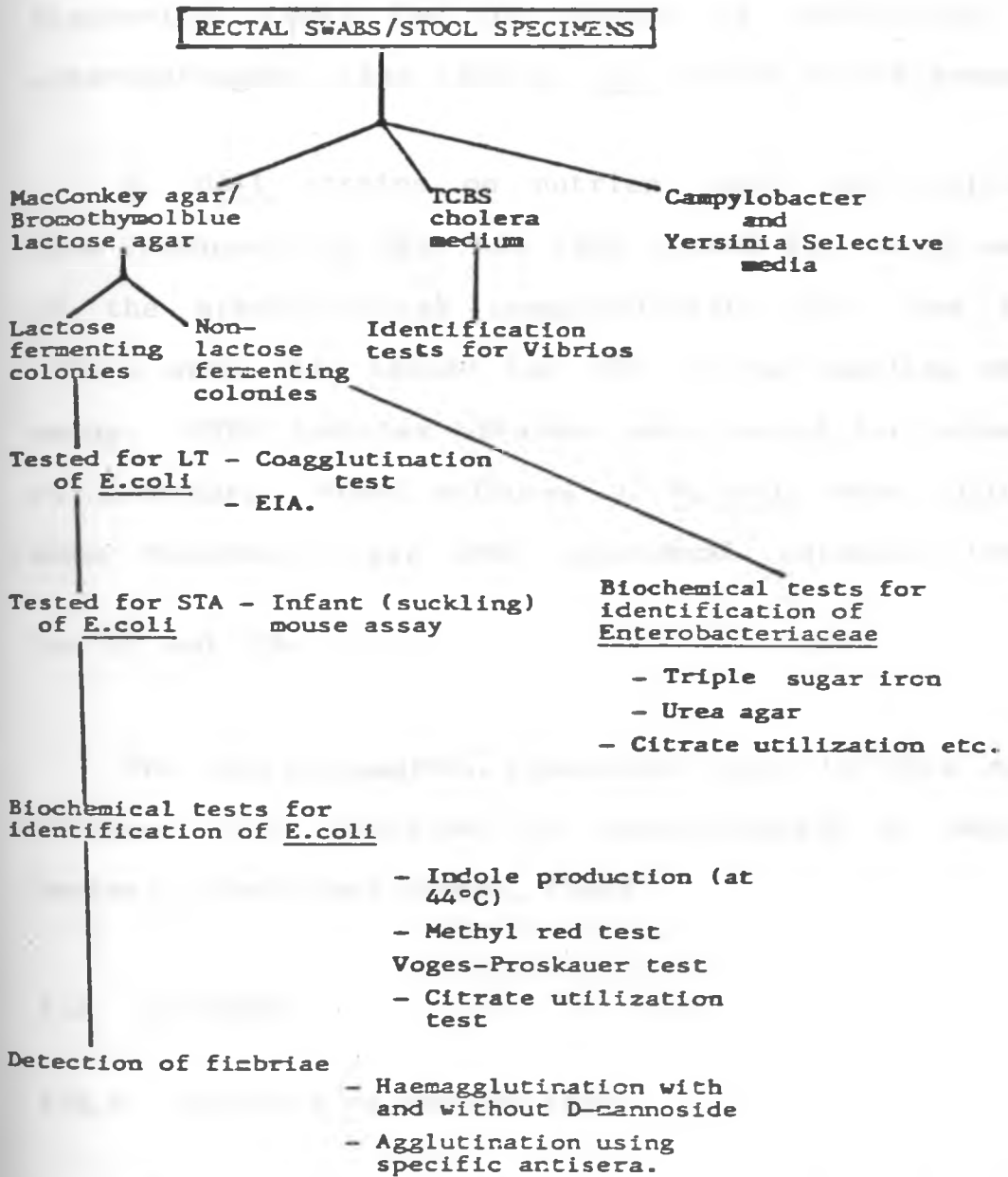

Fig 1: Scheme used in microbiological investigations

Non-lactose fermenting colonies visible on the primary isolation agar plates were subjected to various biochemical tests for the purpose of identifying any enteropathogens other than E. coli which may be present.

E. coli strains on nutrient agar were cultured onto trypticase soy agar and then tested for LT by means of the staphylococcal coagglutination test and EIA. These were also tested for STA in the suckling mouse assay. ETEC isolates obtained were tested for presence of fimbriae. Mixed cultures of E. coli were cultured onto MacConkey agar and individual colonies tested for LT and STA.

The bacteriological procedures used in this study followed those described in identification of Medical bacteria (Cowan and Steele, 1970).

3.4 ANTISERA

3.4.1 Antisera to cholera toxin.

Two adult New Zealand large white rabbits (Ear tag N°s 160 and 142) were bled prior to immunisation. Each rabbit was injected with 250. ug of cholera toxin (Sigma Chemical Company, U.S.A.) reconstituted in 1.0ml sterile distilled water and emulsified in 1.0ml

Freund's complete adjuvant (Gibco Laboratories, Grand Island N.Y., U.S.A.). Injections were made intramuscularly and into superficial lymph nodes as described by Newbould (1965). Each rabbit was given boosters of cholera toxin (50 to 100 ug) in Freund's complete adjuvant at weekly intervals in the same sites described. Blood was collected in vaselinized tubes from each rabbit prior to immunisation.

An adult female goat of the galla breed (Ear tag N° 884) and an adult female sheep of local breed (Ear tag N° 886) were bled several times prior to immunisation till 250 mls of preimmune serum per animal was obtained. Each animal was injected intramuscularly and intranodally in the superficial lymph nodes with 500 ug cholera toxin (Sigma Chemical Company, U.S.A.) reconstituted in 1.0 mls distilled water and emulsified in a 2.0 mls Freund's complete adjuvant (Gibco Laboratories, Grand Island, N.Y. U.S.A.), as described by Newbould (1965). Each animal received three subsequent boosters of between 500 ug and 800 ug of cholera toxin mixed with Freund's complete adjuvant at two week intervals. The sheep and goat were bled prior to each immunisation.

Antisera were harvested and stored following the method described by Campbell et al. (1970), with

some modifications. Briefly, freshly drawn blood was allowed to stand for a few hours at 37°C. The clot which formed was retracted from the sides of the tubes with applicator sticks. After an overnight stay at room temperature, the blood was centrifuged at 2000 x g for 15 minutes to sediment the red blood cells. Clear serum was decanted into clean plastic bottles and 0.1% sodium azide added as a preservative. Serum was stored at -20°C till required for tests.

3.4.2 Antisera to fimbrial antigens

Antisera to F2, F3, F4 F5 and F6 fimbriae of E. coli produced in rabbits were kindly provided by Dr. E. Liven of the Veterinary Institute,Oslo, Norway.

3.4.3 Goat anti-rabbit IgG serum.

Goat anti-rabbit serum available in the Department of Public Health, Pharmacology and Toxicology, immunology section.

3.5. TESTING OF ANTISERA

Antisera to cholera toxin were tested in immuno-diffusion. The microtechnique of Ouchterlony double diffusion as described by Crowle (1973) was used with slight modifications.

Briefly, 1%(W/V) of purified Oxoid agar was dissolved in 100 ml distilled water amd PBS in the ratio 3 to 1 to obtain a medium for diffusion. Sodium azide, 0.1%, was added as a preservative to the agar. Each microscope glass slide (76 x 26mm) was flooded with molten agar to give an appropriate depth of 3mm. Wells of 4.0mm diameter, placed 5.0mm apart were cut in a hexagonal pattern with a central well, using a gel-punch (Gelman Instrument Co. Michigan, U.S.A.). The agar in the punched wells was removed by suction.

Each central well was filled with 20 ug of cholera toxin while the peripheral walls were each filled with serum obtained from different bleedings of rabbits N° 160 and 142, sheep N° 886 and goat N° 884 immunized with cholera toxin. This pattern was repeated with one change, 20ug of subunit A of cholera toxin replaced cholera toxin in the central well. In a third pattern, 20ug of subunit B of cholera toxin was put into the central well while the peripheral wells received serum obtained from the immunized rabbits, sheep and goats.

Diffusion was allowed to take place in a humid chamber at room temperature and over a period of 24 hours. Precipitin lines were visible before and after staining of slides.

The gel was pressed as described by Axelsen et al. (1973) and washed in 3% (W/V) trisodium citrate (Koch-Light Laboratories Ltd., England) buffer of pH 8.5, overnight. Slides were rinsed in water, pressed a second time, air dried and stained with Coomassie brilliant blue dye (Sigma Chemicals, St. Louis, U.S.A.) for 20 minutes. The slides were then destained using coomassie destaining solution until the background was clear. For composition of coomassie blue stain and destaining solution see appendix 3.

3.6 PREPARATION OF GOAT ANTI-RABBIT GLUCOSE OXIDASE CONJUGATE

3.6.1 Isolation of IgG fraction.

An IgG fraction of a goat anti-rabbit IgG serum was prepared using the method described by Fey et al., (1976) and incorporating some modifications as follows: 25mls of 100% saturated ammonium sulphate solution was added slowly under magnetic stirring to an equal amount of goat anti-rabbit IgG antiserum. The resulting mixture was kept at room temperature (25°C) for 15 minutes and then centrifuged at 2000xg for 10 minutes. The precipitate or sediment so obtained was washed twice with 35% saturated ammonium sulphate, dissolved in PBS, after which the precipitated immunoglobulin fraction was dialysed for 18 hours at 4°C

against eluting buffer consisting of 0.02M phosphate of pH 8.0 and containing as preservative 0.02% sodium azide.

The dialysate was then passed through diethylaminoethyl cellulose (DEAE-Cellulose, Cellex D Biorad Laboratories, California, U.S.A.) column pre-equilibrated with 0.0175M phosphate buffer of pH 8.0. The flow-through fraction was collected and concentrated by ultrafiltration using a DIAFLO PM30 millipore filter with a cut-off point of 30,000 daltons.

The optical density(O.D) of the concentrated goat anti-rabbit IgG was read on a spectrophotometer (Beckman Model 25, U.S.A.) at 280nm wavelength. The amount of IgG was estimated, using the formula of Givol and Hurwithz (1969) given below:

$$E^{1\%} = 13.5 \text{ at } 280nm$$

3.6.2 Conjugation of IgG and glucose oxidase

The procedure adopted was that of Wilson and Nakane (1978) with some modifications. IgG fraction of DEAE-cellulose chromatography of the goat anti-rabbit IgG antiserum used. A conjugation efficiency of 80% was assumed resulting in a 1:1 molar ratio of IgG to glucose oxidase.

One hundred milligrams glucose oxidase enzyme (Type VII, Sigma Chemical Co. St Louis, U.S.A.) was dissolved in 9.0mls distilled water. To this solution

of glucose oxidase was added 1.0ml of freshly prepared 0.15M sodium periodate solution (32mg periodate per ml distilled water). The resulting mixture was kept at room temperature in the dark for 30 minutes under slow magnetic stirring. This solution was dialysed against cold 1mM acetate buffer of pH 4.0 with buffer changes at 30 minute intervals. The first two changes of dialysing buffer contained 0.2ml ethyleneglycol. Dialysing buffer was changed a further four times.

A volume of IgG solution (6.73mls) equivalent to 100mg IgG was added to 2.0mls of carbonate buffer pH 9, 0.2M under magnetic stirring. The dialysed glucose oxidase solution was slowly added to IgG solution in carbonate buffer. Stirring of the mixture continued in the dark for 2 hours at room temperature to allow conjugation of glucose oxidase to IgG. The pH of the conjugate was adjusted to 7.5 using 1M HCL. Conjugation was allowed to continue at 4°C overnight.

Forty milligrams lysine was added to the IgG-glucose oxidase conjugate and the pH was again adjusted to 7.5 using 1M HCL. Further conjugation was allowed to proceed at room temperature under magnetic stirring for 2 hours. The conjugate was centrifuged and the supernatant collected. Normal goat serum was added to the conjugate upto 10%(V/W) and conjugate divided into two portions. One portion was kept at -20°C, mixed

with an equal volume of glycerol, while the second portion was lyophilized in small volumes of 1.0ml and kept at -20°C. Each of the portions of goat anti-rabbit IgG glucose oxidase conjugate was titrated out in a double layer enzyme immunoassay (EIA) to determine the optimal dilutions for use.

3.7 COAGGLUTINATION TEST FOR THE DETECTION OF HEAT-LABILE TOXIN (LT)

3.7.1 Growth and stabilisation of Staphylococcus aureus

The method adapted was that of Kronvall (1973) incorporating some modifications. S. aureus (strain Cowan I) was cultured in tryptic soy broth (Gibco, Paisley, Scotland) at 37°C with some agitation to obtain a luxuriant growth over a 24 hour incubation period. The bacterial suspension was then centrifuged in a refrigerated centrifuge (Minifuge, Hereus Christ, Federal Republic of Germany) at 4°C and 2000xg for 15minutes. The supernatant was discarded and the bacterial sediment resuspended in PBS. This sediment was washed 3 times in PBS and finally resuspended in 0.5%(V/V) of 10% formaldehyde solution and kept at room temperature for 3 hours.

The suspension was again centrifuged as before and washed 4 times in PBS. After resuspension in PBS to 10% (v/v), the suspension was heated to 80°C and maintained at that temperature for 10 minutes to kill the bacteria. The killed bacteria suspension was washed twice in PBS and a final suspension of 10%(v/v) in 0.5% formaldehyde solution was made and stored at 4°C till required for coating with antiserum.

3.7.2 Coating of staphylococci with rabbit antiserum

The coating of staphylococci with antiserum was done following the method of Kronvall (1973) with some changes.

Briefly, 2.0mls of a well mixed 10% suspension of stabilized S. aureus from the stock suspension was centrifuged and the supernate discarded. The staphylococcal sediment was washed 5 times in PBS and finally resuspended in 2.0mls PBS. Two hundred microlitres of rabbit anti-cholera serum was slowly added to the staphylococcal suspension while shaking the suspension on a whirlmixer (Fissons Scientific apparatus, Leciestershire, England). After 5 minutes of mixing to ensure optimal coating of rabbit serum immunoglobulins to the staphylococci, the suspension was centrifuged and both supernatant and sediment saved. The supernate which contained immunoglobulins was used in coating another batch of staphylococci.

The bacterial sediment was washed 4 times in PBS to remove unbound serum proteins and the antibody coated staphylococcal sediment resuspended in 2% (v/v) PBS. Sodium azide was added to 0.1% (w/v) as a preservative and the reagent kept at 4°C till needed for co-agglutination test.

3.7.3 Coagglutination test

Reagents used in this test were a 2% suspension of killed stabilized Staphylococcus aureus Cowan I strain, coated with rabbit N°142 anti-cholera toxin serum and 0.05%(v/v) dilution of the non-ionic detergent Triton X-100, in 4% carbol fuchsin dye.

A drop of 0.05% Triton X-100 containing carbol fuchsin dye was placed on a clean dry microscope glass slide. Using a sterile bacteriological loop, part of a colony of E. coli to be examined was picked off a MacConkey agar or bromothymolblue lactose agar or trypticase soy agar plate and emulsified in the detergent on the slide to obtain a uniform suspension. To this was added a drop (0.25ul) of well mixed 2% suspension of stabilized S. aureus coated with rabbit anti-cholera serum. The slide was gently rocked from side to side for up to 5 minutes and the result read against a dark background, in adequate lighting.

E. coli strains H10407, LT positive and H10407-1, LT negative were used as control strains.

3.8 ENZYME IMMUNOASSAY FOR THE DETECTION OF LT.

3.8.1 Preparation of E. coli Supernates for EIA.

A colony of E. coli was inoculated into 5.0mls of Biken broth (See Appendix 2) containing 100 ug/ml lincomycin (Sigma Chemical Co. St. Louis, U.S.A.) and incubated at 37°C overnight under slow agitation on a roller drum. Each tube received polymyxin B sulphate solution at the rate of 10 ug/ml and was kept under agitation at 37°C for 30 minutes.

Each culture was subjected to 100 watt bursts of sonication for 10 seconds in an ice bath using a Braunsonic 1510 (Braun Messungen Ag. Germany). Culture broths were then centrifuged at 2000xg and for 15 minutes in order to sediment the bacteria. Clear supernate was collected using sterile pipettes into clean tubes and kept frozen at -20°C till required for testing.

3.8.2 Four layer sandwich enzyme immunoassay for detection of LT.

The method adopted here was that of Yolken et al. (1977), with modifications. Disposable microtitration plates (Falcon, U.S.A.) were coated with 100ul of a dilution of 1 in 4000 of sheep anti-cholera toxin serum per well (determined from checkerboard titration).

The diluent used was 1 in 100 PBS containing as preservative 0.1% sodium azide. Plates were incubated in a humid chamber at room temperature overnight and subsequently frozen at -20°C till required.

A plate was allowed to thaw at room temperature washed 3 times with a standard wash solution (0.15 PBS with 0.5%(v/v) Tween 20) at one minute intervals. One hundred microlitres of supernates obtained from growth of E. coli in Biken broth (Section 3.9.1) was added in duplicate, to wells and the plate again incubated at room temperature in a humid chamber overnight. Positive and negative control supernates obtained from growth in Biken broth of E. coli strains H10407-1 (Serotype 078:H19), LT positive and H10407 (Serotype 078:H19) LT negative were also included. The plate was washed using the standard wash solution and 100ul per well of rabbit N° 142 anti-cholera serum, the second antibody layer, diluted 1 in 5000 in PBS/Tween added to wells.

Following a further incubation for 2 hours at 37°C in a humid chamber, the plate was then washed as before and 100ul of a goat anti-rabbit glucose oxidase conjugate diluted 1 in 5000 in KCL/EDTA with 0.5% Tween 80 added. This dilution was determined through a checkerboard titration. The plate was incubated at 37°C in humid chamber for 1 hour and after washing in the standard way 100ul of a substrate solution was added to each well. The plate was kept

at room temperature in the dark for 1 hour to enable enzyme and substrate to react, after which results were read by visual observation.

3.9 DETECTION OF STA BY SUCKLING MOUSE ASSAY.

A modification of the method of Dean et al. (1972) was followed in this bioassay.

Supernates of E. coli obtained from growth of E. coli in biken broth and already tested for LT were allowed to attain a temperature of 37°C in an incubator. To 2.0ml supernate, 0.1mls of a 2% sterile solution of Evans Blue dye was added and thoroughly mixed.

One to three day old Balb/C mice were separated from their mothers and kept for at least 2 hours. A 1.0cc tuberculin syringe containing E. coli supernate mixed with Evan's Blue dye and fitted with a flexible plastic tubing of external diameter of 0.5mm, was gently introduced into the mouth of a mouse and the mouse allowed to swallow the rubber tubing. An amount of 0.1ml of supernate/dye mixture was then introduced into the stomach of the mouse, by gentle depression of the plunger of the syringe. Each test supernate was inoculated into two mice.

The mice, following intragastric inoculation with E. coli supernates, were kept at room temperature in suitable containers for a period of 3 hours. Each mouse was sacrificed, the abdominal cavity exposed

and the entire small intestines dissected out and weighed (IW). The rest of the body weight (RBW) was also recorded for each mouse. Ratios of IW and RBW were calculated and recorded. Means of IW/RBW for each pair of mice which received a given supernate were calculated.

Mice which were found dead after incubation period as well as mice whose stomachs burst in the process of being fed with supernate were discarded. The corresponding supernates were retested.

3.10 AGGLUTINATION OF ERYTHROCYTES IN THE PRESENCE/ABSENCE OF D-MANNOSE FOR IDENTIFICATION OF FIMBRIAE.

Colonization factors I and II (CFA/I and CFA/II), also termed F2 and F3 fimbria antigens were identified by mannose resistant agglutination of human group A and bovine erythrocytes as described by Evans and Evans (1978). Somatic type 1 and F1 fimbriae of E. coli were identified by their ability to cause the agglutination of guinea pig erythrocytes which is inhibited by mannose (Salit and Gotschilch, 1977). In these experiments α-methyl D-mannoside was used for the inhibition of haemagglutination by fimbriae.

3.10.1 Preparation of erythrocyte suspensions.

Freshly drawn human group A blood, bovine blood and guinea pig blood in anticoagulant were obtained, washed several times in physiological saline (0.085% NaCl (w/v). Each erythrocyte sediment was finally resuspended to 3% in physiological saline. Erythrocyte suspensions were kept at 4°C till needed, but storage never exceeded one week.

3.10.2 Haemagglutination tests.

Haemagglutination tests were performed as described by Levine et al. (1983) with some modifications.

Several colonies of E. coli of a particular strain grown overnight on trypticase soy agar were emulsified in 200ul of haemagglutination buffer to make a uniform suspension in a well on a white ceramic tile. Two hundred microlitres of 3% suspension of erthrocyte of a particular species was added to the suspension of E. coli. The mixture was swirled for five minutes using a clinical rotator (Arthur Thomas Company, Philadelphia, U.S.A.). The tile was then observed visually for any haemagglutination. The experiment was repeated but this time up to 100ul of 0.1% α-methyl D-mannoside was added to erythrocyte/E. coli mixture before observing for any agglutination.

Evidence of agglutination was recorded. Control strains of E. coli used were BAM 1 which is somatic type 1 or F1 positive, H10407, CFA/I or F2 positive and strain PB176, CFA/II or F3 positive.

3.11 IDENTIFICATION OF FIMBRIAE BY MEANS OF A COAGGLUTINATION TEST

Staphylococcal suspensions (2%) coated with antisera against F2, F3, F4, F5 and F6 produced in rabbits were used. Coating of staphylococci with each antiserum was done as described for coagglutination test for detection of LT (Section 3.7.2).

Coagglutination test was done as described by Kronvall (1973) with modifications. A colony of E. coli was mixed in a drop (25ul) of physiological saline on a clean microscope glass slide to obtain a uniform suspension. To the bacterial suspension was added, a drop (25ul) of 2% suspension of Staphylococcus aureus coated with antiserum to a fimbrial antigen. The slide was gently rocked from side to side for about five minutes and the reaction read visually and recorded. Each isolate of E. coli was tested against each of the fimbrial types.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 THE STAPHYLOCOCCAL COAGGLUTINATION TEST FOR THE DETECTION OF HEAT-LABILE TOXIN OF E. COLI

4.1 Characterization of antisera to LT.

The antisera produced in rabbit N°142, sheep N°886 and goat N°884 gave precipitin reactions of identity with cholera toxin (CT) as shown in Figure 2. These antisera also gave precipitin reactions with subunits A and B of CT in immunodiffusion tests. Heat-labile enterotoxin of E. coli is known to have antigenic determinants common to the subunits A and B of cholera toxin (Clements and Fingelstein, 1978). Therefore, rabbit N°142 and sheep N°886 anti-CT sera were selected for use in immunoassays for the detection of LT of E. coli.

Because protein A of Staphylococcus aureus binds better to the immunoglobulins of rabbits (Richman et al., 1982), the anti-CT serum produced in rabbit N°142 was selected for use in the coagglutination (CAG) test for the detection of LT. Sheep N°886

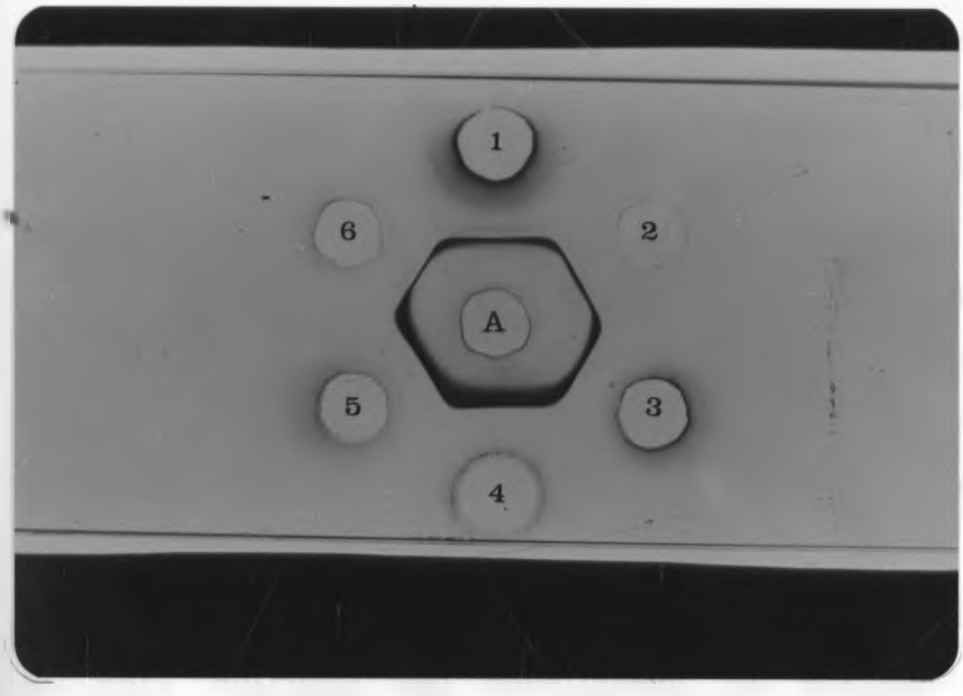

Figure 2. Immunodiffusion test showing the reactions between cholera toxin (A) and antisera produced in a sheep No. 886 (2,5), rabbit No. 142 (1,4) and goat No. 884 (3,6). The reactions of these antisera show identity.

anti-CT serum and rabbit N°142 anti-CT serum were selected for use in the enzyme immunoassay (EIA) for the detection of LT.

4.1.2 Selection of a suitable detergent to enhance enterotoxin release from cultures of E. coli.

Polymyxin B sulphate in a concentration of 0.5mg/ml and the less expensive Triton X-100 at a dilution of 0.05%(v/v) enhanced the release of periplasmic LT from E. coli strain H10407 (serotype 078:H11), LT positive, leading to strong CAG reactions with anti-CT coated staphylococcal suspension as shown in Figure 3. Triton X-100 treated E. coli cultures produced stronger CAG reactions with anti-CT coated staphylococcal suspensions than cultures treated with Tween 20. The time that elapsed between addition of anti-CT coated staphylococcal suspension to detergent-treated E. coli cultures and development of maximum coagglutination reaction was however the same for both Tween 20 and Triton X 100 (Table 5).

Triton X-100 was selected as a detergent suitable for use in CAG test to increase the release of LT from E. coli, since it was as effective as polymyxin B sulphate and also less expensive. It was also more effective than Tween 20.

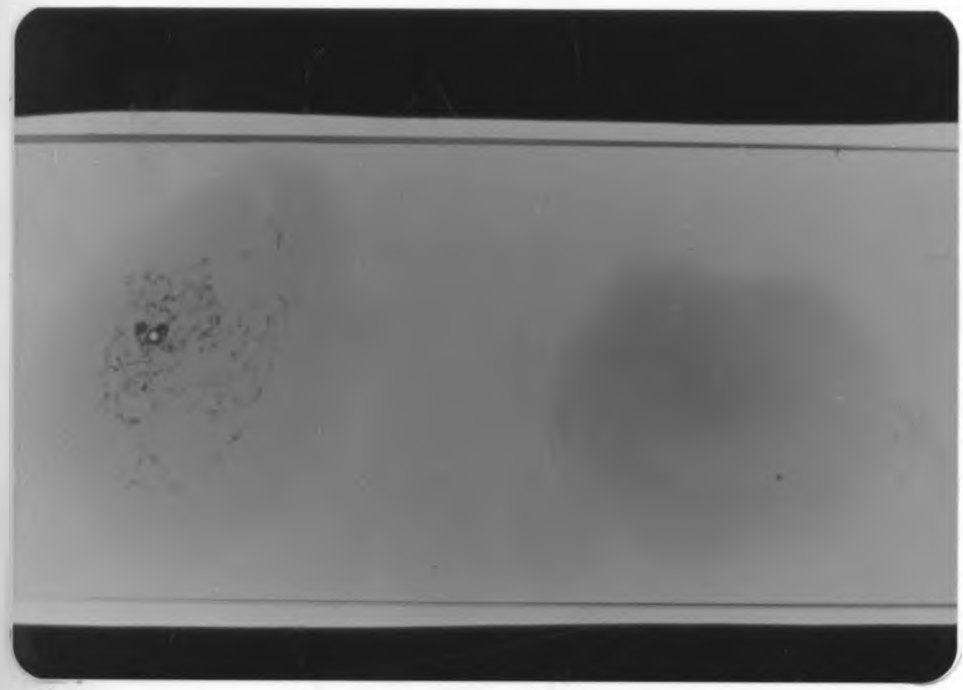

Figure 3 Slide coagglutination test for heat-labile toxin of E. coli showing a positive reaction (left) and a negative reaction (right).

Table 5. DETERMINATION OF OPTIMAL CONCENTRATIONS OF POLYMYXIN B SULPHATE, TRITON X-100 AND TWEEN 20 IN THE COAGGLUTINATION TEST FOR THE DETECTION OF HEAT-LABILE ENTEROTOXIN OF E.COLI.

A. Polymyxin B Sulphate

Concentration	Reaction observed	
	LT Positive	LT Positive
1mg/ml	+++	-
0.5mg/ml	+++	-
0.1mg/ml	++	-
0.05mg/ml	++	-
0.01mg/ml	+	-

B. Triton X-100

Concentration	Reaction observed	
	LT Positive	LT negative
0.1%(v/v)	+++	-
0.05%(v/v)	+++	-
0.025%(v/v)	++	-
0.01%(v/v)	+	-

C. Tween 20

Concentration	Reaction observed	
	LT Positive	LT NEGATIVE
0.1%(v/v)	++	-
0.05%(v/v)	++	-
0.025%(v/v)	++	-
0.01%(v/v)	+	-

4.1.3 Results of enzyme immunoassay and the coagglutination test for the detection of heat-labile toxin of *E. coli*

One hundred and seventy one strains of *E. coli* isolated from children with diarrhoeal disease, attending the Kenyatta National Hospital, Nairobi were tested for LT by means of the CAG test and EIA. Of these strains, 5.3% (9/171) were found positive for LT while 94.7%(162/171) were negative. All the strains which were positive for LT in EIA were also positive for LT in CAG and vice versa. However with regard to 17 *E. coli* strains from children with diarrhoeal disease at the Muhimbili Medical Centre, Dar es Salaam, Tanzania, there was a difference, with the EIA detecting 17.6%(3/17)LT positive *E. coli* compared with 11.8%(2/17) LT positive *E. coli* detected by the CAG.

A total of 103 *E. coli* cultures obtained from children with diarrhoeal disease attending the Kenyatta National Hospital, were cultured on trypticase soy agar and as many individual colonies as possible examined for LT by means of the CAG test and EIA. The total number of colonies investigated with regard to LT was 2083 with an average of 20 colonies, tested per primary culture. Of the 2083 colonies, 5.9%(123/2083) were positive for LT in the CAG as compared with 7.1% (147/2083) in the EIA. All the colonies which were positive for LT in CAG were also positive for LT in EIA.

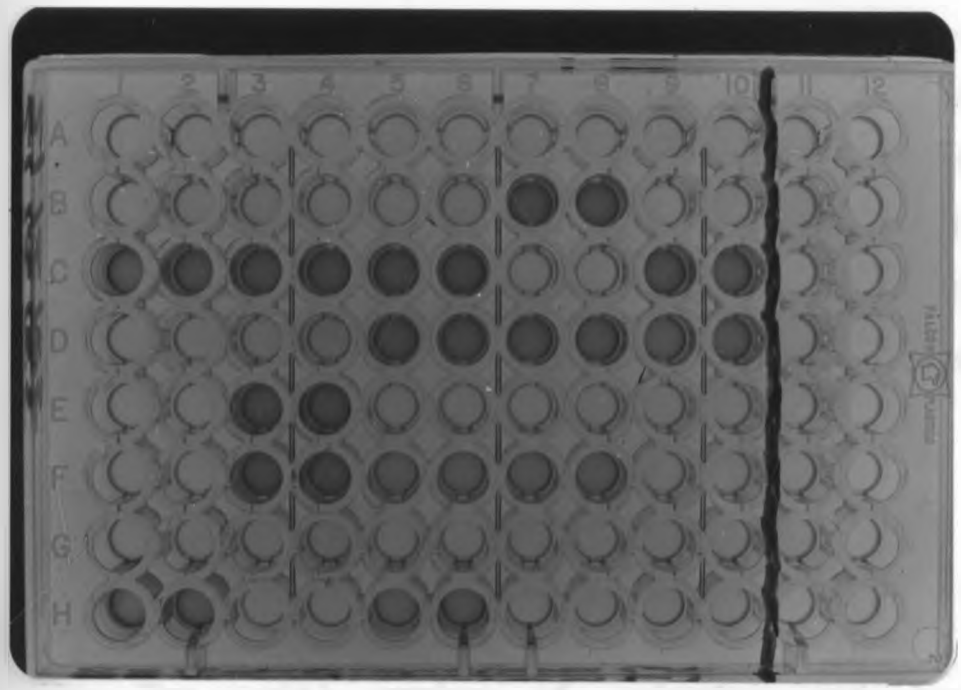

Fig 4. A microtitre plate showing results of a representative EIA of LT. Wells C3 and 4 show the positive (bluish green colour) Control (E. coli strain H10407 cell-free supernate), while E5 and 6 show the negative (colourless) Control (E. coli strain H10407-1). The 11th and 12th columns of wells (colourless) represent substrate, conjugate and LT controls.

The other wells contain cell-free supernates from LT positive (bluish green) and negative (colourless) E. coli cultures of human and animal origins. The tests were performed in duplicate.

E. coli colonies from primary stool cultures, obtained from children and domestic animals were examined with the CAG test and EIA for LT. A total of 8438 colonies were examined. Upto 26 colonies per sample and an overall average of 20 colonies per primary stool culture were tested. There was a difference in the results of the two assays, with 9.8% (825/8438) of these colonies being found positive for LT in CAG as compared with 10.3% (867/8438) being positive in the EIA (Table 6).

4.1.4 Comparison between EIA and CAG test for the detection of heat-labile toxin of E. coli.

The total number of colonies and isolates or strains of E. coli investigated for LT by means of the EIA and CAG test was 10,709. The number of colonies positive for LT by means of the CAG test was 958, while 1025 were positive for LT in the EIA. The CAG test for LT thus missed 6.6% (67/1025) of the E. coli isolates or colonies which were positive in the EIA.

The CAG test for LT was found to have a sensitivity of 93.3% and a specificity of 100% compared with the EIA. These results are summarised in table 7.

Table 6. RESULTS OF EIA AND CAG TEST FOR THE DETECTION OF HEAT-LABILE TOXIN OF *E. COLI*.

N°. OF CULTURES OR STRAINS	SOURCE	TOTAL N°. OF STRAINS OR COLONIES TESTED	CAG		EIA	
			+	-	+	-
17	Muhimbili Medical Centre (children with diarrhoea)	17	1	16	2	15
171	KNH (Children with diarrhoea)	171	9	162	9	162
103	KNH (children with diarrhoea)	2083	123	1960	147	1936
84	KNH (children with diarrhoea	1557	345	1212	377	1180
110	Sheep (healthy)	2210	72	2138	73	2137
73	Goats (healthy)	1394	10	1384	10	1384
54	Pigs (healthy)	1074	52	1022	55	1019
55	Cattle (healthy)	1109	50	1059	52	1057
1	Calf (diarrhoea)	12	12	0	12	0
TOTAL		10,709	958	9751	1025	9684

Table 7. EVALUATION OF THE COAGGLUTINATION TEST FOR THE DETECTION OF HEAT-LABILE ENTEROTOXIN OF E.COLI

		EIA for detection of LT		
		+	-	Total
Coagglutination (CAG) test for detection of LT	+	958	0	958
	-	67	9684	9751
Total		1025	9684	10,709

Sensitivity of CAG = 93.3%(958/1025)

Specificity of CAG = 100%(9684/9684)

The CAG test missed 6.6%(67/1025) of LT positive colonies.

4.1.5 Discussion

In recent years, attention has been focused on the use of several immunological techniques for the detection of heat-labile enterotoxin of E. coli as replacement for the expensive and demanding bioassay. The immunoassays are less cumbersome than the bioassays and are sensitive enough to detect low levels of LT, but may not be applicable in routine laboratory investigations, especially in many developing countries. Immunoassays such as radioimmunoassay and enzyme immunoassay generally require sophisticated equipment and expensive reagents. There has thus been a need to develop a simple, inexpensive as well as quick method of detecting enterotoxins.

The application of the staphylococcal coagglutinatin technique of Kronvall (1973) appears to be a promising immunoassay for the detection of LT because of its simplicity, potentially high sensitivity, and reproducibility.. A tube coagglutination test was originally used for the detection of LT (Brill et al., 1979) and later, the simpler but less sensitive slide coagglutination test was introduced (Wadstrom and Ronnberg, 1983; Sen et al., 1984). Despite the apparent suitability of this test for routine laboratory detection of LT, no extensive evaluation had been done.

The evaluation of the test, presented in this study, showed that the CAG test for LT was approximately 7% less sensitive compared with EIA, but the specificity of both tests was found to be the same. This is similar to the report of Brill et al. (1979) who showed that the sensitivity of the tube coagglutination test for the detection of LT was roughly comparable to sensitivities reported for the immunoassays for the detection of LT, such as passive immune hemolysis (Evans and Evans, 1977) radioimmunoassay (Greenberg et al., 1977) and enzyme-linked immunosorbent assay (Yolken et al., 1977).

An average of 20 E. coli colonies from each stool specimen were examined for LT in the present study. In most stool specimens where the EIA detected LT-producing E. coli, nearly half of the approximately 20 colonies were positive for LT. In a few healthy individuals however only 1 to 3 LT positive colonies were found among an average of 20 colonies. These few colonies were detected by both tests.

A maximum of 26 colonies per stool specimen were examined for LT. This however does not preclude testing upto 30 or even 50 colonies if the number

of isolated colonies from a primary culture allows. By examining as many colonies as possible for LT, the chances of missing an otherwise positive isolate of E. coli because it could not be subjected to the CAG test at all, is thus greatly minimised, especially when handling specimens from healthy individuals or carriers who are likely to harbour few toxigenic strains in a largely non-toxigenic population of E. coli.

It has been suggested that when 6 colonies are chosen randomly from a stool culture, there is a 99% chance that at least one of these isolates will represent the predominant strain of aerobic gram-negative bacteria in faeces (Lidin-Janson et al., 1977). This has formed the basis for deciding on the number of colonies which should be tested in order to obtain reliable results for LT-producing E. coli in a sample.

Earlier reports on colonization of the gastro-intestinal tract of man and animals by E. coli indicate that it is likely that in any individual the E. coli population consists of a majority serotype and a number of minority serotypes (Wallick and Stuart, 1943). Therefore by selecting as many individual colonies as possible for assay for LT by means of CAG, one

does not only surpass the chances of testing the most predominant strains of E. coli but one might very well be testing the minority serotypes of E. coli present in a faecal sample.

The pooling of many colonies by simply scooping them off a primary isolation culture before testing for enterotoxins has been suggested as a means of overcoming this controversy over the number of colonies to be tested in order to arrive at a diagnosis of ETEC. However some doubt has been placed on the reliability of this method of screening for ETEC (Back, 1979). Consequently, the testing of as many individual colonies as possible per primary isolation culture remains the most useful way of identifying ETEC, especially where these ETEC strains are overwhelmed in numbers by non-toxigenic strains of E. coli. The very short time it takes to examine a colony for LT by means of the CAG makes this test ideal for such a purpose.

In the CAG test, 0.1ml of antiserum is used with staphylococcal suspension to produce a reliable reagent for LT. This reagent may be used to examine as many as 200 colonies of E. coli as compared with using the same volume of antiserum (0.1ml) to test only 4 colonies in the less sensitive gel immunodiffusion test (Biken test) of Honda et al. (1981). In addition, there was no need to isolate anti-cholera gammaglobulins

for use in the CAG since during coating of staphylococci with antiserum, the protein A of staphylococci preferentially binds to the Fc portion of the gamma-globulins (Kronvall, 1973).

Most LT activity has been detected in cell lysates with only 10% of total LT activity being detected in cultures (Wadstrom et al., 1974). There is a strong indication that most LT resides in the periplasmic space of the organism (Evans et al., 1974). For this reason bacterial cells have often been treated with polymyxin B sulphate or sonication to effect the release of LT (Yolken et al., 1977; Honda et al., 1981). In this study, the non-ionic detergent Triton x-100 was found to be a suitable replacement for the more expensive antibiotic polymyxin B sulphate, in facilitating the release of LT and improving the ability of the CAG test to detect LT.

The antibody-coated staphylococcal suspension retained its activity for a year when kept at 4°C with 0.1% sodium azide as preservative. With 0.1% sodium azide as preservative, staphylococcal coagglutination reagent will keep at room temperature for at least a month. The present study therefore shows the very good keeping quality of the staphylococcal coagglutination reagent, a quality that further emphasises the suitability of this test for use in

field investigations of diarrhoeal diseases.

An unusual feature of the CAG test for LT is the appearance in the form of a filmy floating mass of a positive reaction. This is different from a typical coagglutination reaction appearance, described as granular in appearance in contrast to a negative reaction which is milky in appearance (Sen et al., 1984; Brill et al., 1979). The cause of the unusual appearance of CAG test for LT in this study maybe attributable to the use of a non-ionic detergent.

This evaluation of the staphylococcal coagglutination test for LT has shown that this test is highly specific for LT and has a sensitivity nearly equal to that of the EIA for LT. The ease with which the CAG test can be used for the screening of a large number of colonies from a primary culture may more than compensate for the lower sensitivity as compared with an EIA. The CAG test serves as a better replacement for other more sophisticated immunoassays especially in studies on populations aimed at determining carrier status of ETEC disease.

4.2 PREVALENCE OF ENTEROTOXIGENIC E.COLI IN HUMAN AND ANIMAL POPULATIONS.

4.2.1 LT positive E. coli isolates from children and domestic animals.

The results of EIA for detection of LT were used for the determination of LT-producing E.coli isolates from children and domestic animals.

Of the 329 children from Kenyatta National Hospital, whose stool samples were examined for LT-producing E. coli, 10.6%(35/329) were positive for this enterotoxin. E. coli positive for LT were identified in 23.5% (4/17) of children with diarrhoea from Muhimbili Medical Centre, Tanzania.

In the domestic animal species examined, the isolation rates of E. coli positive for LT were as follows: healthy pigs, 9.2%(5/54), diarrhoeic pigs, 29.0%(16/55), healthy cattle, 10.9%(6/55), healthy sheep, 7.3% (8/110) and healthy goats, 1.4% (1/73). A calf with diarrhoea was also positive for LT-producing E. coli. (Table 8)

4.2.2 ST positive E. coli isolates from children and domestic animals.

Results of ST-producing E. coli were based on the suckling mouse assay. The ratio of intestinal

Table 8: PREVALENCE RATES OF ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC) IN DOMESTIC ANIMALS AND MAN.

	Diarrhoeic animals		Healthy animals	
	Number of Samples	Number of Isolates	Number of samples	Number of ETEC isolates.
Pigs	55	18(32.7%)	54	10(18.5%)
Cattle	1	1	55	12(21.8%)
Sheep	-	-	110	8(7.3%)
Goats	-	-	73	1(1.4%)
Humans	329	135(41.0%)	-	-

weight (IW) to the rest of body weight (RBW) of mice regarded as indicating presence of STA in test supernate was 0.083. The results of IW, RBW and IW/RBW ratios are presented in Appendices I, II, III and IV.

E. coli isolates positive for STA were identified in 33.4%(110/329) of stool samples from children presented at Kenyatta National Hospital with diarrhoea. A higher figure of 41.2%(7/17) was obtained of STA positive E. coli in children with diarrhoea at the Muhimbili Medical Centre in Tanzania.

In the domestic animals, STA-positive E. coli were present in 13.0% (7/54) of healthy pigs, 20.0%(11/55) of diarrhoeic pigs and 16.4%(9/55) of healthy cattle. A calf with diarrhoea also harboured STA positive E. coli. E. coli from stool specimens collected from 110 healthy sheep and 73 healthy goats were examined and found negative for STA. (Table 8).

4.2.3 Isolation rates of ETEC in domestic animals and children.

E. coli positive for either LT, ST or LT/ST were isolated from 41.0%(135/329) of children with diarrhoea from Kenyatta National Hospital and 52.9%(9/17) of children with diarrhoea from the Muhimbili Medical Centre in Tanzania.

For the domestic animals examined, ETEC comprised 18.5%(10/54) of healthy pigs, 32.7%(18/55) of diarrhoeic pigs, 21.8% (12/55) of healthy cattle, 7.3%(8/110) of healthy sheep and 1.4%(1/73) of healthy goats. E. coli positive for LT/ST was isolated from a calf with diarrhoeal disease.

4.2.4 Types of enterotoxin produced by E. coli isolates from humans and domestic animals.

Table 9 shows the results of LT, ST and LT/ST positive E. coli isolated from diarrhoeic children from Kenya and Tanzania as well as healthy and diarrhoeic domestic animals. The predominant enterotoxin type detected was ST.

4.2.5 Distribution of ETEC among children of age and sex.

All the children with diarrhoeal disease from Kenyatta National Hospital were 5 years old or less, and 79.3%(261/329) of these children were in the age group 0-2 years. Of all the children with ETEC, 88.9% were 2 years and below. Of all the ETEC isolates found, 53.3%(72/153) were from boys while 45.1%(61/135) were from girls. (Table 9)

Table 9. DISTRIBUTION OF LT, ST AND LT/ST POSITIVE E.COLI IN CHILDREN AND DOMESTIC ANIMALS

SOURCE OF STOOL OR CULTURE SPECIMENS	HEALTH STATUS	TOTAL NUMBER OF STOOL OR CULTURE SPECIMENS	ENTEROTOXINS PRODUCED		
			LT	ST	LT/ST
HUMAN	DIARRHOEA (Children, Kenyatta National Hospital)	329	25 (7.5%)	100 (30.4%)	10 (3.0%)
	DIARRHOEA (Muhimbili Medical Centre, Tanzania).	17	3 (17.6%)	6 (35.3%)	1 (5.9%)
PIG	HEALTHY	54	3 (5.5%)	5 (9.2%)	2 (3.7%)
	DIARRHOEA	55	7 (12.7%)	2 (3.6%)	9 (16.4%)
CATTLE	HEALTHY	55	3 (5.4%)	6 (10.9%)	3 (5.4%)
	DIARRHOEA	1			1
SHEEP	HEALTHY	110	8 (7.3%)	0	0
GOATS	HEALTHY	73	1 (1.4%)	0	0

Table 10. DISTRIBUTION OF LT POSITIVE, ST POSITIVE AND LT/ST POSITIVE E.COLI ISOLATES FROM CHILDREN OF DIFFERENT AGES.

AGE GROUP	TOTAL NUMBER OF CHILDREN	E T E C			
		LT POSITIVE	ST POSITIVE	ST/LT POSITIVE	T O T A L
0-6 months	63	2(3.2%)	26(41.3%	4(6.3%)	32(50.8%)
7-12 months	103	1(0.9%)	15(14.6%)	3(2.9%)	19(18.4%)
13-18 months	59	14(23.7%)	33(55.9%)	1(1.7%)	48(81.3%)
19-23 months	36	5(13.9%)	16(44.4%)	0	21(58.3%)
24-29 months	22	1(4.5%)	4(18.2%)	0	5(22.7%)
2½-3 years	15	1(6.7%)	0	0	1(6.7%)
> 3 years	2	0	2(100%)	0	
AGE UN-RECORDED OR NOT AVAIL-ABLE	29	1(3.4%)	4(13.8%)	2(6.9%)	7(24.1%)

The ages and sexes of children with diarrhoea from Muhimbili Medical Centre, Tanzania were unavailable.

4.2.6 Discussion

Isolation rates of ETEC from humans vary considerably for different parts of the world (Table 3). This wide variation may be due to true geographical differences, although many other factors may explain it.

The isolation rate of ETEC of 41% reported for children in the present study is well within the range of what has been found in some developing countries, such as 55% in Bangladesh (Nalin et al., 1975).

In an earlier study ETEC was isolated from 8.3% of children with diarrhoea attending Kenyatta National Hospital, a figure which is far less than that reported for the present study. The reasons for the two different rates are likely due to a more sensitive test for the detection of LT, the EIA and the use of the co-agglutination test which allowed the examination of an average of 20 colonies per patient.

Prolonged time of storage as well as repeated subculturing on agar of E. coli may lead to loss of plasmids which encode for production of enterotoxins (Evans et al., 1977). The use of an adaptation of

the staphylococcal coagglutination test of Kronvall (1973) enabled the testing of E. coli from a primary culture of stool specimens without the need for sub-culturing. When it was found necessary to subculture, trypticase soy agar was the medium of choice, having been found to preserve enterotoxin plasmids. The ease and rapidity with which the CAG test could be performed also enabled the testing of E. coli within a very short time thus avoiding any prolonged storage which is thought to cause loss of plasmids encoding for enterotoxin production. The precautions taken in handling and testing of specimens may have contributed to the high isolation rate of ETEC in children in this study as compared with earlier investigations.

Although E. coli has been known as a pathogen in domestic animals for a long time, the lack of an easy and inexpensive test for the detection of entero-toxins has represented a limitation on the available information as regards the prevalance rates of ETEC in the domestic animals. According to the Ministry of Agriculture, Fisheries and Food Laboratories in England and Wales, 35% of all the diseases diagnosed in pigs between 1975 and 1980 were neonatal diarrhoea due to E. coli. Similarily, nenonated calf and lamb diarrhoea due to E. coli comprised 26% and 17% of all the diseases recorded in these two species (cited by Morris and Sojka, 1985). In the study

presented here, ETEC was isolated from 32.7% of pigs with diarrhoea. Pigs and cattle appear to be important sources of ETEC (18.5% and 21.8%) compared with sheep and goats (7.3% and 1.4%) which are of less importance as healthy carriers. Adetosoye (1980) identified ETEC as the main pathogen involved in diarrhoea in all calves, piglets and lambs he examined. The low isolation rate of ETEC from goats obtained confirmed the known fact that goats are not important hosts of ETEC (Sojka, 1965; Sojka 1971).

ETEC disease in animals follows initial ingestion of ETEC strains by susceptible animals, colonization of the intestines of these animals made possible by the presence on ETEC of appropriate fimbriae and elaboration of enterotoxins (Nielsen et al., 1969). The risk of diseases is greatest where there are large numbers of animals which harbour ETEC strains. The healthy carriers of ETEC found in the present study especially among pigs and cattle may serve as a source of ETEC for the infection of other animals.

ETEC strains that cause diarrhoeal disease in humans belong to a heterogeneous array of O:K:H serotypes and also produce different types of enterotoxins. Generally, strains that are positive for LT/ST tend to cause more severe diarrhoeal syndromes than strains positive for either LT alone of ST alone. It is also

recognised that in certain geographical areas LT/ST strains predominate, while strains positive for either LT or ST alone are rare. In the present study, only 7.4% of all the ETEC isolated from children with diarrhoeal disease from Kenyatta National Hospital were positive for both toxins. Of the 17 E. coli isolates from Tanzania children, only one was a producer of both LT and ST. These findings are similar to those of Shimotori et al. (1984, Personal Communication) who studied villagers in the South Nyanza District of Kenya and found that 9.5% of all ETEC isolates were positive for LT/ST.

E.coli positive for ST was predominant over LT positive strains in the present study, a finding that is in contrast to a study in Ethiopian children where such strains were rarely found (Stintzing et al., 1981). These findings are, however, in agreement with those from Bangladesh (Black et al., 1980; Black et al., 1981; Merson, et al., 1979). Schoub et al. (1977) found equal numbers of LT positive and ST E. coli in black South African infants.

ST-producing E. coli were predominant over LT positive strains also in the domestic animals. The only exception was in the case of sheep and goat isolates of E. coli which were all positive for LT only. This is in contrast to the observation that all ETEC strains in these species do produce heat-stable enterotoxin

(Smith and Gyles, 1970). Since the test used was capable of detecting only STA, the possibility of existence of other types of heat-stable enterotoxins can not be ruled out. It is generally accepted that all three variants of enterotoxin-producing E. coli (LT, ST and LT/ST) occur among humans and animals in all geographical areas of the world (WHO, 1980).

The observation that children of upto 2 years old comprised the majority of patients in the present study as well as the age group from which most ETEC were isolated is in agreement with the results obtained by Stintzing et al. (1981).

It is generally accepted that ETEC diarrhoeal disease is most prevalent in calves, piglets and lambs. The ages of most of the domestic animals examined were unavailable, although most pigs with diarrhoea were less than 12 weeks old. The age group which is most vulnerable to ETEC diarrhoea in the domestic animals examined in this study can therefore not be defined.

The production of enterotoxins by E. coli is encoded for by transferable DNA plasmids, with different plasmids governing production of LT alone, LT and ST production or ST alone (Gyles et al., 1974; Wachsmuth et al., 1976). It is also known that the stability

of plasmids that encode for enterotoxin production is related to O:H serotype with certain O:H types displaying considerable stability (Evans et al.,1977). The spread of enterotoxin plasmids will therefore depend on the availability of serotypes of E. coli capable of acquiring these plasmids. In the study presented here, E. coli were not serotyped and it is therefore not possible to conclude on the ease with which ETEC populations may expand through plasmid transfer. It is also not possible to determine whether serologically identical strains of ETEC were harboured by man and animals.

4.3 IDENTIFICATION OF FIMBRIAL ANTIGENS OF ETEC FROM HUMAN AND ANIMAL ORIGINS.

4.3.1 Distribution of F1 (Somatic type 1), F2(CFA/I) and F3(CFA/II) fimbriae in ETEC and non-ETEC.

The characteristic haemagglutination reactions considered as indicating presence of F1, F2 and F3 fimbriae are illustrated in Figures 5, 6 and 7.

A total of 185 strains (135 ETEC and 50 non-ETEC) isolated from children and 100 strains (50 ETEC and 50 non-ETEC) isolated from sheep, goats, cattle and pigs were examined for the presence of the F1, F2 and F3 fimbriae. Of the toxigenic strains of E. coli

Figure 5. Agglutination of guinea pig erythrocytes by E. coli, inhibited by α-methly D-mannoside a pattern indicating the presence of F1 (Somatic type 1) fimbriae. Bovine and human (group A) erythrocytesare unaffected.

B - Bovine erythrocytes

Bm - Bovine erythrocytes with α-methly D-mannoside

H - Human group A erythrocytes

Hm - Human group A crythrocytes with α-methly D-mannoside

GP - Guinea pig erythrocytes

GPm - Guinea pig erythrocytes with α-methly D-mannoside.

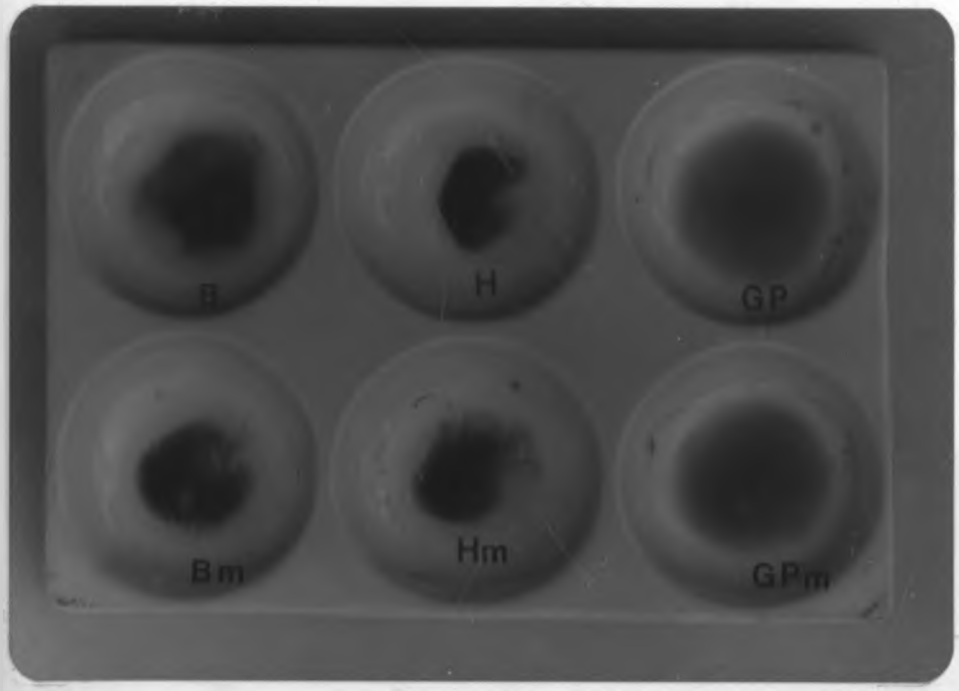

Figure 6. Agglutination of bovine and human (group A) erythrocytes by E. coli, resistant to the presence of α-methyl D-mannoside. This pattern of haemagglutination indicates presence of F2 (CFA/I) fimbriae. Guinea pig erythrocytes are unaffected.

B - Bovine erythrocytes

Bm - Bovine erythrocytes with α-methyl D-mannoside.

H - Human group A erythrocytes

Hm - Human group A erythrocytes with α-methyl D-mannoside.

GP - Guinea pig erythrocytes

GPM - Guinea pig erythrocytes with α-methly D-mannoside.

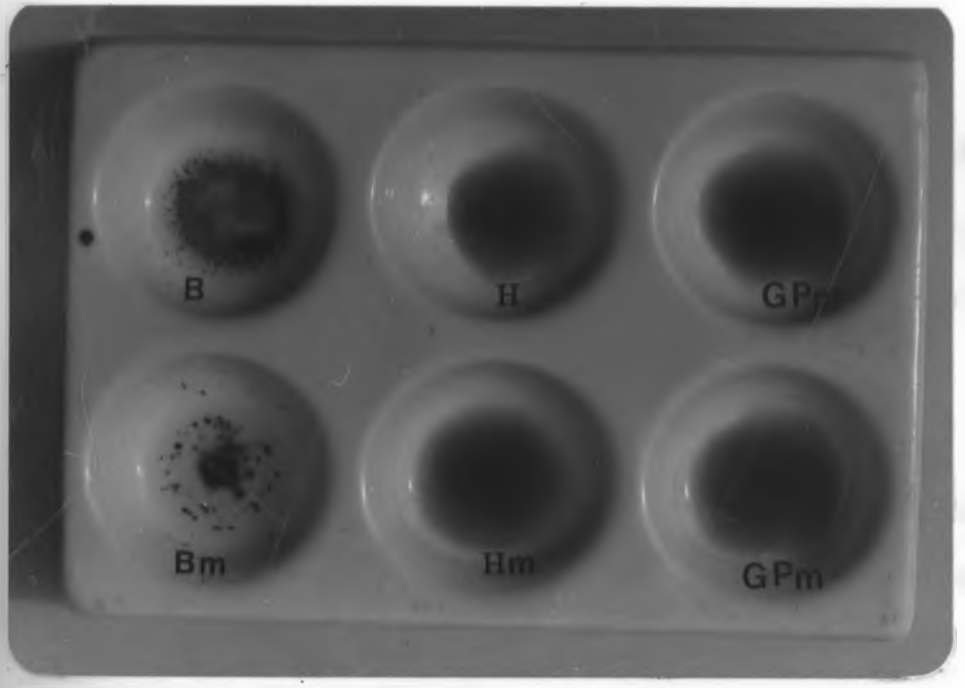

Figure 7 Agglutination of bovine erythrocytes by E. coli resistant to α-methyl D-mannoside. This haemagglutination pattern indicates presence of F3 (CFA/II) fimbriae. Human group A and guinea pig erythrocytes are unaffected.

B - Bovine erythrocytes
Bm - Bovine erythrocytes with α-methyl D-mannoside
H - Human erythrocytes
Hm - Human erythrocytes with α-methyl D-mannoside
Gp - Guinea pig erythrocytes
GPm - Guinea pig erythrocytes with α-methyl D-mannoside.

from children, 47.4% (64/135) were positive for F1, 18.5%(25/135) were positive for F2 and 10.4%(14/135) were positive for F3. In the case of non-toxigenic strains from children, 54%(27/50) were positive for F1. None of the non-ETEC strains were positive for F2 and F3 fimbriae. E. coli lacking any of these fimbrial antigens comprised 23.7%(32/135) of the ETEC and 46%(23/50) of the non-ETEC isolates from children.

None of the fimbrial antigens, F1, F2 and F3 were identified in the 50 ETEC and 50 non-ETEC isolates from sheep, goats, cattle and pigs.

Of the LT only producing E. coli from children, 8% were also positive for F2 and 4% positive for F3, while of the ST only producing E. coli, 19% were positive for F2 and 11% were positive for F3. Table 11 summarizes these findings.

4.3.2 Distribution of F4(K88), F5(K99) and F6(987P) fimbriae in ETEC and non-ETEC.

The E. coli strains examined for F1, F2 and F3 fimbriae, comprising 135 ETEC and 50 non-ETEC of human origin and 50 ETEC and 50 non-ETEC of animal origin were also examined for F4, F5 and F6 fimbriae by means of an agglutination test employing specific antisera to these fimbriae.

Table 11. DISTRIBUTION OF FIMBRIAE IN ETEC/NON-ETEC OF HUMAN AND ANIMAL ORIGINS

SOURCE	ENTEROTOXIN	TOTAL NUMBER	FIMBRIAL TYPES						
			F1 Somatic type 1	F2 CFA/I	F3 CFA/II	F4 K88	F5 K99	F6 987P	No FIMBRIAE Detected
HUMAN ETEC	LT POSITIVE	25	17 (68%)	2 (8%)	1 (4%)	0	0	0	5 (20%)
	ST POSITIVE	100	46 (46%)	19 (19%)	11 (11%)	0	0	0	24 (24%)
	LT/ST POSITIVE	10	1 (10%)	4 (40%)	2 (20%)	0	0	0	3 (30%)
HUMAN NON-ETEC	-	50	27 (54%)	0	0	0	0	0	23 (46%)
PIGS	LT POSITIVE	10	0	0	0	0	0	2 (20%)	2 (80%)
	ST POSITIVE	7	0	0	0	0	2 (28.5%)	3 (42.8%)	2 (28.5%)
	LT/ST POSITIVE	11	0	0	0	8 (72.7%)	3 (27.2%)	0	0

CATTLE	LT POSITIVE	3	0	0	0	0	1 (33.3%)	0	2 (66.6%)
	ST POSITIVE	6	0	0	0	0	5 (83.3%)	0	0
	LT/ST POSITIVE	4	0	0	0	0	4 (100%)	0	0
SHEEP	LT POSITIVE	8	0	0	0	0	3 (37.5%)	0	5 (62.5%
	ST POSITIVE	0	0	0	0	0	0	0	0
	LT/ST POSITIVE	0	0	0	0	0	0	0	0
GOATS	LT POSITIVE	1	0	0	0	0	1 (100%)		0
	ST POSITIVE	0	0	0	0	0	0		0
	LT/ST POSITIVE	0	0	0	0	0	0		0
ANIMAL NON-ETEC	-	50							50 (100%)

F4, F5 and F6 fimbrial antigens were absent in all E. coli strains of human origin.

Of all the ETEC isolates from pigs, 28.6%(8/28) were positive for F4, 17.8%(5/28) were positive for F5 and 17.8%(5/28) were positive for F6 fimbriae.

F5 was the only fimbrial type detected in the ruminants and was found in 76.9%(10/13) of ETEC isolates from cattle, 37.5%(3/8) of ETEC from sheep and one isolate from a goat. None of the non-toxigenic strains of E.coli of animal origin were positive for F4, F5 and F6 fimbriae. Fimbriae could not be detected in 10 ETEC isolates from pigs, 3 from cattle and 5 from sheep. These results are presented in table 10.

4.3.3 Discussion

The adhesive properties of E. coli were recognized by Guyot in 1908 (cited by Parry and Rooke, 1985), who observed the agglutination of erythrocytes of certain species by strains of E. coli. Fimbriae are adhesive structures on the surface of E. coli which enable the organism to attach to mucosal cells and release enterotoxins in close proximity to target cells, a prerequisite for causation of disease. These attachment factors show considerable host specificity, with F1, F2 and F3 fimbriae previously referred to as somatic type, CFA/I and CFA/II being associated

with ETEC of human origin only. F4, F5 and F6 (also referred to as K88, K99 and 987P) have only been found in ETEC pathogenic for animals (Levine, 1981; Moon et al., 1977; Isaacson et al., 1977).

In the present study F1, F2 and F3 fimbriae were observed only in ETEC of human origin while F4, F5 and F6 were found in ETEC isolates from domestic animals. F5 was present in ETEC from pigs, sheep, goats and cattle. This confirms earlier reports on the distribution of these fimbriae (Evans et al., 1975; Evans and Evans, 1978; Ørskov and Ørskov, 1977). A number of ETEC of human and animal origin were found without any of the characterised fimbriae. The role of these apparently non-fimbriate E. coli in diarrhoeal disease is not clearly understood, especially in the light of reports that non-fimbriate E. coli may be capable of attaching to surfaces (Ip et al., 1981; Sussman et al., 1982).

F2 and F3 fimbriae are encoded for by plasmids which also encode for the synthesis of heat-labile and heat-stable toxins (Evans et al., 1975; Evans and Evans, 1978; Smith et al.,1979; Penaranda et al., 1980). Similarily the production of F4 and F5 fimbriae is also plasmid-determined (Ørskov and Ørskov, 1966; Smith and Linggood, 1972). Enterotoxin plasmids have been experimentally transferred between E. coli strains from different host species (Gyles, et al.,

1978; Franklin and Mollby, 1981; Franklin et al., 1981). Plasmids that encode for antibiotic resistance are easily transferrable within as well as between species and genera of enteric bacteria (O'Brien et al., 1980; Charbbert et al., 1979). The ease with which plasmids which encode for the production of enterotoxins and host-specific fimbriae may be transferred between E. coli isolates from different species in nature is largely unknown.

Strains of E. coli which colonize an individual at infancy and become established as a part of the normal microflora are non-toxigenic. It is however conceivable that these non-toxigenic E. coli which possess appropriate attachment factors, may acquire the ability to produce enterotoxins through plasmid transfer occurring in vivo following the ingestion of ETEC of animal origin. The extent to which this may occur will depend on whether the ingested E. coli of animal origin are capable of transferring these plasmids, as well as the capability of non-toxigenic humans strains for acquiring the plasmids which encode for enterotoxin production.

The non-fimbriate ETEC isolates from man and animals, in the present study, were not serotyped and the question of serologically identical strains of ETEC being present in both man and animals could therefore not be answered. It was also not possible

to determine whether the ETEC strains isolated in the present study belong to any of the serogroups often associated with ETEC.

F1 fimbriae have been found in both ETEC and non-ETEC of human origin (Levine et al., 1980). The study presented here confirmed this finding. Although the role of this fimbrial type in ETEC disease has been questioned, its widespread distribution (F1 was presented in 47% of all ETEC from children) means it might play a role in a future vaccine for ETEC diseases.

The distribution of F5 among ETEC from pigs, cattle, sheep and goats means the possible interspecies spread of ETEC among the domestic animals.

Accordingly, the pattern which has emerged from this study indicates that ETEC strains exhibit strict host specificity, the only exception being F5-fimbriated ETEC which were found in pigs, sheep, goats and cattle. It can therefore be concluded that cross-infections are unlikely to occur between man and animals. Nevertheless, the possibility of interchange of infective plasmids between strains of animal and human origin resulting in interspecies spread of ETEC disease cannot be ignored.

5. GENERAL CONCLUSIONS

The staphylococcal coagglutination technique (CAG) for the detection of LT of E. coli was found to have the same specificity as an enzyme immuno-assay (EIA) for LT but a 7% less sensitivity compared with the EIA. The ease and rapidity with which the test may be performed for as many colonies as possible from a primary stool culture may more than compensate for the slightly lower sensitivity compared with EIA. The CAG test employs inexpensive reagents and requires little skill in setting up and reading results, factors which make it suitable for routine field investigations of LT-producing E. coli.

The present study also confirms the frequent occurrence of ETEC in children and domestic animals as a cause of diarrhoea as well as in healthy domestic animals as a part of their usual intestinal flora. Among the domestic animal species, pigs and cattle harbour ETEC more than sheep and goats.

Children upto 2 years old constitute the age group most affected by ETEC. ETEC was also more frequently encountered in pigs within 12 weeks of age. Age distribution of ETEC in other domestic animals could not be determined.

On the basis of attachment factors, ETEC show

a considerable degree of host-specificity, with one exception being F5-fimbriated ETEC which are common to pigs, cattle, sheep and goats. It is therefore unlikely that cross-infection between man and animals of ETEC can take place. However the possibility of interchange of infective plasmids between strains of human and animal origin, resulting in interspecies spread of ETEC disease cannot be overlooked.

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Appendix 1.1 Results of tests for LT and STA of *E. coli* isolates from children with diarrhoea at Muhimbili Medical Centre, Dar es Salaam, Tanzania.

All 17 strains were negative for LT in the co-agglutination test except strain 13/8. When the same strains were tested by means of the EIA for LT, strains 13/8 and 26/8 were found positive while the rest were negative.

Strains 63/7, 14/8, 26/8, 27/8, 28/8 and 35/8 were positive for STA by the infant (suckling) mouse assay.

Appendix 1.2 Results of EIA and Coagglutination test for LT of E. coli strains from humans.

E. COLI STRAIN	T E S T F O R LT		TEST FOR STA Infant (Suckling) Mouse Assay
	COAGGLUTINATION	EIA	
361	Pos	Pos	Not Tested
369	Pos	Pos	" "
188	Pos	Pos	" "
092	Pos	Pos	" "
255	Pos	Pos	" "
P379	Pos	Pos	" "
308	Pos	Pos	" "
399	Pos	Pos	" "
255A	Pos	Pos	" "
387	Pos	Pos	" "
396	Pos	Pos	" "
408	Pos	Pos	" "
394	Pos	Pos	" "
379	Pos	Pos	" "
P369	Pos	Pos	" "
m/meat	Pos	Pos	" "
H10407 (Serotype 078:H11)	Neg	Neg	" "
H10407-1 (Serotype 078:H11)	Neg	Neg	" "
404	Neg	Neg	" "
403	Neg	Neg	" "
402	Neg	Neg	" "
407	Neg	Neg	" "
409	Neg	Neg	" "
381	Neg	Neg	" "
400	Neg	Neg	" "
395	Neg	Neg	" "

Appendix 1.3 Results of EIA and Coagglutination test for LT of E. coli isolates from humans.

171 E. coli isolates 198/83 to 369/83) were tested in both coagglutination test and EIA for LT.

9 Strains (234/83, 236/83, 271/83, 275/85, 289/83, 309/83, 333/83, 335/83, 344/83), were positive for LT in both EIA and Coagglutination test.
The rest (162) were negative for LT in both tests.

Appendix 1.4 Results of tests for LT of individual colonies from mixed cultures, obtained from children with diarrhoea.

CULTURE NUMBER	NUMBER OF COLONIES TESTED	TEST FOR LT			
		COAGGLUTINATION		EIA	
		+	−	+	−
1/83	15	0	15	0	15
2/83	25	2	23	2	23
3/83	19	0	19	0	19
4/83	22	0	22	0	22
5/83	18	0	18	0	18
6/83	10	0	10	0	10
7/83	23	0	23	0	23
8/83	20	0	20	0	20
9/83	18	0	18	0	18
10/83	25	23	2	25	0
11/83	18	0	18	0	18
12/83	18	0	18	0	18
13/83	19	0	19	0	19
14/83	22	0	22	0	22
15/83	21	0	21	0	21
16/83	20	0	20	0	20
17/83	24	0	24	0	24
18/83	23	0	23	0	23
19/83	21	18	3	19	2
20/83	10	0	10	0	10
21/83	17	0	17	0	17
22/83	18	0	18	0	18
23/83	21	0	21	0	21

	NUMBER OF COLONIES TESTED	COAGGLUTINATION		EIA	
		+	-	+	-
24/83	19	19	0	19	0
25/83	21	0	21	0	21
26/83	22	0	22	0	22
27/83	23	0	23	0	23
28/83	25	0	25	0	25
29/83	24	0	24	0	24
30/83	26	3	23	3	23
31/83	25	15	10	15	10
32/83	21	0	21	0	21
33/83	22	0	22	0	22
34/83	15	0	15	0	15
35/83	18	0	18	0	18
36/83	22	0	22	0	22
37/83	21	0	21	0	21
38/83	19	0	19	0	19
39/83	18	0	18	0	18
40/83	20	0	20	0	20
41/83	21	0	21	0	21
42/83	22	22	0	22	0
43/83	21	0	21	0	21
44/83	21	0	21	0	21
45/83	20	0	20	0	20
46/83	20	0	20	0	20
47/83	20	0	20	0	20
48/83	18	0	18	0	18
49/83	21	0	21	0	21
50/83	18	0	18	0	18
51/83	17	0	17	0	17
52/83	19	0	19	0	19
53/83	19	0	19	0	19
54/83	21	0	21	0	21
55/83	21	0	21	0	21

		COAGGLUTINATION		EIA	
		+	-	+	-
56/83	20	0	20	0	20
57/83	20	0	20	0	20
58/83	20	0	20	0	20
59/83	20	0	20	0	20
60/83	21	0	21	0	21
61/83	21	0	21	0	21
62/83	23	0	23	0	23
63/83	18	0	18	0	18
64/83	15	0	15	0	15
65/83	19	0	19	0	19
66/83	20	0	20	0	20
67/83	21	0	21	0	21
68/83	23	0	23	0	23
69/83	25	0	25	0	25
70/83	20	0	20	0	20
71/83	21	0	21	0	21
72/83	23	22	1	23	0
73/83	15	0	15	0	15
74/83	19	0	19	0	19
75/83	21	0	21	0	21
76/83	20	0	20	0	20
77/83	20	0	20	0	20
78/83	25	0	25	0	25
79/83	18	0	18	0	18
80/83	21	0	21	0	21
81/83	18	0	18	0	18
82/83	25	0	25	0	25
83/83	19	0	19	0	19
84/83	20	0	20	0	20
85/83	22	0	22	0	22
86/83	20	0	20	0	20
87/83	22	0	22	0	22

		COAGGLUTINATION		EIA	
		+	-	+	-
88/83	22	0	22	0	22
89/83	21	0	21	0	21
90/83	23	0	23	0	23
91/83	21	0	21	0	21
92/83	20	0	20	0	20
93/83	22	0	22	0	22
94/83	21	0	21	0	21
95/83	23	0	23	0	23
96/83	21	0	21	0	21
97/83	20	0	20	0	20
98/83	17	0	17	0	17
99/83	18	0	18	0	18
100/83	18	0	18	0	18
101/83	20	0	20	0	20
102/83	19	19	0	19	0
103/83	18	0	18	0	18

Appendix 1.5 Results of tests for LT of individual colonies from primary stool cultures obtained from children with diarrhoea.

CULTURE NO.	NUMBER OF COLONIES TESTED	TESTS FOR LT			
		COAGGLUTINATION		EIA	
		+	-	+	-
1/84	21	18	3	21	0
2/84	15	0	15	0	15
3/84	19	0	19	0	19
4/84	22	0	22	0	22
5/84	18	1	17	1	17
6/84	21	0	21	0	21
7/84	17	0	17	0	17
8/84	8	0	8	0	8
9/84	21	0	21	0	21
10/84	15	15	0	15	0
11/84	10	10	0	10	0
12/84	23	0	23	0	23
13/84	20	19	1	20	0
14/84	18	17	1	18	0
15/84	10	10	0	10	0
16/84	21	21	0	21	0
17/84	16	0	16	0	16
18/84	21	0	21	0	21
19/84	20	0	20	0	20
20/84	19	18	1	19	0
21/84	17	0	17	0	17

	NUMBER OF COLONIES TESTED	COAGGLUTINATION		E I A	
		+	-	+	-
22/84	15	15	0	15	0
23/84	10	0	10	0	10
24/84	12	0	12	0	12
25/84	15	12	3	12	3
26/84	18	0	18	0	18
27/84	20	0	20	0	20
28/84	20	0	20	0	20
29/84	19	0	19	0	19
30/84	21	0	21	0	21
31/84	20	0	20	0	20
32/84	21	0	21	0	21
33/84	18	0	18	0	18
34/84	17	0	17	0	17
35/84	19	0	19	0	19
36/84	18	0	18	0	18
37/84	21	0	21	0	21
38/84	20	0	20	0	20
39/84	20	0	20	0	20
40/84	18	0	18	0	18
41/84	20	0	20	0	20
42/84	21	0	21	0	21
43/84	23	0	23	0	23
44/84	23	21	2	23	0
45/84	17	17	0	17	0
46/84	9	9	0	9	0
47/84	22	0	22	0	22
48/84	16	16	0	16	0
49/84	22	0	22	0	22

	NUMBER OF COLONIES TESTED	COAGGLUTINATION		E I A	
		+	-	+	-
50/84	15	15	0	15	0
51/84	18	18	0	18	0
52/84	20	20	0	20	0
53/84	23	0	23	0	23
54/84	23	22	1	23	0
55/84	25	0	25	0	25
56/84	18	0	18	0	18
57/84	20	0	20	0	20
58/84	21	0	21	0	21
59/84	19	0	19	0	19
60/84	20	0	20	0	20
61/84	22	0	22	0	22
62/84	21	3	18	3	18
63/84	15	0	15	0	15
64/84	16	0	16	0	16
65/84	12	0	12	0	12
66/84	20	0	20	0	20
67/84	18	0	18	0	18
68/84	10	0	10	0	10
69/84	8	0	8	0	8
70/84	10	0	10	0	10
71/84	21	0	21	0	21
72/84	23	21	2	23	0

CULTURE NUMBER	NUMBER OF COLONIES TESTED	COAGGLUTINATION		E I A	
		+	-	+	-
73/84	20	0	20	0	20
74/84	20	0	20	0	20
75/84	21	0	21	0	21
76/84	22	0	22	0	22
77/84	22	0	22	0	22
78/84	23	0	23	0	23
79/84	21	0	21	0	21
80/84	25	24	1	25	0
81/84	23	23	0	23	0
82/84	18	0	18	0	18
83/84	19	0	19	0	19
84/84	18	0	18	0	18

Appendix 1.6 Results of infant(suckling) mouse assay for STA of E. coli strains from children with diarrhoea.

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
1/83	0.19	1.69	0.112	+
2/83	0.10	2.04	0.049	-
3/83	0.13	2.00	0.065	-
4/83	0.12	1.95	0.061	-
5/83	0.08	1.57	0.050	-
6/83	0.16	1.68	0.095	+
7/83	0.14	1.82	0.076	-
8/83	0.13	2.03	0.064	-
9/83	0.11	1.98	0.055	-
10/83	0.09	1.59	0.056	-
11/83	0.10	1.62	0.061	-
12/83	0.13	1.86	0.069	-
13/83	0.12	1.71	0.070	-
14/83	0.14	1.80	0.077	-
15/83	0.11	1.51	0.072	-
16/83	0.09	1.39	0.064	-
17/83	0.10	1.59	0.062	-
18/83	0.14	1.72	0.081	-
19/83	0.13	1.87	0.069	-
20/83	0.08	1.32	0.060	-
21/83	0.11	1.37	0.080	-
22/83	0.11	1.42	0.077	-
23/83	0.12	1.70	0.070	-
24/83	0.10	1.90	0.052	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATION IW/RBW	STA
25/83	0.10	1.57	0.063	-
26/83	0.10	1.43	0.069	-
27/83	0.09	1.41	0.070	-
28/83	0.13	2.10	0.061	-
29/83	0.14	1.97	0.071	-
30/83	0.12	1.50	0.080	-
31/83	0.14	1.60	0.087	+
32/83	0.12	1.31	0.091	+
33/83	0.10	1.26	0.079	-
34/83	0.10	1.35	0.074	-
35/83	0.11	1.77	0.062	-
36/83	0.10	1.25	0.080	-
37/83	0.13	1.53	0.085	+
38/83	0.11	1.72	0.063	-
39/83	0.11	1.69	0.065	-
40/83	0.10	1.42	0.070	-
41/83	0.12	1.32	0.090	+
42/83	0.13	1.82	0.071	-
43/83	0.13	1.68	0.077	-
44/83	0.15	1.51	0.10	+
45/83	0.10	1.53	0.065	-
46/83	0.09	1.57	0.057	-
47/83	0.08	1.31	0.061	-
48/83	0.10	1.92	0.052	-
49/83	0.11	1.82	0.060	-
50/83	0.12	1.31	0.091	+
51/83	0.11	1.30	0.084	+
52/83	0.11	1.32	0.083	+
53/83	0.12	1.69	0.071	-
54/83	0.10	1.75	0.057	-
55/83	0.09	1.83	0.049	-
56/83	0.15	1.07	0.14	+
57/83	0.08	1.86	0.043	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
58/83	0.10	1.23	0.081	-
59/83	0.11	1.25	0.088	+
60/83	0.10	1.38	0.072	-
61/83	0.12	1.26	0.095	+
62/83	0.10	1.35	0.074	-
63/83	0.12	1.73	0.069	-
64/83	0.09	1.55	0.058	-
65/83	0.12	2.00	0.060	-
66/83	0.10	2.07	0.053	-
67/83	0.10	2.17	0.046	-
68/83	0.11	1.37	0.080	-
69/83	0.12	1.81	0.066	--
70/83	0.13	1.44	0.090	+
71/83	0.10	1.38	0.072	--
72/83	0.11	1.61	0.068	-
73/83	0.14	1.55	0.090	+
74/83	0.12	1.51	0.079	-
75/83	0.08	1.05	0.076	-
76/83	0.12	1.25	0.096	+
77/83	0.12	1.18	0.101	+
78/83	0.11	1.89	0.058	-
79/83	0.11	2.07	0.053	--
80/83	0.11	2.29	0.048	-
81/83	0.08	1.15	0.069	-
82/83	0.14	1.25	0.112	+
83/83	0.12	1.55	0.077	-
84/83	0.10	1.81	0.055	-
85/83	0.13	2.32	0.056	--
86/83	0.12	1.90	0.063	-
87/83	0.10	1.38	0.072	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
88/83	0.10	1.42	0.070	-
89/83	0.14	1.45	0.096	+
90/83	0.09	1.80	0.050	-
91/83	0.10	1.78	0.056	-
92/83	0.12	1.69	0.071	-
93/83	0.08	1.21	0.066	-
94/83	0.08	1.15	0.069	-
95/83	0.07	1.20	0.058	-
96/83	0.10	1.20	0.083	+
97/83	0.09	1.47	0.061	-
98/83	0.12	2.10	0.057	-
99/83	0.15	1.44	0.104	+
100/83	0.13	1.62	0.080	-
101/83	0.11	1.39	0.079	-
102/83	0.12	1.76	0.068	-
103/83	0.13	1.38	0.094	+
104/83	0.14	1.75	0.080	-
105/83	0.11	1.42	0.077	-
106/83	0.11	1.59	0.069	-
107/83	0.12	1.26	0.095	+
108/83	0.13	1.49	0.087	+
109/83	0.12	1.79	0.067	-
110/83	0.11	1.69	0.065	-
111/83	0.15	1.53	0.098	+
112/83	0.12	2.26	0.053	-
113/83	0.14	1.89	0.074	-
114/83	0.09	1.30	0.069	-
115/83	0.09	1.20	0.075	-
116/83	0.11	1.54	0.071	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
117/83	0.18	2.46	0.073	-
118/83	0.14	1.50	0.093	+
119/83	0.18	2.04	0.088	+
120/83	0.15	1.45	0.103	+
121/83	0.13	1.56	0.083	+
122/83	0.11	1.29	0.085	+
123/83	0.09	1.32	0.068	-
124/83	0.15	1.63	0.092	+
125/83	0.15	1.66	0.090	+
126/83	0.14	1.60	0.087	+
127/83	0.11	1.15	0.095	+
128/83	0.15	1.53	0.098	+
129/83	0.15	1.50	0.100	+
130/83	0.14	1.55	0.090	+
131/83	0.14	1.81	0.077	-
132/83	0.13	1.52	0.085	+
133/83	0.09	1.30	0.069	-
134/83	0.12	2.10	0.057	-
135/83	0.12	2.14	0.056	-
136/83	0.14	1.62	0.086	+
137/83	0.08	1.17	0.068	-
138/83	0.09	0.97	0.092	+
139/83	0.13	1.71	0.076	
140/83	0.10	1.61	0.062	-
141/83	0.13	1.42	0.091	+
142/83	0.12	1.84	0.065	-
143/83	0.12	1.69	0.071	-
144/83	0.13	1.49	0.087	+

STRAIN NUMBER	WEIGHT OF	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
145/83	0.08	1.42	0.056	-
146/83	0.11	1.89	0.058	-
147/83	0.14	2.50	0.056	-
148/83	0.11	1.69	0.065	-
149/83	0.10	1.63	0.061	-
150/83	0.10	1.42	0.070	-
151/83	0.12	1.42	0.084	+
152/83	0.10	1.36	0.073	-
153/83	0.13	1.88	0.069	-
154/83	0.11	1.66	0.066	-
155/83	0.13	1.73	0.075	-
156/83	0.12	1.69	0.071	-
157/83	0.10	1.47	0.068	-
158/83	0.11	1.50	0.073	-
159/83	0.12	1.00	0.120	+
160/83	0.10	0.96	0.104	+
161/83	0.13	1.41	0.092	+
162/83	0.11	1.37	0.080	-
163/83	0.15	2.02	0.074	-
164/83	0.09	1.36	0.066	-
165/83	0.14	2.37	0.059	-
166/83	0.11	1.80	0.061	-
167/83	0.11	1.17	0.094	+
168/83	0.13	1.47	0.088	+
169/83	0.13	1.30	0.100	+
170/83	0.13	1.18	0.110	+
171/83	0.09	1.45	0.062	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
172/83	0.11	1.57	0.070	-
173/83	0.12	1.48	0.081	-
174/83	0.08	1.06	0.075	-
175/83	0.10	1.44	0.069	-
176/83	0.13	1.51	0.086	+
177/83	0.09	1.57	0.057	-
178/83	0.08	1.33	0.060	-
179/83	0.12	1.25	0.096	+
180/83	0.16	1.77	0.090	+
181/83	0.14	1.50	0.093	+
182/83	0.11	1.41	0.078	-
183/83	0.14	1.89	0.074	-
184/83	0.13	1.47	0.088	+
185/83	0.13	1.26	0.103	+
186/83	0.13	1.49	0.087	+
187/83	0.12	1.76	0.068	-
188/83	0.14	1.64	0.085	+
189/83	0.13	1.64	0.079	-
190/83	0.14	1.50	0.093	+
191/83	0.14	1.53	0.091	+
192/83	0.12	1.42	0.084	+
193/83	0.10	1.29	0.077	-
194/83	0.23	2.16	0.106	+
195/83	0.13	1.44	0.090	+
196/83	0.13	1.36	0.095	+
197/83	0.13	1.66	0.078	-
198/83	0.10	1.61	0.062	-
199/83	0.12	1.34	0.089	+
200/83	0.14	1.79	0.078	-
201/83	0.16	1.45	0.10	+

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
202/83	0.15	1.59	0.094	+
203/83	0.11	2.11	0.052	-
204/83	0.12	2.18	0.055	-
205/83	0.09	1.80	0.050	-
206/83	0.12	1.90	0.063	-
207/83	0.10	1.66	0.060	-
208/83	0.11	1.89	0.058	-
209/83	0.20	1.66	0.120	+
210/83	0.14	1.29	0.108	+
211/83	0.14	1.57	0.089	+
212/83	0.12	1.64	0.073	-
213/83	0.09	1.18	0.076	-
214/83	0.14	1.62	0.086	+
215/83	0.11	1.77	0.062	-
216/83	0.12	1.78	0.084	+
217/83	0.10	1.63	0.061	-
281/83	0.10	1.53	0.065	-
219/83	0.11	1.67	0.066	-
220/83	0.11	1.83	0.060	-
221/83	0.12	1.62	0.074	-
222/83	0.12	1.90	0.063	-
223/83	0.11	1.54	0.071	-
224/83	0.13	1.56	0.083	+
225/83	0.12	1.60	0.075	-
226/83	0.09	1.34	0.067	-
227/83	0.13	1.91	0.068	-
228/83	0.10	1.72	0.058	-
229/83	0.14	1.66	0.084	+
230/83	0.10	1.63	0.061	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
231/83	0.11	1.86	0.059	-
232/83	0.12	1.44	0.083	+
233/83	0.12	1.41	0.085	+
234/83	0.11	1.26	0.087	+
235/83	0.12	1.44	0.083	+
236/83	0.11	1.50	0.073	-
237/83	0.11	1.59	0.069	-
238/83	0.13	1.42	0.091	+
239/83	0.09	1.76	0.051	-
240/83	0.14	1.50	0.093	+
241/83	0.11	1.74	0.063	-
242/83	0.12	1.71	0.070	-
243/83	0.10	1.53	0.065	-
244/83	0.10	1.47	0.068	-
245/83	0.13	1.47	0.088	+
246/83	0.10	1.47	0.068	-
247/83	0.10	1.42	0.070	-
248/83	0.10	1.44	0.069	-
249/83	0.09	1.55	0.058	-
250/83	0.14	1.38	0.101	+
251/83	0.13	1.52	0.085	+
252/83	0.12	1.44	0.083	+
253/83	0.08	1.50	0.053	-
254/83	0.08	1.25	0.064	-
255/83	0.10	1.69	0.059	-
256/83	0.12	1.71	0.070	-
257/83	0.08	1.17	0.068	-
258/83	0.14	1.62	0.086	+
259/83	0.10	1.11	0.090	+

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
260/83	0.10	1.53	0.065	-
261/83	0.09	1.52	0.059	-
262/83	0.16	1.55	0.103	+
263/83	0.08	1.11	0.072	-
264/83	0.09	1.60	0.056	-
265/83	0.10	1.75	0.057	-
266/83	0.10	1.75	0.057	-
267/83	0.11	1.71	0.064	-
268/83	0.14	2.33	0.060	-
269/83	0.12	1.44	0.083	+
270/83	0.12	1.66	0.072	-
271/83	0.11	1.57	0.070	-
272/83	0.19	2.34	0.081	-
273/83	0.10	1.38	0.072	-
274/83	0.10	1.42	0.070	-
275/83	0.11	1.59	0.069	-
276/83	0.08	1.48	0.054	-
277/83	0.16	1.42	0.112	+
278/83	0.14	1.48	0.094	+
279/83	0.13	1.62	0.080	-
280/83	0.13	1.68	0.077	-
281/83	0.12	1.79	0.067	-
282/83	0.13	2.32	0.050	-
283/83	0.12	2.10	0.057	-
284/83	0.14	1.62	0.086	+
285/83	0.14	1.52	0.092	+
286/83	0.10	1.49	0.067	-
287/83	0.09	1.45	0.062	-
288/83	0.08	1.26	0.063	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
289/83	0.10	1.51	0.066	-
290/83	0.11	1.30	0.084	+
291/83	0.12	1.17	0.102	+
292/83	0.13	1.36	0.095	+
293/83	0.13	1.38	0.094	+
294/83	0.14	1.60	0.087	+
295/83	0.11	1.74	0.063	-
296/83	0.11	1.46	0.075	-
297/83	0.14	1.66	0.084	+
298/83	0.15	1.36	0.110	+
299/83	0.13	1.52	0.085	+
300/83	0.11	1.50	0.073	-
301/83	0.09	1.30	0.069	-
302/83	0.11	1.32	0.083	+
303/83	0.10	1.63	0.061	-
304/83	0.12	1.37	0.087	+
305/83	0.12	1.33	0.090	+
306/83	0.13	1.42	0.091	+
307/83	0.13	1.60	0.081	-
308/83	0.14	1.48	0.094	+
309/83	0.11	1.50	0.073	-
310/83	0.10	1.66	0.060	-
311/83	0.15	1.33	0.112	+
312/83	0.14	1.42	0.098	+
313/83	0.14	1.41	0.099	+
314/83	0.12	1.66	0.072	-
315/83	0.12	1.71	0.070	-
316/83	0.11	1.59	0.069	-
317/83	0.16	1.65	0.097	+

STRAIN NUMBER	(IW)	RBW	IW/RBW	S T A
318/83	0.10	1.75	0.057	-
319/83	0.11	1.74	0.063	-
320/8	0.11	1.69	0.065	-
321/83	0.10	1.44	0.069	-
322/83	0.09	1.28	0.070	-
323/83	0.09	1.47	0.061	-
324/83	0.12	2.06	0.058	-
325/83	0.14	1.60	0.087	+
326/83	0.11	1.37	0.080	-
327/83	0.11	1.46	0.075	-
328/83	0.09	1.13	0.079	-
329/83	0.10	1.58	0.063	-
CGH 352 (ST positive E. coli from patient with diarr-hoea Coast General Hospital Mombasa	0.14	1.13	0.123	+
E. coli strain PSLM 004 HB101 STA +	0.16	1.23	0.130	+

Appendix 1.7 Results of tests for LT of individual colonies from primary stool cultures obtained from cattle

CULTURE NUMBER	NUMBER OF COLONIES TESTED	TESTS FOR LT			
		COAGGLUTINATION		EIA	
		+	-	+	-
1/83	20	0	20	0	20
2/83	18	0	18	0	18
3/83	22	0	22	0	22
4/83	25	2	23	2	23
5/83	18	0	18	0	18
6/83	19	0	19	0	19
7/83	21	0	21	0	21
8/83	22	0	22	0	22
9/83	15	0	15	0	15
10/83	21	0	21	0	21
11/83	23	0	23	0	23
12/83	16	9	7	9	7
13/83	24	0	24	0	24
14/83	17	0	17	0	17
15/83	19	0	19	0	19
16/83	20	0	20	0	20
17/83	21	0	21	0	21
18/83	22	0	22	0	22
19/83	20	10	10	10	10
20/83	22	0	22	0	22
21/83	21	10	11	10	11
22/83	19	0	19	0	19
23/83	19	0	19	0	19
24/83	20	0	20	0	20

		+	-	+	-
25/83	20	0	20	0	20
26/83	21	0	21	0	21
27/83	21	0	21	0	21
28/83	17	0	17	0	17
29/83	17	0	17	0	17
30/83	18	0	18	0	18
31/83	20	0	20	0	20
32/83	20	9	11	9	11
33/83	23	0	23	0	23
34/83	24	0	24	0	24
35/83	21	0	21	0	21
36/83	25	0	25	0	25
37/83	22	0	22	0	22
38/83	26	0	26	0	26
39/83	21	0	21	0	21
40/83	18	0	18	0	18
41/83	19	0	19	0	19
42/83	15	0	15	0	15
43/83	17	0	17	0	17
44/83	20	0	20	0	20
45/83	18	10	8	12	6
46/83	21	0	21	0	21
47/83	20	0	20	0	20
48/83	16	0	16	0	16
49/83	18	0	18	0	18
50/83	20	0	20	0	20
51/83	21	0	21	0	21
52/83	25	0	25	0	25
53/83	22	0	22	0	22
54/83	18	0	18	0	18
55/83	21	0	21	0	21
1/83	12	12	0	12	0

Appendix 1.8 Results of the infant (suckling) mouse assay for STA of E. coli strains from cattle.

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
1/83	0.11	1.74	0.063	-
2/83	0.10	1.42	0.070	-
3/83	0.14	2.37	0.059	-
4/83	0.10	1.58	0.063	-
5/83	0.12	1.66	0.072	-
6/83	0.10	1.78	0.056	-
7/83	0.10	1.72	0.058	-
8/83	0.11	2.15	0.051	-
9/83	0.09	1.57	0.057	-
10/83	0.12	1.45	0.082	-
11/83	0.16	2.00	0.080	-
12/83	0.10	1.20	0.083	+
13/83	0.10	2.08	0.048	-
14/83	0.12	2.40	0.050	-
15/83	0.11	1.80	0.061	-
16/83	0.13	1.68	0.077	-
17/83	0.14	1.75	0.080	-
18/83	0.12	1.69	0.071	-
19/83	0.14	1.60	0.087	+
20/83	0.13	1.68	0.077	-
21/83	0.13	1.36	0.095	+
22/83	0.14	2.25	0.062	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
23/83	0.11	1.74	0.063	-
24/83	0.10	1.72	0.058	-
25/83	0.10	1.66	0.060	-
26/83	0.09	1.28	0.070	-
27/83	0.08	1.09	0.073	-
28/83	0.10	1.72	0.058	-
29/83	0.12	2.10	0.057	-
30/83	0.14	2.29	0.061	-
31/83	0.12	1.44	0.083	+
32/83	0.11	1.52	0.072	-
33/83	0.18	1.55	0.116	+
34/83	0.11	1.44	0.076	-
35/83	0.15	1.87	0.080	-
36/83	0.11	1.39	0.079	-
37/83	0.13	1.52	0.085	+
38/83	0.09	1.80	0.055	-
39/83	0.12	1.79	0.067	-
40/83	0.16	1.58	0.101	+
41/83	0.14	2.02	0.069	-
42/83	0.08	1.40	0.057	-
43/83	0.13	1.40	0.090	+
44/83	0.14	1.62	0.086	+
45/83	0.10	1.36	0.073	-
46/83	0.11	1.41	0.078	-
47/83	0.11	1.41	0.075	-
48/83	0.14	1.94	0.072	-
49/83	0.13	1.60	0.081	-
50/83	0.10	1.51	0.066	-

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
51/83	0.09	1.21	0.074	-
52/83	0.11	1.80	0.061	-
53/83	0.12	1.56	0.076	-
54/83	0.10	1.45	0.068	-
55/83	0.14	1.94	0.072	-
1/83	0.15	1.61	0.093	+

Appendix 1.9 Results of tests for LT of individual colonies from primary stool cultures obtained from healthy sheep

CULTURE NUMBER	NUMBER OF COLONIES TESTED	COAGGLUTINATION		EIA	
		+	-	+	-
1/83	21	0	21	0	21
2/83	18	0	18	0	18
3/83	20	0	20	0	20
4/83	23	0	23	0	23
5/83	15	0	15	0	15
6/83	10	0	10	0	10
7/83	12	0	12	0	12
8/83	12	0	12	0	12
9/83	18	0	18	0	18
10/83	22	5	17	5	17
11/83	22	0	22	0	22
12/83	23	0	23	0	23
13/83	20	0	20	0	20
14/83	20	0	20	0	20
15/83	19	0	19	0	19
16/83	19	0	19	0	19
17/83	20	0	20	0	20
18/83	21	0	21	0	21
19/83	18	0	18	0	18
20/83	21	11	10	11	10
21/83	20	0	20	0	20
22/83	20	0	20	0	20
23/83	18	0	18	0	18
24/83	20	0	20	0	20
25/83	20	0	20	0	20

CULTURE NUMBER	NUMBER OF COLONIES TESTED	COAGGLUTINATION		E I A	
		+	-	+	-
26/83	17	0	17	0	17
27/83	17	0	17	0	17
28/83	22	0	22	0	22
29/83	22	0	22	0	22
30/83	21	0	21	0	21
31/83	23	0	23	0	23
32/83	15	0	15	0	15
33/83	22	0	22	0	22
34/83	21	0	21	0	21
35/83	22	0	22	0	22
36/83	21	0	21	0	21
37/83	19	0	19	0	19
38/83	20	0	20	0	20
39/83	20	0	20	0	20
40/83	20	8	12	8	12
41/83	21	9	12	10	11
42/83	20	0	20	0	20
43/83	20	0	20	0	20
44/83	20	0	20	0	20
45/83	18	11	7	11	7
46/83	24	0	24	0	24
47/83	12	0	12	0	12
48/83	18	0	18	0	18
49/83	21	0	21	0	21
50/83	22	0	22	0	22
51/80	20	0	20	0	20
52/80	20	0	20	0	20
53/83	20	0	20	0	20
54/83	21	0	21	0	21
55/83	20	0	20	0	20

		+	-	+	-
56/83	20	0	20	0	20
57/83	23	0	23	0	23
58/83	24	0	24	0	24
59/83	25	0	25	0	25
60/83	22	0	22	0	22
61/83	15	8	7	8	7
62/83	18	0	18	0	18
63/83	21	0	21	0	21
64/83	23	0	23	0	23
65/83	21	0	21	0	21
66/83	20	0	20	0	20
67/83	22	0	22	0	22
68/83	23	0	23	0	23
69/60	20	0	20	0	20
70/83	21	0	21	0	21
71/83	22	0	22	0	22
72/83	21	0	21	0	21
73/83	20	0	20	0	20
74/83	20	0	20	0	20
75/83	20	0	20	0	20
76/83	20	0	20	0	20
77/83	21	0	21	0	21
78/83	19	0	19	0	19
79/83	20	0	20	0	20
80/83	25	0	25	0	25
81/83	22	0	22	0	22
82/83	23	0	23	0	23
83/83	23	0	23	0	23
84/83	22	0	22	0	22

		+	−	+	−
85/83	22	0	22	0	22
86/83	22	0	22	0	22
87/83	21	0	21	0	21
88/83	20	0	20	0	20
89/83	20	0	20	0	20
90/83	20	0	20	0	20
91/83	19	0	19	0	19
92/83	20	0	20	0	20
93/83	21	0	21	0	21
94/83	21	0	21	0	21
95/83	21	0	21	0	21
96/83	20	0	20	0	20
97/83	21	0	21	0	21
98/83	22	0	22	0	22
99/83	20	0	20	0	20
100/83	20	0	20	0	20
101/83	21	10	11	10	11
102/83	21	0	21	0	21
103/83	18	0	18	0	18
104/83	22	0	22	0	22
105/83	20	0	20	0	20
106/83	15	0	15	0	15
107/83	16	0	16	0	16
108/83	20	0	20	0	20
109/83	20	0	20	0	20
110/83	20	10	10	10	10

Appendix 1.10 Results of infant (suckling) mouse assay for STA of E. coli strains from healthy sheep.

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	STA
1/83	0.11	1.75	0.062	-
2/83	0.13	1.70	0.076	-
3/83	0.10	1.53	0.065	-
4/83	0.11	1.60	0.068	-
5/83	0.11	1.55	0.070	-
6/83	0.12	1.67	0.071	-
7/83	0.12	1.82	0.065	-
8/83	0.14	2.12	0.066	-
9/83	0.10	1.62	0.061	-
10/83	0.10	1.70	0.058	-
11/83	0.11	1.65	0.066	-
12/83	0.10	1.68	0.059	-
13/83	0.12	1.67	0.071	-
14/83	0.15	2.37	0.063	-
15/83	0.09	1.49	0.060	-
16/83	0.09	1.43	0.062	-
17/83	0.10	1.41	0.070	-
18/83	0.09	1.30	0.069	-
19/83	0.10	1.45	0.068	-
20/83	0.11	1.42	0.077	-
21/83	0.13	1.62	0.080	-
22/83	0.12	1.60	0.075	-
23/83	0.10	1.50	0.066	-
24/83	0.11	1.47	0.074	-
25/83	0.14	1.82	0.076	-
26/83	0.08	1.21	0.066	-

STRAIN NUMBER	(IW)	(RBW)	IW/RBW	STA
27/83	0.15	2.02	0.074	-
28/83	0.11	1.40	0.078	-
29/83	0.13	1.65	0.078	-
30/83	0.10	1.46	0.068	-
31/83	0.11	1.43	0.076	-
32/83	0.13	1.71	0.076	-
33/83	0.14	1.73	0.080	-
34/83	0.12	1.67	0.071	-
35/83	0.13	1.66	0.078	-
36/83	0.13	1.68	0.077	-
37/83	0.10	1.58	0.063	-
38/83	0.15	1.88	0.079	-
39/83	0.11	2.15	0.051	-
40/83	0.10	1.72	0.058	-
41/83	0.13	1.78	0.073	-
42/83	0.12	1.80	0.066	-
43/83	0.12	1.75	0.068	-
44/83	0.11	1.71	0.064	-
45/83	0.10	1.48	0.067	-
46/83	0.14	1.82	0.076	-
47/83	0.10	1.51	0.066	-
48/83	0.09	1.37	0.065	-
49/83	0.09	1.28	0.070	-
50/83	0.10	1.35	0.074	-
51/83	0.13	1.68	0.077	-
52/83	0.08	1.26	0.063	-
53/83	0.09	1.29	0.069	-
54/83	0.09	1.31	0.068	-
55/83	0.10	1.32	0.075	-

STRAIN NUMBER	(IW)	(RBW)	IW/RBW	STA
56/83	0.11	1.43	0.076	-
57/83	0.14	1.76	0.079	-
58/83	0.13	1.78	0.073	-
59/83	0.13	1.73	0.075	-
60/83	0.12	1.69	0.071	-
61/83	0.14	1.81	0.077	-
62/83	0.14	1.78	0.078	-
63/83	0.11	1.69	0.065	-
64/83	0.12	1.85	0.064	-
65/83	0.15	2.41	0.062	-
66/83	0.16	2.39	0.066	-
67/83	0.12	2.10	0.057	-
68/83	0.10	2.05	0.048	-
69/83	0.14	2.17	0.064	-
70/83	0.11	1.95	0.056	-
71/83	0.09	1.45	0.062	-
72/83	0.13	1.69	0.076	-
73/83	0.12	1.77	0.067	-
74/83	0.10	1.72	0.058	-
75/83	0.11	1.79	0.061	-
76/83	0.11	1.85	0.059	-
77/83	0.12	1.69	0.071	-
78/83	0.09	1.53	0.058	-
79/83	0.10	1.66	0.060	-
80/83	0.09	1.34	0.067	-
81/83	0.10	1.28	0.078	-
82/83	0.10	1.37	0.072	-
83/83	0.16	2.43	0.065	-

STRAIN NUMBER	(IW)	(RBW)	IW/RBW	STA
84/83	0.15	2.10	0.071	-
85/83	0.10	1.80	0.055	-
86/83	0.11	1.57	0.070	-
87/83	0.13	1.63	0.079	-
88/83	0.12	1.52	0.078	-
89/83	0.11	1.48	0.074	-
90/83	0.10	1.35	0.074	-
91/83	0.12	1.45	0.082	-
92/83	0.12	1.68	0.071	-
93/83	0.13	1.75	0.074	-
94/83	0.11	1.67	0.065	-
95/83	0.10	1.52	0.065	-
96/83	0.10	1.58	0.063	-
97/83	0.09	1.36	0.066	-
98/83	0.11	1.71	0.064	-
99/83	0.14	1.82	0.076	-
100/83	0.15	1.98	0.075	-
101/83	0.11	1.39	0.079	-
102/83	0.10	1.45	0.068	-
103/83	0.10	1.41	0.070	-
104/83	0.10	1.33	0.075	-
105/83	0.13	1.77	0.073	-
106/83	0.11	1.38	0.079	-
107/83	0.10	1.48	0.067	-
108/83	0.11	1.39	0.079	-
109/83	0.11	1.62	0.067	-
110/83	0.15	1.97	0.076	-

Appendix 1.11 Results of tests for LT of individual colonies from primary stool cultures obtained from healthy goats.

CULTURE NUMBER	NUMBER OF COLONIES TESTED	TEST FOR LT			
		COAGGLUTINATION		EIA	
		+	-	+	-
1/83	20	0	20	0	20
2/83	18	0	18	0	18
3/83	21	0	21	0	21
4/83	20	0	20	0	20
5/83	22	10	12	10	12
6/83	23	0	23	0	23
7/83	22	0	22	0	22
8/83	15	0	15	0	15
9/83	16	0	16	0	16
10/83	10	0	10	0	10
11/83	20	0	20	0	20
12/83	21	0	21	0	21
13/83	17	0	17	0	17
14/83	19	0	19	0	19
15/83	22	0	22	0	22
16/83	23	0	23	0	23
17/83	24	0	24	0	24
18/83	18	0	18	0	18
19/83	19	0	19	0	19
20/83	19	0	19	0	19
21/83	20	0	20	0	20
22/83	21	0	21	0	21
23/83	10	0	10	0	10
24/83	18	0	18	0	18
25/83	16	0	16	0	16
26/83	15	0	16	0	15
27/83	9	0	9	0	9

CULTURE NUMBER	NUMBER OF COLONIES TESTED	T E S T F O R L T			
		COAGGLUTINATION		E I A	
		+	-	+	-
28/83	18	0	18	0	18
29/83	20	0	20	0	20
30/83	21	0	21	0	21
31/83	23	0	23	0	23
32/83	20	0	20	0	20
33/83	20	0	20	0	20
34/83	21	0	21	0	21
35/83	23	0	23	0	23
36/83	18	0	18	0	18
37/83	21	0	21	0	21
38/83	23	0	23	0	23
39/83	22	0	22	0	22
40/83	18	0	18	0	18
41/83	20	0	20	0	20
42/83	21	0	21	0	21
43/83	17	0	17	0	17
44/83	16	0	16	0	16
45/83	15	0	15	0	15
46/83	17	0	17	0	17
47/83	18	0	18	0	18
48/83	19	0	19	0	19
49/83	20	0	20	0	20
50/83	20	0	20	0	20
51/83	20	0	20	0	20
52/83	21	0	21	0	21
53/83	21	0	21	0	21
54/83	23	0	23	0	23

CULTURE NUMBER	NUMBER OF COLONIES TESTED	TEST FOR COAGGLUTINATION		LT E I A	
		+	-	+	-
55/83	21	0	21	0	21
56/83	8	0	8	0	8
57/83	21	0	21	0	21
58/83	22	0	22	0	22
59/83	23	0	23	0	23
60/83	24	0	24	0	24
61/83	22	0	22	0	22
67/83	21	0	21	0	21
63/83	20	0	20	0	20
64/83	20	0	20	0	20
65/83	18	0	18	0	18
66/83	15	0	15	0	15
67/83	16	0	16	0	16
68/83	12	0	12	0	12
69/83	21	0	21	0	21
70/83	22	0	22	0	22
71/83	10	0	10	0	10
77/83	23	0	23	0	23
73/83	21	0	21	0	21

Appendix 1.12 Results of Infant (Suckling) mouse assay for STA of E. coli strains from healthy goats

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	IW/RBW	STA
1/83	0.12	1.71	0.070	-
2/83	0.10	1.49	0.067	-
3/83	0.11	1.37	0.080	-
4/83	0.10	1.61	0.062	-
5/83	0.10	1.81	0.055	-
6/83	0.09	1.80	0.050	-
7/83	0.11	1.83	0.060	-
8/83	0.13	1.83	0.071	-
9/83	0.14	1.79	0.078	-
10/83	0.11	2.24	0.049	-
11/83	0.10	1.88	0.053	-
12/83	0.12	1.87	0.064	-
13/83	0.15	1.89	0.079	-
14/83	0.11	1.39	0.079	-
15/83	0.10	1.61	0.062	-
16/83	0.08	1.32	0.060	-
17/83	0.09	1.36	0.066	-
18/83	0.11	1.61	0.068	-
19/83	0.10	1.33	0.075	-
20/83	0.12	1.57	0.076	-
21/83	0.11	1.42	0.077	-
22/83	0.11	1.64	0.067	-
23/83	0.10	1.43	0.069	-
24/83	0.13	2.01	0.064	-

STRAIN NUMBER	(IW)	RBW	IW/RBW	
25/83	0.12	2.09	0.057	-
26/83	0.14	1.91	0.073	-
27/83	0.12	1.60	0.075	-
28/83	0.09	1.32	0.068	-
29/83	0.10	1.51	0.066	-
30/83	0.10	1.88	0.053	-
31/83	0.10	2.00	0.050	-
32/83	0.13	2.45	0.053	-
33/83	0.11	1.96	0.056	-
34/83	0.11	1.54	0.071	-
35/83	0.08	1.33	0.060	-
36/83	0.09	1.52	0.059	-
37/83	0.10	1.63	0.061	-
38/83	0.11	1.69	0.065	-
39/83	0.12	1.50	0.080	-
40/83	0.13	1.60	0.081	-
41/83	0.13	1.78	0.073	-
42/83	0.14	2.05	0.068	-
43/83	0.11	1.57	0.070	-
44/83	0.11	1.54	0.071	-
45/83	0.11	1.57	0.070	-
46/83	0.10	1.44	0.069	-
47/83	0.13	1.96	0.066	-
48/83	0.11	2.07	0.053	-
49/83	0.13	2.36	0.055	-
50/83	0.15	2.02	0.074	-
51/83	0.07	1.48	0.047	-

STRAIN NUMBER	(IW)	RBW	IW/RBW	STA
52/83	0.09	1.28	0.070	-
53/83	0.10	1.53	0.065	-
54/83	0.12	1.73	0.069	-
55/83	0.12	2.06	0.058	-
56/83	0.12	2.00	0.060	-
57/83	0.11	1.80	0.061	-
58/83	0.15	2.30	0.065	-
59/83	0.13	1.88	0.069	-
60/83	0.10	1.66	0.060	-
61/83	0.10	1.51	0.066	-
62/83	0.10	1.49	0.067	-
63/83	0.11	1.41	0.078	-
64/83	0.12	2.10	0.057	-
65/83	0.11	1.57	0.070	-
66/83	0.11	1.59	0.069	-
67/83	0.14	1.86	0.075	-
68/83	0.13	1.80	0.072	-
69/83	0.12	1.71	0.070	-
70/83	0.13	1.62	0.080	-
71/83	0.10	2.00	0.050	-
72/83	0.09	1.40	0.064	-
73/83	0.14	1.72	0.081	-

Appendix 1.13 Results of tests for LT of individual colonies from primary stool cultures obtained from healthy pigs

STRAIN NUMBER	NUMBER OF COLONIES TESTED	TEST FOR LT			
		COAGGLUTINATION		ELISA	
		+	-	+	-
1/83	18	0	18	0	18
2/83	21	0	21	0	21
3/83	20	0	20	0	20
4/83	23	0	23	0	23
5/83	25	0	25	0	25
6/83	21	0	21	0	21
7/83	20	0	20	0	20
8/83	18	0	18	0	18
9/83	21	0	21	0	21
10/83	22	0	22	0	22
11/83	22	0	22	0	22
12/83	20	0	20	0	20
13/83	19	0	19	0	19
14/83	19	0	19	0	19
15/83	10	0	10	0	10
16/83	20	11	9	11	9
17/83	16	0	16	0	16
18/83	23	0	23	0	23
19/83	18	0	18	0	18
20/83	17	3	14	3	14
21/83	16	0	16	0	16
22/83	15	0	15	0	15
23/83	9	0	9	0	9
24/83	23	0	23	0	23
25/83	22	0	22	0	22

STRAIN NUMBER	NUMBER OF COLONIES TESTED	COAGGLUTINATION		E I A	
		+	-	+	-
26/83	21	0	21	0	21
27/83	21	0	21	0	21
28/83	22	0	22	0	22
29/83	23	0	23	0	23
30/83	25	0	25	0	25
31/83	18	0	18	0	18
32/83	25	15	10	16	9
33/83	25	0	25	0	25
34/83	22	0	22	0	22
35/83	20	0	20	0	20
36/83	21	0	21	0	21
37/83	20	0	20	0	20
38/83	20	0	20	0	20
39/83	19	0	19	0	19
40/83	18	0	18	0	18
41/83	21	0	21	0	21
42/83	17	0	17	0	17
43/83	19	0	19	0	19
44/83	20	0	20	0	20
45/83	20	0	20	0	20
46/83	20	0	20	0	20
47/83	22	13	9	15	7
48/83	22	0	22	0	22
49/83	21	0	21	0	21
50/83	22	0	22	0	22
51/83	18	0	18	0	18
52/83	15	0	15	0	15
53/83	21	0	21	0	21
54/83	18	10	8	10	8

Appendix 1.14 Results of infant (Suckling) mouse assay for STA of E. coli strains from healthy pigs

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	S T A
1/83	0.10	1.36	0.073	-
2/83	0.12	1.62	0.074	-
3/83	0.13	1.85	0.070	-
4/83	0.11	1.61	0.068	-
5/83	0.15	1.45	0.103	+
6/83	0.14	1.75	0.080	-
7/83	0.10	1.81	0.055	-
8/83	0.09	1.80	0.050	-
9/83	0.08	1.66	0.048	-
10/83	0.10	1.72	0.058	-
11/83	0.11	1.74	0.063	-
12/83	0.13	1.34	0.097	+
13/83	0.12	1.51	0.079	-
14/83	0.11	1.57	0.070	-
15/83	0.09	2.00	0.045	-
16/83	0.10	1.61	0.062	-
17/83	0.14	2.12	0.066	-
18/83	0.13	2.16	0.060	-
19/83	0.11	1.61	0.068	-
20/83	0.15	2.50	0.060	-
21/83	0.14	1.41	0.099	+
22/83	0.13	1.52	0.085	+
23/83	0.11	1.50	0.073	-

STRAIN NUMBER	(IW)	RBW	IW/RBW	S T A
24/83	0.12	2.26	0.053	-
25/83	0.13	2.24	0.058	-
26/83	0.10	1.96	0.051	-
27/83	0.14	2.54	0.055	-
28/83	0.09	1.80	0.050	-
29/83	0.10	1.23	0.081	-
30/83	0.08	1.90	0.042	-
31/83	0.10	2.00	0.050	-
32/83	0.09	2.04	0.044	-
33/83	0.10	1.42	0.070	-
34/83	0.11	1.57	0.070	-
35/83	0.12	1.69	0.071	-
36/83	0.11	1.64	0.067	-
37/83	0.14	2.22	0.063	-
38/83	0.10	1.66	0.060	-
39/83	0.15	2.45	0.061	-
40/83	0.11	1.89	0.058	-
41/83	0.10	1.63	0.061	-
42/83	0.10	1.31	0.076	-
43/83	0.12	1.79	0.067	-
44/83	0.11	1.92	0.057	-
45/83	0.11	1.71	0.064	-
46/83	0.13	1.96	0.066	-
47/83	0.12	1.44	0.083	+
48/83	0.14	2.00	0.070	-
49/83	0.11	1.22	0.090	+
50/83	0.13	2.70	0.048	-
51/83	0.10	1.88	0.053	-
52/83	0.09	1.26	0.071	-
53/83	0.11	1.42	0.077	-
54/83	0.13	1.42	0.091	+

Appendix 1.15 Results of tests for LT of individual colonies from primary stool cultures obtained from pigs with diarrhoea.

CULTURE NUMBER	NUMBER OF COLONIES TESTED	TESTS FOR LT			
		COAGGLUTINATION		ELISA	
		+	-	+	-
55/83	20	0	20	0	20
56/83	19	0	19	0	19
57/83	20	19	1	19	1
58/83	21	0	21	0	21
59/83	21	0	21	0	21
60/83	17	0	17	0	17
61/83	16	16	0	16	0
62/83	15	0	15	0	15
63/83	16	0	16	0	16
64/83	20	18	2	18	2
65/83	21	0	21	0	21
66/83	14	0	14	0	14
67/83	16	0	16	0	16
68/83	10	10	0	10	0
69/83	20	0	20	0	20
70/83	23	0	23	0	23
71/83	18	0	18	0	18
72/83	21	0	21	0	21
73/83	20	19	1	19	1
74/83	15	15	0	15	0
75/83	22	0	22	0	22
76/83	21	0	21	0	21
77/83	19	17	2	19	0
78/83	20	0	20	0	20
79/83	22	0	22	0	22

CULTURE NUMBER	NUMBER OF COLONIES	TESTS FOR LT			
		COAGGLUTINATION		EIA	
		+	-	+	-
80/83	23	21	2	21	2
81/83	20	0	20	0	20
82/83	21	0	21	0	21
83/83	24	0	24	0	24
84/83	22	0	22	0	22
85/83	25	0	25	0	25
86/83	20	0	20	0	20
87/83	20	18	2	18	2
88/83	18	0	18	0	18
89/83	19	0	19	0	19
90/83	19	19	0	19	0
91/83	20	0	20	0	20
92/83	21	19	2	20	1
93/83	21	0	21	0	21
94/83	20	0	20	0	20
95/83	18	0	18	0	18
96/83	15	0	15	0	15
97/83	21	0	21	0	21
98/83	23	14	9	14	9
99/83	16	0	16	0	16
100/83	18	18	0	18	0
101/83	23	0	23	0	23
102/83	21	0	21	0	21
103/83	23	20	3	20	3
104/83	22	20	2	21	1
105/83	19	0	19	0	19
106/83	22	0	22	0	22
107/83	20	0	20	0	20
108/83	21	21	0	21	0
109/83	20	0	20	0	20

Appendix 1.16 Results of infant (Suckling) mouse assay for STA of E. coli strains from pigs with diarrhoea.

STRAIN NUMBER	WEIGHT OF INTESTINES (IW)	REST OF BODY WEIGHT (RBW)	RATIO IW/RBW	S T A
55/83	0.12	1.84	0.065	-
56/83	0.10	1.72	0.058	-
57/83	0.09	1.63	0.055	-
58/83	0.10	1.88	0.053	-
59/83	0.11	2.20	0.050	-
60/83	0.11	1.86	0.059	-
61/83	0.12	1.90	0.063	-
62/83	0.13	1.78	0.073	-
63/83	0.11	1.59	0.069	-
64/83	0.12	1.44	0.083	+
65/83	0.15	1.87	0.080	-
66/83	0.13	1.42	0.091	+
67/83	0.10	1.61	0.062	-
68/83	0.16	1.92	0.083	+
69/83	0.13	1.64	0.079	-
70/83	0.11	1.61	0.068	-
71/83	0.10	1.51	0.066	-
72/83	0.10	1.58	0.063	-
73/83	0.09	1.16	0.077	-
74/83	0.13	1.83	0.071	-
75/83	0.11	1.57	0.070	-
76/83	0.12	1.71	0.070	-
77/83	0.12	1.84	0.065	-
78/83	0.13	2.03	0.064	-

STRAIN NUMBER	(IW	RBW	IW/RBW	S T A
79/83	0.12	1.87	0.064	-
80/83	0.14	1.62	0.086	+
81/83	0.15	1.85	0.081	-
82/83	0.10	1.78	0.056	-
83/83	0.08	1.63	0.049	-
84/83	0.09	1.76	0.051	-
85/83	0.11	2.20	0.050	-
86/83	0.11	1.64	0.067	-
87/83	0.12	1.33	0.090	+
88/83	0.10	1.38	0.072	-
89/83	0.10	1.33	0.075	-
90/83	0.13	1.36	0.095	+
91/83	0.13	2.24	0.058	-
92/83	0.16	1.70	0.094	+
93/83	0.15	2.34	0.064	-
94/83	0.11	1.22	0.090	+
95/83	0.12	1.93	0.062	-
96/83	0.10	1.44	0.069	-
97/83	0.14	1.75	0.080	-
98/83	0.15	1.50	0.100	+
99/83	0.13	1.66	0.078	-
100/83	0.16	1.77	0.090	+
101/83	0.13	1.64	0.079	-
102/83	0.12	1.81	0.066	-
103/83	0.10	1.58	0.063	-
104/83	0.14	1.64	0.085	+
105/83	0.11	1.64	0.067	-
106/83	0.12	1.96	0.061	-
107/83	0.11	1.71	0.064	-
108/83	0.10	1.40	0.071	-
109/83	0.09	1.69	0.053	-

Appendix 2 Bacteriological media used.

2.1 Cary-Blair's transport medium base (Gibco, Europe, Paisley, Scotland)

12.5g of powder was weighed into 1000mls distilled water. This was brought to the boil to dissolve the powder by heating. The medium was dispensed into screwcapped tubes and autoclaved at 121°C for 15 minutes.

2.2 Bromothymol-blue Lactose agar Merck, W. Germany).

41g of powder was added to 1000mls of distilled water and heated to dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes and dispensed into sterile petri dishes.

2.3 MacConkey agar (Oxoid, England)

50g of powder was weighed out and added to 1000mls distilled water. This was heated till it boiled to allow the powder to dissolve. The medium was sterilized by autoclaving at 121°C for 15 minutes. Media was dispensed into sterile petri dishes.

2.4 Tryptic soy broth (Gibco, Europe, Paisley, Scotland)

30g of powder was suspended in 1000ml distilled water and allowed to dissolve. The broth was then dispensed into flasks and sterilized by autoclaving at 121°C for 15 minutes.

2.5 Trypticase soy agar (Oxoid, England).

40g of powder was suspended in 1000mls distilled water, brought to the boil and sterilized at 121°C for 15 minutes. Media was dispensed into sterile petri dishes.

2.6 Triple Sugar Iron agar (Lab M, London)

65g of powder was weighed out and mixed with 1000mls distilled water. The mixture was heated till it boiled, allowing the powder to dissolve completely. Media was distributed into tubes and sterilized by autoclaving at 121°C for 15 minutes. The tubes were allowed to solidify at room temperature in slanted position.

2.7 MRVP medium (Oxoid, England)

15g of powder was suspended in 1000mls of distilled water and heated to allow to dissolve. The media was dispensed into screwcapped tubes and sterilized by autoclaving at 121°C for 15 minutes.

2.8 Proteose peptone (Oxoid, England)

10g of powder (proteose peptone) and 5g NaCl were weighed and dissolved in 1000mls distilled water. The media was dispensed into screwcapped tubes and sterilized by autoclaving at 121°C for minutes.

2.9 Urea agar (Oxoid, England)

0.9g of urea agar base (Oxoid, England), was suspended in 95mls distilled water. This was sterilized by autoclaving for 20 mins at 115°C 10lb per sq inch pressure). 5ml of sterile 40% urea solution (Oxoid, England) was asceptically introduced into the agar base and mixed. The urea agar medium was distributed in 10ml amounts into sterile bijou bottles and allowed to solidify.

2.10 Simmons citrate agar (Oxoid, England)

23g of powder was suspended in 1000mls of distilled water and heated to the boil to dissolve the powder. The media was dispensed into bottles and sterilized by autoclaving at 121°C for 15 minutes.

2.11 T.C.B.S. Cholera medium (Oxoid, England)

88g of media was weighed out and suspended in 1000mls distilled water. The media was boiled to dissolve completely and poured into sterile plates. the agar was allowed to dry before use.

2.12 Tryptone (Oxoid, England)

10g of powder was weighed and suspended in 1000mls distilled water. Media was dispensed into tubes and was sterilized by autoclaving at 121°C for 15 mins.

2.13 Trace salt solution for Biken broth.

To about 80 ml of distilled water the following were added.

$MgSO_4 . 7H_2O$	10.2g
$CoCl_2 . 6H_2O$	2.0g
Ferric chloride solution 60% (w/v)	0.83ml

Distilled water was added upto 100ml.

2.14 Biken broth (Casamino, Yeast extract broth)

Distilled water	1000mls
Yeast extract (Oxoid, England)	10g
Hydrolysed casein (Oxoid,England)	20g
NaCl	2.5g
K_2HPO_4	15g
Glucose	5.0g
Trace salts solution	0.5ml

Dispensed in 5.0mls into screwcapped tubes and autoclaved at 121°C for 15 minutes.

Appendix 3.0 Protein staining and destaining solutions:

3.1 Coomassie Brilliant blue stain

Coomassie Brilliant blue 250R	10g
Ethanol	900 ml
Distilled water	900ml
Glacial acetic acid	200ml

The stain was filtered through Whatman No. 1 filter paper to remove any insoluble material.

3.2 Destaining solution for Coomassie Brilliant blue stain.

Glacial acetic acid	200ml
Ethanol	900ml
Distilled water	900ml

3.3 Ponceau "S" stain

Ponceau "S"	2g
1M acetic acid	1000ml
0.1M sodium acetate	1000ml

3.4 Destaining solution for Ponceau "S"

3%(v/v) of glacial acetic acid in distilled water.

Appendix 4.0 Buffers and Solutions used in immunodiffusion

4.1 PBS for preparation of agar gel for immunodiffusion and other purposes.

PBS (0.15M, pH 7.4)

21.2g Di-sodium hydrogen phosphate (anhydrous) was dissolved in 800ml of distilled water, the pH adjusted to 7.4 by using dilute HCl acid (1NHCl), the volume was then made up to 1000ml in a volumetric flask.

4.2 PBS pH 7.4

1 volume of 0.15M phosphate buffer pH 7.4 was added to 9 volumes of saline (0.9% sodium chloride in distilled water).

4.3 Agar used in immunodiffusion

1% Agar in PBS pH 7.4 for immunodiffusion

Purified Oxoid agar	2g
PBS	50ml
Distilled water	150ml
Sodium azide (NaN_3)	0.02g

0.1%(w/v) sodium azide served as a preservative to prevent microbial growth on the agar during immunodiffusion.

Appendix 5 Buffers, diluents and solutions used in enzyme immunoassays.

5.1 Standard wash solution

Phosphate buffered saline pH 7.4	100ml
Distilled water	9900ml
Tween 20	5ml
Sodium azide	1g

5.2 Diluent for serum - 1/100 PBS with 0.01% sodium azide

Phosphate buffered saline pH 7.4	10ml
Distilled water	990ml
Sodium azide	0.1g

5.3 KCL/EDTA Diluent for conjugate.

0.05M phosphate buffer pH 8.0	1000ml
KCl	75g
EDTA	1g
Benzoic acid	2.5g
Tween 80	5ml

pH was adjusted to 7.5 using 4M NaOH.

5.4 Glucose Oxidase Substrate

5.4.1 Substrate buffer (0.05M ammonium acetate/0.05M citrate buffer pH 5.0 with 0.1% benzoic acid).

5.4.2 Glucose oxidase substrate solution

Substrate buffer	10mls
20% β D-Glucose	1ml (17.85mg/ml)
Horseradish peroxidase solution	0.1ml (8.93ug/ml)
2, 2'-Azino-bis (3-Ethylbenzthiazolinesulphonic Acid (ABTS)	0.1ml (0.22mg/ml)