STUDIES ON COLIFORMS OF RAW MILK AND THEIR TOXIGENIC POTENTIAL IN DAIRY ENVIRONMENT

THESIS

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BY

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DEDICATED

To my family whose co operation, patience and encouragement which had helped me to make this work possible.

II

C E R T I F I C A T E

This is to certify that the thesis entitled "STUDIES ON COLIFORMS OF RAW MILK AND THEIR TOXIGENIC POTENTIAL IN DAIRY ENVIRONMENT" submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology to the Tamil Nadu Agricultural University, Coimbatore, is a record of bonafide research work carried out by K.S. PALANISWAMI, under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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PLAN OF STUDY

CHAPTER I

PLAN OF STUDY

A greater understanding and appreciation of the bacteriological quality of dairy foods had developed in the last decade particularly in relation to product quality (Mabbit, 1980a) as well as its potential food-borne health hazard (Busta, 1979; Richard et al., 1981). The ideal aim in producing high quality milk is that it should be produced in an aesthetic environment using suitable sanitary practices permitting only minimal bacterial contamination of the milk (Palmer, 1980). The implementation of quality milk production programme requires not only routine laboratory assessment of milk quality but also periodic farm inspection for enforcement of milk hygiene during production (Hartley et al., 1968; Hartley et al., 1969). Any evaluation of microbiological problems related to dairy foods must include problems related to the metabolic process of microorganisms. The typical types of food poisoning and their causes were well understood and could be controlled by proper sanitation, processing and storage techniques (Richter, 1981).

In developing countries, the wide spread collection of unchilled raw milk in tropical climate from small farms where insanitary practices exist had brought in bacteriological

problems at these milk sheds and in the dairy industry as a whole.

The bacteriological problems in dairy industry could be classified as those associated with product quality deterioration, spoilage and public health and nutrition concerns of dairy products (Richter, 1981). Though all these problems existed with increased use of milk and milk products as wholesome food in our country with stepped up "Operation flood" programmes they had become the primary concern for a veterinary microbiologist to concentrate on food-borne illness.

Though the presence of coliforms in milk and milk products was considered hitherto as indicator organisms of faecal pollution and insanitary practices, the occurrence of diarrhoeal illness due to coliforms had opened up a newer thinking with regard to the significance of coliforms (Goel et al., 1971; Ranganathan, 1973; Marrier et al., 1973; Singh and Ranganathan, 1974; Misra and Rao, 1975; Mehlmen et al., 1976; Indian Standards Specification No. 1479 - 1977; Twedt and Boutin, 1979; Glatz and Brudvig, 1980a,b, and Batish et al., 1981a,b).

Milk borne infections, intoxications and toxi-infections due to organisms such as Staphylococcus, Bacillus cereus,

Yersinia enterocolitica, Campylobacter fetus spp. jejuni Escherichia coli, Clostridium and Aspergillus had gained an increasing attention in recent years (Batish et al., 1981a,b, Richard et al., 1981; Todd, 1978). New directions in research on enteropathogenic E.coli and other such organisms had been internationally discussed and proposed for the decade of 1980's (Busta, 1979; Richter, 1981; Kornacki and Marth, 1982b, Winslow, 1982; and Kraft, 1982).

Apart from being incriminated recently as public health hazard, the E.coli being a lactose fermenter producing lactic acid and other products like indole, ethanol, acetic acid succinic acid, carbon dioxide and hydrogen (Foster et al., 1958) has got a major role to play in milk and milk product spoilage.

Further the other coliforms like Enterobacter aerogenes Enterobacter cloacae, Klebsiella pneumoniae and Citrobacter freundii also produce lactic acid, gassy defects, and off flavour (Foster et al., 1958; Mikolajcik, 1979). Although these bacteria could ferment lactose to acid and gas at 37°C, research had indicated that psychrotrophic coliforms could also contribute to proteolytic spoilage of refrigerated milk The coliforms constituted 5 to 33% of the psychrotrophic counts (Cousins, 1982). Species of Enterobacter and

Klebsiella are the members of the family Enterobacteriaceae most frequently isolated from refrigerated raw milk (Thomas, 1958; Jayasankar et al., 1966)

So a greater understanding and appreciation of sources of potentially hazardous coliforms in the farm environment, level of them at various stages of raw milk handling, would facilitate not only clean milk production but also check diarrhoeal illness, due to toxigenic E.coli, Klebsiella Enterobacter and Citrobacter sp. While research effort to date had been primarily concerned only with enterotoxin production by E.coli isolated from milk and milk products, they had not advanced to the potentially toxigenic other members of coliform group like Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae and Citrobacter freundii. There is dearth of information of enterotoxigenic coliforms from dairy farm environment.

Hence a detailed study on the presence of coliform group from the healthy udder, the surface of the teat of a healthy cow, milking environment and its total level in the commingled milk was envisaged and their significance in the dairy sectors of Tamil Nadu are discussed. An attempt was also made to detect the toxigenic coliforms from E.coli, Klebsiella, Enterobacter and Citrobacter sp. isolated

from the farm environment. The production of heat stable enterotoxin by enterotoxigenic coliforms in laboratory condition was analysed and the possibility of its production and implications in field condition was discussed.

In an applied aspect, a rapid reliable screening test to assess the Gram negative bacterial load of milk and to develop rapid methods to identify the isolates preliminarily before the release for processing and sale of milk and milk products were made by employing Limulus amoebolysate test for Gram negative endotoxin estimation and diagnostic microtube kit systems for the identification of coliforms in the dairy industry.

The entire study could conceivably provide insights in the applicability of present specification on the coliform quality and quantity of raw milk in the tropical climate of our country especially in the Anand pattern of milk collection suggesting ways and means of control in a cursory manner.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

1. Factors Affecting Bacteriological Quality of Raw Milk

The purpose of clean milk production with good bacteriological quality is to ensure that the product when delivered remains fresh under domestic conditions of storage as long as it can and also be safe to the consumers. Clean milk is achieved by the application of general hygienic principles to the production, processing and the distribution of milk. The term "Safe milk" implies that the milk is free from pathogenic organisms. This is achieved in part by cleanliness, in part by heat treatment and in part by eradication of bovine disease (McCoy, 1966).

The epidemics of infantile diarrhoea, summer diarrhoea etc. had been brought down in England due to improvement in the cleanliness of the milk supply, modern packaging of the fluid milk, substitution of heated milk for infant feeding and fly control (McCoy, 1966). Hence the initial bacteriological quality of fresh raw milk has a marked influence on the keeping quality of raw milk and on the quality of products made from it (Gehriger, 1980).

It had been emphasized that in order to obtain ex-farm bulk milk of high bacteriological quality with total desirable bacterial load of 10,000 per mL, considerable care is needed in its production (Mabbit, 1980a). A critical survey on the sources of contamination of bacteria in milk in farms, where refrigerated storage is not available is presented.

(A) Environmental factors influencing the quality of individual and pooled milk at farms:

(1) Farm air:

The microbial flora of air, its ecology and analysis were extensively studied by Verstrete and Votes (1974). Air would be highly contaminated with many types of viruses, bacteria and fungi either free in the air or bound to dust particles (Verstrete and Votes, 1974). Bacterial contamination of indoor air of dairy cattle sheds, the technological factors and their influence on quantitative bacterial contamination were studied (Beer et al., 1976). The percentage of coliform count at the mobile house for feeding cows and at milking stall were estimated to be at 2.5% and 5.6% respectively, of the total microbial count (Fiser and Svitavsky, 1975).

In a survey of air borne bacteria in typical milking premises in U.K., it was found that the air borne contamination during milking was no worse in the modern

integrated unit than in the cowshed (Benham and Egdell, 1970). Mean microbial count per litre of air throughout the entire year was 39 for the early and 35 for the later part of the year. In isolated and free standing milking parlours, air borne bacterial levels were marked by lower counts (17 per litre of air). The aerial contamination in all types of milking premises when the cows were out at grass was only one quarter of the contamination during the period when the cows were housed.

The bacterial levels in air of cow sheds were reported as 6-45 per litre. The distribution percentage of microorganisms was Gram positive cocci, 50-70; Gram positive rods, 10-40; Gram negative rods, 2-8; aerobic spore formers, 7-9; and moulds 4-10 (Boer and Melhom, 1974). At milking time in a fairly clean barn 1×10^3 to 1×10^4 psychrotrophs per square feet of exposed surface were collected during 10 minutes exposure (Thomas, 1958).

The specific types of bacteria contaminating the milk from the air may be of some importance. For instance, aerobic spore formers such as Bacillus cereus causes "bitty cream" in pasteurized milk and anaerobic spore formers such as Clostridia could cause spoilage of Swiss type of cheese (Palmer, 1980). In a study of chemotypes and antibiotypes of coliform bacteria isolated in a Normandy

dairy farm with calf septicaemia, E.coli constituted 80% of the isolates with Citrobacter (8%), Klebsiella (4%) and Enterobacter (6-9%) (Jacquet and Chiki, 1977). While concluding a study on the control of environmental hygiene, the cowsheds were found to have standard bacterial counts of 5333/cfu/M^3; coliforms 191/cfu/M^3; yeasts 645/cfu/M^3 and moulds 536/cfu/M^3 (Rondinini, 1982).

The aerosolized microorganisms were measured by fallout, exposing appropriate media for five minutes, 10 minutes and 15 minutes (Anon, 1972; Cruickshank et al., 1975; Harrigan and McCance 1976). Anderson samplers connected to a portable field vaccum source aspirates required quantity of air per minute (28.3 litres or 1 C.ft per minute) and impinges the collected organisms on a nutrient medium placed on the petridish (Adams and Spendlove, 1970). Casitone agar was used for collection and growth of general microbes. Endoagar and eosine methylene blue agar were used for selective growth of coliform organism (Adams and Spendlove, 1970).

If the test was conducted during day light hours, solar radiation had a deleterious effect. Overcast skies could be expected to reduce somewhat the effect of solar radiation. High wind velocity, high relative humidity, darkness and low temperatures, would be expected to give the greatest

recoveries or fallout. Relative humidity, thunderstorm were known to have a pronounced effect on the survival of air bone E.coli (Adams and Spendlove, 1970).

Standards with regard to levels of air infection are available. The number of organisms per C.ft. per minute should not exceed one, for satisfactory atmosphere (ICAR, 1972). Normal settling of 4.5 to 50 bacteria carrying particles per 15 minutes exposure had also been proposed for dairy industry by American Public Health Association (Anon, 1972). Levels of air infection in temperate climate, for various places like hospitals, factories offices etc. had been reported (Cruickshank et al., 1975).

(ii) Farm Water:

Water consumption in industrial milk production was at an average of 121.8 L/cow or 12.7 L/kg of milk of which around 40 L was for drinking purposes and the balance was for washing (Koallick and Holke, 1980).

Unless properly protected, farm water supplies might be contaminated at source with a wide variety of microorganisms including coliforms, psychrotrophic pseudomonas which are indeed human pathogens. Contaminated water might not be a major source of bacterial contamination in milk unless such water

was added directly into milk. Lactose fermenting and milk spoiling organisms had been isolated from farm water supplies (Palmer, 1980). The use of such water supply for udder washing and for rinsing of cleaned equipment could cause contamination of milk. Furthermore when milk residues remained on dairy equipment, residual bacteria including those from the water would grow and cause contamination, when the equipment was next used (Cuthbert, 1960). Many of the strongly lipolytic and caseolytic psychrotrophs found in milk had their origin from the water supply (Thomas, 1958).

The psychrotrophic count of water ranged from less than 10 to more than 1,000,000 per ml with a median of 10 to 560 per ml (Morse et al., 1968). A good quality water supply should have a low colony count with no faecal organisms and should possess a satisfactory field report. Presumptive coli count of less than 50 per 100 ml of water and E.coli absent per 100 ml were recommended as bacteriological standards of farm water (Palmer, 1980).

(iii) Farm equipments and utensils:

It had been generally observed that micrococci, corynebacteria and occasionally streptococci form the dominant flora in low count equipment rinses and that streptococci and Gram negative rods were prominent in high

count equipment rinses (Thomas, 1964 and Palmer, 1980). Gram negative rods dominated even in low count tank rinses forming upto 70% of the total isolates. Although both coliaerogenes organisms and spore forming rods formed only a small proportion of the total isolates, they occasionally constituted an appreciable proportion of the microflora of some utensils (Palmer, 1980). Equipment used for carrying, dispensing or holding foods should bear less than 10 coliform bacteria per 100 sq.cm. Absence of coliform bacteria in 100 sq.cm. had been suggested as a satisfactory standard for coliforms (Harrigan and McCance, 1976).

The milking and milk storage equipments are frequently the main sources of bacteria in farm milk supplies. It had also been well established that where an effective cleaning and sterilising routine was being carried out correctly, the bacterial contamination from the equipment was quite low (less than 1000 bacteria per ml of milk). However, major bacterial contamination of milk occurred from the farm equipments and due to a number of factors such as poorly designed equipment and failure to carryout cleaning instructions as directed or misinterpretation of instructions due to complexity or other reasons (Palmer, 1980; Gehriger, 1980 and Fluckiger et al., 1980)

(iv) Personnel:

Microbial contamination of the milk by the milker might occur in several ways but mainly through contact of the washed teats with unclean hands and poor milking technique (Palmer, 1980). It was found that hospital staphylococci could be transferred to the milk via patients discharged from hospital who had contact with farm animals (Moeller et al., 1963). Pathogenic Klebsiella had been found to survive longer than the non-pathogenic Klebsiella in the finger tips (Cooke et al., 1981). In a study of sources of milk contamination of farms it was found that milkers' hands rinsed in 200 ml of sterile milk added as much as 260 x 10^3 bacteria to the milk (Smythe, 1980). Examples of poor milking techniques giving rise to microbial contamination included moistening the hands with the first drops of milk and touching milk contact surfaces such as the inside of milk storage containers and milking buckets (Palmer, 1980).

(v) Udder and teats:

(a) The apparently healthy mammary gland:

If milk is removed from the healthy gland or teats by aseptic puncture of the wall or by surgically

implanted catheter systems sterile samples can be obtained (Tolle, 1980). Even with machine milking, samples of milk with very low numbers of bacteria could be obtained from healthy udders, if precautions were taken to avoid contaminants. It had been observed that the mean numbers were 138 cfu/ml from non-selected animals and 46 cfu/ml from selected animals consisting of Micrococcus sp. and Corynebacterium bovis (Kleter, 1974).

In practice, however, the teat canal is populated by microorganisms varying greatly in numbers and types. Besides coagulase-negative staphylocci and Corynebacterium bovis there are aesculin-splitting streptococci including Streptococcus uberis, Staphylococcus aureus, Bacillus sp. and Proteus sp.(Cullen and Hebert, 1967). The resulting primary contamination of milk might amount to 10^2 cfu/ml in 41%, between 10^2 and 10^3 cfu/ml in 35%, between 10^3 and 10^4 cfu/ml in 23% and 10^4 cfu/ml in one percent of quarter milk samples (Tolle, 1980). The rarity of reports in cases of subclinical mastitis due to coliforms could be assessed by the fact that no coliforms could be isolated in nearly 500 milk samples tested from apparently healthy quarters in a study made in National Dairy Research Institute, Bangalore (Rao and Nambudripadi, 1978)

(b) Species of the bacteria:

It had been shown that Staphylococcus aureus multiplies on the skin of teat and udder, and was considered to be a true resident of the surface of the udder. It was, however, very difficult to distinguish the resident bacterial flora from the transient ones, originating from bedding, faeces and soil. While the microflora inside the udder was almost exclusively mesophilic, the flora on the surface also included psychrotrophicbacteria, thermoduric bacteria and anaerobic sporeforming species (Joergensen, 1980). From unwashed udders, the major group of bacteria identified were staphylococci, Corynebacterium bovis and coliforms (Cullen and Hebert, 1967). The sources of coliforms and coagulase negative staphylococci were especially pronounced at the teat apex (Schonberg, 1951).

(c) Factors influencing bacterial flora of the teat:

The influence of grazing, cow housing and bedding, effect of udder washing, type of disinfectant used for udder washing and methods for minimizing contamination from teat and udder had been comprehensively reviewed (Joergensen, 1980). The saw dust bedding had been a major source of coliform contamination of teat ends

followed by wood shavings and straw. Among the coliforms the geometric mean of Klebsiella was 11, 2 and 1 with beddings of saw dust, shavings and straw respectively. With fresh bedding the total coliform and Klebsiella counts of teat ends were significantly reduced (Rendos, 1975). E.coli was isolated at 28% level from the buffalo udders using swabs in dextrose infusion broth (Awan and Barya, 1972).

(d) Teat dipping and drying:

Teat dipping with iodine and non-iodine preparation before and after the milking and drying could improve the milk quality and also reduce the mastitis problem (Hansen, 1973; Bigalke, 1978; Hemken et al., 1981; Hubble and Mein, 1981; and Sheldrake and Hoare, 1982).

To ensure that milk is of high bacteriological quality, udder surface need to be dry and teat surfaces clean and dry (Golton et al., 1982)

(B) Factors influencing the commingled milk quality:

(i) Importance of raw milk quality:

The commercial pressures within the dairy industry are tending to extend the time between milk

production and processing. If a consequential deterioration in product quality is to be avoided, the milk ex-farm must be of high bacteriological quality (Mabbit, 1980b).

According to International Dairy Federation monograph on the bacteriological quality of pooled bulk milk and total viable count of ex-farm milk produced under good hygienic conditions should not exceed 10,000 bacteria per mℓ. In order to achieve this quality, it is important to recognize the sources of contamination of the milk supply and to understand how they can be controlled. The main sources are the udder and the milking equipment (Lagrange, 1979 and Mabbit, 1980b).

(ii) Seasonal trends:

The warmer months each year would be highlighted by a reduction in the percentage of samples of class I of high bacterial quality with an increase in samples placed in Class II of low bacterial quality and undergrade (Lagrange, 1979).

(iii) Time:

An important enemy of milk quality would be time. The mesophilic or psychrotrophic bacteria, will continue

to metabolize, reproduce and increase in number at ambient temperature and refrigerated temperature. Even in refrigerated milk, a bacterial count of 500,000 per mL could be one million per mL the next day (Lagrange, 1979; and Gehriger, 1980).

(iv) Temperature:

Another major influence on raw milk quality and one that might be frequently abused would be temperature. Unless milk had been held at temperatures below 5°C milk quality was in jeopardy (Swartling, 1967; Lagrange, 1979 and Gehriger, 1980).

(v) Storage and handling:

Failure to clean and sanitize the milk storage tanks at chilling centre or dairy plant during flush season would add to the quality problems. New milk would be mixed with the old milk in the plant, storage tanks and the dynamic bacterial metabolic chain might not be broken and the counts would got built up to several million per mL (Morse et al., 1968 and Lagrange, 1979).

(C) Antibacterial system in milk:

The lactoperoxidase system is a naturally occurring inhibitory system in raw milk that could reduce the bacterial content in milk (Bjorck et al., 1975; Bjorck, 1978). The lactoperoxidase, thiocyanate and hydrogen peroxide inhibited lactic streptococci and Gram negative rods especially pseudomonads (Bjorck et al., 1975). The lactoperoxidase was present in bovine milk but thiocyanate content depended upon on the feed consumed by the cow (Bjorck, 1978). The antibacterial effect depended on the amount of thiocyanate present and the temperature. When the thiocyanate in milk had been depleted, the bacteria could start to multiply usually within 4 hours at 30°C and 72 hours at 5°C. This antibacterial component was heat labile at 60°C for 15 minutes and not present in pasteurized milk (Bjorck et al., 1975 and Bjorck, 1978)

Certain lactic acid bacteria like Leuconostoc cremoris and Lactobacillus acidophilus produced antibacterial substances and thus inhibited Gram negative bacteria including coliforms (Babel, 1977 and Hosono et al., 1977). However, the practical importance of the antibacterial system of milk probably was very slight. Certainly it could not be depended upon to prevent or even to retard significantly the development of mixed populations of

microorganisms in raw milk (Foster et al., 1958; and Ayres et al., 1980)

(D) Bacteriological Standards:

The coliform organisms because of their prevalence in nature are practically always present in raw milk (Barber, 1962). The Scotland standards regulate that the "premium milk" must contain no coliform bacteria in 0.01 ml and standard milk must not contain coliform bacteria in 0.001 ml (Cruickshank et al., 1975). Guidelines for grading of raw milk based on the coliform content and standard plate count had been suggested by Indian Standards Institution (Indian Standards No.1479 - Part III - 1977). The guidelines suggested, that the satisfactory level would be the absence of coliforms in 0.01 ml of raw milk. There are no existing legal standards for coliforms in raw milk in Prevention of Food Adulteration Act, 1954 and in Prevention of Food Adulteration Rules, 1979.

(i) Achieved levels:

In a wide survey conducted in the Hissar City, the presumptive coliform count varied from 6,400 to 46,200 per ml and did not conform at 100% level to the Indian Standards No.1479 - Part III - 1962 (Gahlot et al., 1975).

In a study at National Dairy Research Institute, Karnal, the coliform count was found to vary from 500 - 50,000/ml in buffalo milk and 50,000 - 1.96 million/ml for cow milk. The samples were collected in the farm as well as from nearby villages (Singh and Ranganathan, 1978). In an organized dairy sector the raw milk samples collected at reception dock indicated coliforms to vary from 500/ml to 25000/ml (Misra and Sinha, 1978). In a study in tropical climate at Mauritius, the coliforms ranged from 1390 to 2670/ml (Rangasamy, 1980). In temperate climate, the coliform titre were found to range from 100000 to 10 million/ml of raw milk, contamination being higher in summer months (Kaloyanov and Gogov, 1977).

(ii) Improvement opportunities for Bacteriological standards

Since raw milk quality did have a significant influence on the quality of all dairy products, efforts might need to be continued to improve milk quality not only on the farm but also in the processing plant. Quality assurance programmes should include careful evaluation of initial numbers of bacteria in individual farm milk samples coupled with studies of the time and temperature profile of milk from the farm to the processing vat. Testing milk for bacterial numbers at critical points in the chain from farm to plant would reveal links where opportunity existed for improvement (Lagrange, 1979).

2. Coliforms in raw Milk

The large group called the Enterobacteria contains various genera of small Gram negative non sporing rods which ferment dextrose producing acid or acid and gas and are oxidase negative (Collins and Lyne, 1970). The family received its name because most genera are commensals or parasites in the animal or human intestine. For laboratory purposes it would be convenient to divide the Enterobacteria into two groups according to the fermentation of lactose. This was an historical division from the time when bacteriology was almost exclusively a medical science and the lactose fermenters were considered to include mostly saprophytic and commensal organisms while the non-lactose fermenters included the pathogens (Collins and Lyne, 1970; and Edwards and Ewing, 1972).

The lactose fermenters produce acid and gas rapidly from the sugar lactose. The genera Escherichia, Klebsiella, Citrobacter and Enterobacter are collectively known as coliform bacilli (Harrigan and McCance, 1976).

The non-lactose fermenters fail to ferment the sugar lactose at all or ferment it late or irregularly. Included in this group primarily are the genera. salmonella, some members of Arizona , Shigella, Edwardsiella, few Citrobacter

Proteus, Serratia, Providencia and Plesiomonas (Collins and Lyne, 1970; Edwards and Ewing, 1972; and Cruickshank et al., 1975).

The coliforms which are lactose fermenters would be completely undesirable in milk and its products (Foster et al., 1958; Ayres et al., 1980; and Cousins, 1982).

(A) Bacteriology of Coliforms:

The name 'coliform' was derived from the Latin word 'Colon' meaning large intestine, with reference to the common occurrence there. Among them, Escherichia coli is the chief member of the group (Foster et al., 1958) The other members include Enterobacter aerogenes, E.cloacae, Klebsiella pneumoniae and Citrobacter freundii (Edwards and Ewing, 1972; and Buchanan et al., 1974).

The coliform bacteria can grow in the presence of bile salts or other equivalent selective agents and produce acid and gas from lactose at 35°C or 37°C. This group belongs to family Enterobacteriaceae, the members of which grow in the presence of bile salts and produce acid from glucose as determined by the use of violet red bile glucose agar (VRBA). They are Gram negative rods, motile with peritrichous flagella or non-motile. With few exceptions they reduce nitrate to nitrite. Faecal

coliforms are bacteria which in the presence of bile salts or other equivalent selective agents can grow and produce acid and gas from lactose when incubated at 44-45°C (Harrigan and McCance, 1976).

(B) Principal differentiating characters of coliform group:

While all the five principal members of coliforms hydrolyse O-nitrophenyl- β-D-galactopyranoside by β-galactosidase they differentiate themselves in arginine dihydrolase activity (E.cloacae and Citrobacter freundii 94% and 34% positive respectively), lysine decarboxylase test (E.coli, Klebsiella pneumoniae and E.aerogenes are positive by 83%, 75% and 99% respectively), Ornithine decarboxylase test (E.coli, Enterobacter aerogenes and E.cloacae are positive by 76%, 99% and 97% respectively), Citrate utilization (Citrobacter freundii 61% and all others except E.coli 90% to 95%), H_2S production (only Citrobacter freundii produce at 60% level), urease production (K.pneumoniae positive by 64%), inositol acid fermentation (K.pneumoniae and E.aerogenes 97% and 93% positive respectively) and motility (all motile except Klebsiella pneumoniae), (Anon, 1979).

The colony characters on eosin methylene blue agar (EMB agar) are the primary differentiating character between E.coli and other group of organisms. In case of

lack of accuracy other conventional system could be adopted (Edwards and Ewing, 1972; Fung and Cox, 1981).

(C) Presence of coliforms in milk:

(i) Indicator organisms:

This group of bacteria was considered important in quality control of milk as it was indicative of possible faecal contamination and due to its ability to produce acid and taints in milk. The presence of this group in milk indicated degree of insanitary practices during production, processing or storage and was intended to measure general care taken in handling this product (Indian Standards No. 1479 - 1977).

Coliforms were often associated with organisms of intestinal origin but other groups may act as indicators for other situations (Harrigan and McCance, 1976). Escherichia coli is the so called "Faecal coli" which occurred normally in the human and animal intestines and the presence of this organism in milk indicated recent contamination with faeces. E.coli being, fairly wide spread in nature and although most strains originate in faeces, its presence particularly in small numbers did not necessarily mean that the substance from which it

was isolated contained faecal matter (Collins and Lyne, 1970).

In water analysis, the presence of *Escherichia coli* indicated faecal pollution of the water, there being a positive correlation between the concentration of the organisms and the amount and/or the recency of the pollution. In carrying out the test for coliform bacilli in water it would be therefore advisable to determine whether the strains present are *E.coli* (typical) or other non *E.coli* coliforms (atypical) (Harrigan and McCance, 1969).

(ii) *Distribution of coliforms*:

The distribution of coliform in the organized dairy sector were *E.coli* (40%), *Enterobacter cloacae* (8%); *E.aerogenes* (23%); *C.freundii* (17%) and *K.aerogenes* (7%) (Misra and Sinha, 1978).

In another study *Klebsiella aerogenes*, *Enterobacter aerogenes*, *E.cloacae* and *Citrobacter* sp. constituted 11.3%, 35.0%, 23.9% and 29.8% respectively of the coliforms (Kaloyanov and Gogov, 1977).

(iii) *Effect on the final product quality*:

The production of lactic acid and the intermediate metabolites of coliforms reduce the product quality and

nutritive value and also cause spoilage of milk and its products (Muier et al., 1978; Kraft and Rey, 1979; Mikolajcik, 1980). The presence of high counts of coliforms in raw milk might cause irreversible changes, which could result in a decreased shelf life of the finished product. As these organisms are psychrotrophs they are able to grow in the refrigerated milk and cause irreversible change. Enterobacter sp., E.coli and Streptococcus liquefaciens formed the majority of psychrotrophs (Juven et al., 1981).

(iv) Potential role in public health hazard:

The holding of milk at 143°F (61°C) for 30 minutes (low temperature, high time) or 161°F (71°C) for 15 seconds (high temperature, short time) and immediate chilling to 4°C was called pasteurization (Foster et al., 1958). At this time temperature combination non-sporing pathogenic bacteria such as Mycobacterium sp., Brucella abortus, Salmonella and E.coli could be destroyed (Foster et al., 1958; Kay, 1962; Kaplan et al., 1962). The thermal death point of E.coli was 60°C if held for 125 seconds (Kaplan et al., 1962).

Ability to resist 63°C for 30 minutes by coliforms is extremely uncommon (Foster et al., 1958; Kaplan et al., 1962), though heat injured E.coli might be present in the

pasteurized milk (Natarajan et al., 1983). The E.coli had been attributed in large number of milk borne diseases (Marrier et al., 1973; Batish et al., 1981a & Kornacki and Marth, 1982b).

Heat stable enterotoxins are produced by E.coli, Klebsiella pneumoniae, Enterobacter cloacae and these toxins might withstand pasteurization temperature as they were stable at 60°C for 30 minutes or 65°C for 15 minutes (Evans et al., 1973a, Sack et al., 1975; Klipstein and Engert, 1976a,b, Mehlman et al., 1976; Burgess et al., 1978; Clements and Finkelstein, 1979). A heat stable toxin of a porcine strain of E.coli had been positively assayed after heat treatment at 87°C for 20 minutes (Olsson, 1982). It had also been reported that exposure of E.coli, K.pneumoniae and E.cloacae stable enterotoxin to a temperature of 100°C for 30 minutes did not reduce the activity (Klipstein and Engert, 1976a,b, Klipstein et al., 1977).

The stable toxin (ST) of E.coli had been classified into STa and STb depending upon the thermal stability and other characters. STb was comparatively more stable than STa (Burgess et al., 1978). Extracellular presence of ST had been reported (Olsson, 1982).

(D) Rapid Screening tests:

A large number of objective tests had been developed and proposed for assessing the microbial quality of food (Jay et al., 1979). Standardized rapid dye reduction tests using methylene blue thiocyanate indicator, resazurin and tetrazolium dyes had been in practice in the dairy industry (Foster et al., 1958, Indian Standards No.1479 - Part I - 1960; Cruickshank et al., 1975; Indian Standard No. 1479 - Part III - 1977; Cousins, 1982). The method was adoptable to the examination of a large number of samples in a comparatively short time with minimum equipment and materials and had been used widely for the grading of raw milk (Foster et al., 1958; Indian Standards No.1479 - Part II - 1960).

A method for detecting the proteolysis in refrigerated raw milk on storage upto three days by interpreting the tyrosine value in relation to bacterial numbers was evolved.(Juffs, 1973). This was found suitable only to store raw refrigerated bulk milk. Benzalkon-crystal violet ATP method comprising rapid determination of the bacterial ATP could after addition of benzalkon and crystal violet to the pasteurized milk was developed but yet to be standardized (Waes and Bossuyt, 1982).

The measurement of electrical impedence using Bactrometer 32 offered a definite time advantage approximately 7 versus 48 hours for the standard plate count when examining raw milk samples. This test involved a procedure in which microorganisms growing in liquid medium produced electrical resistance of the solution (Gnan and Luedecke, 1982).

(i) Limulus amoebolysate test (LAL)

The amoebocyte was the only blood cell of Limulus polyphemus (horse shoe crab) and the clottable blood protein came from this amoebocyte. The LAL developed from an observation that a vibrio caused infection resulted in clotting of Limulus blood (Levin and Bang, 1964). In the presence of endotoxins of Gram negative bacterial (GNB) cells, the lysate gelled and gave the appearence of a positive coagulase tube test (Jay, 1977, Culbertson and Osburn, 1980).

(a) Chemical composition and biological effects of endotoxin:

Lipopolysaccharide (LPS) molecule attached to the outer wall of the GNB and once thought to be released only upon their death was now known to be a product of normal growth. The biological effect of endotoxin would

have pyrogenic effect, haemodynamic effect, disseminated intra vascular coagulation, effect on complement system, induction of generalized Shwartzman phenomenon, granulocytopaenia and effect on immune, nervous and hepatic systems (Culbertson and Osburn, 1980).

(b) Application:

The most widely used though somewhat controversial assay was LAL test (Culbertson and Osburn, 1980). The Limulus amoebolysate endotoxin assay had been used in assessing microbial quality of ground beef as a rapid sensitive indicator system (Jay and Margitic, 1979; Jay et al., 1979; Jay, 1981).

Limulus amoebolysate test, Easicult method and direct sampling method for surveillence of operating nebulizers were studied. The LAL and Easicult method were sensitive enough to detect 10^3 colony forming units (cfu) per mL. The LAL detected the endotoxin content of GNB but not the viable bacteria. The Limulus test was considered as a valuable procedure in clinical microbiology for it could detect moderate to heavy microbial contamination within one hour of testing(Reinhart et al., 1981). In screening urine for GNB, LAL test had been applied (Nachum and Shambrom, 1981).

The Limulus in vitro endotoxin assay was evaluated as a possible method for the prompt detection and measurement of endotoxins of GNB in milk (Luzio and Friedman, 1973; Hartman et al., 1976). The endotoxin was resistant to pasteurization temperature (Luzio and Friedmann, 1973).

(c) Sensitivity of the test:

The test detected 0.001 µg of E.coli endotoxin in 0.1 ml solution. Commercial milk samples contained 30-130 µg/ml with a 16 fold increase in samples held at 24 hours at room temperature (Luzio and Friedmann, 1973).

As the LAL test was described a simple rapid and reliable method for assessment of endotoxins of GNB. This method was preferred among other rapid screening tests which measured both GNB and other microbes. This test required one hour for assessing the quality (Hartman et al., 1976; Jay et al., 1979). At concentration of 1×10^5 to 2×10^5 of E.coli, Klebisiella sp. and E.cloacae this test required only 10 - 15 minutes for assay (Nachum and Shambrom, 1981).

(E) Detection and Identification of coliforms:

(i) Standard conventional system:

The procedure for collection of samples for microbiological analysis (Indian Standards No.1479 -

Part I - 1960, Indian Standards No.5404 - 1969, ICMSF - 1974) and the collection of samples on random and non-random basis had been suggested (Harrigan and McCance, 1976). The swab method and rinse method of collection of material from dairy utensils for bacteriological examination and the standards for the grading had been documented (Indian Standards No.5253 - 1969).

Detection and enumeration of coliforms in the milk could be done by employing presumptive test (Multitube technique) in liquid medium, presumptive test in solid medium (colony counts) and complete test in solid medium (Indian Standards No.5402 - 1969; Indian Standards 1479 - Part III - 1962). Statistical methods for selection picking and examination of colonies from plate culture, of a pour plate method had been narrated (Harrison, 1938; Harigan and McCance, 1976). Procedure for enumeration, isolation and identification of Escherichia coli responsible for food poisoning had been described (Indian standards No.5887 - 1976).

The conventional procedure for differentiation of all the members of Enterobacteriaceae with diagnostic key or tables had been in existence (Edwards and Ewing, 1972; Buchanan et al., 1974; Cowan, 1974; Harrigan and McCance, 1976). These conventional methods of identification of

bacteria consumed minimum of 96 hours and as such, time consuming and laborius. It took a lot of time for the preparation of glassware, media and washing after the test. Hence the food and clinical microbiologists did not prefer, as the results were not available before processing or sale of milk and its products (Swanson and Collins, 1980; Fung and Cox, 1981).

(ii) Rapid tests for Identification of the Isolates:

(a) Diagnostic Kits:

Industrious microbiologists, always interested in finding ways to quickly identify bacterial isolates from clinical food and environmental samples, had developed miniaturized microbiological techniques and diagnostic kits in the last decade to meet this need (Cruickshank et al., 1975; Fung and Cox, 1981; Mac Faddin, 1980). Diagnostic kits evolved for clinical isolates had been currently used for food isolates also. Many systems of diagnostic kits are commercially available which include API 20E, Enterotube, Micro ID, Entero-set, Minitek and R-B systems (Cruickshank et al., 1975; Fung and Cox, 1981)

Conventional test results were correlated and it was observed that food isolates tested with API 20E, Enterotube and Minitek systems correlated at 99% (Poelma et al., 1977).

In the accuracy aspect the Conventional Association of Official Analytical Chemists' procedure gave the accuracy (96.1%) in identifying 440 cultures, followed by Minitek (91.6%), Enterotube 89.8%, API 20E (89.5%) and pathotec 84.8% (Poelma et al., 1978).

In a study on isolates from meat using API 20E and R-B systems the API 20E and R-B system correlated at 82% and 72% to 80% respectively with conventional system. A study could be completed in four hours with Micro ID system (Cox and Mercuri, 1978; Mercuri and Cox, 1979; Cox and Mercuri, 1979).

Beneficial use of API 20E system to identify clinical isolates of Enterobacteriaceae had also been made (Swanson and Collins, 1980). It had been reported that no shortened mini identification scheme could ever be as accurate as conventional system. However with all this lack in accuracy the food microbiologists continue to seek rapid methods to identify isolates from food for its easiness, simplicity and low cost as it is 4-5 times cheaper than conventional methods and as the quality and safety of food must be determined as quickly as possible before the ingredients and foods are released for processing or sale (Fung and Cox, 1981).

(b) Other tests:

An automated rapid test using auto analyzer II system for Escherichia coli in milk by measuring the presence of glutamate decarboxylase was studied. This system was capable of detecting as few as 50,000 E.coli organisms per mℓ of milk (Moran and Witter, 1976).

3. Role of coliforms as a milk borne Enteric pathogen:

New socio-economic and political demands for conservation of energy, water and food products coupled with changes in life style including more meals eaten away from home would require new approaches to milk food handling. These modifications might increase or uncover new hazards and potential opportunities for food borne health hazards. Microbial hazards will remain a major problem in the food protection for the 80's (Busta, 1979). A number of recent advances and a recognition of enteric pathogens and their mechanisms of virulence had revolutionized enteric microbiology in the last decade (Richard et al., 1981). In the public health significance of milk borne infections and intoxications, Staphylococcus, Clostridium, E.coli, B.cereus, Streptococcus and Aspergillus were incriminated (Batish et al., 1981a,b).

Milk and milk products like ice cream, kulfi, dried milk and cheese could be contaminated with toxigenic strains of Escherichia coli which elaborate the enterotoxins under favourable conditions. Contaminations of milk and its products with E.coli serotypes capable of producing infections and enterotoxins could take place at any stage from production through other stages of processing (Ranganathan, 1973; Batish et al., 1981a). Ingestion of microbial toxins already synthesized in the milk or milk products (i.e. preformed) bring about diarrhoeal syndromes in the consumers. This was called "intoxication". The toxins affecting gastro intestinal tract were known as enterotoxins. On the other hand ingestion of pathogenic microorganisms along with milk foods leads to their lodgement and establishment in the consumer's organ. This was termed as "infection". There are yet other organisms which infest intestine when ingested along with the milk foods, produce toxin in situ to bring about symptoms of poisoning. This was called as "toxi-infection" (Batish et al., 1981a)

(A) Escherichia coli:

Gastrointestinal illness caused by E.coli is the diarrhoeal disease which may result from ingestion of large numbers (10^6-10^9) of enteropathogenic Escherichia coli. Because of the greater likelihood of poor sanitation,

this illness is more common in the developing nations than in developed countries. E.coli had been responsible for the death of young children and the discomfort of many vacationers. Sporadic diarrhoeal outbreaks in nurseries, a severe cholera-like syndrome clinically indistinguishable from that caused by Vibrio cholerae and a shigella-like illness were reported. These problems might sometimes result from consumption of food or water containing large numbers of these bacteria (Sack, 1980; Sack, 1981). Enteric diseases caused by enteropathogenic E.coli could be classified into four major categories.

Classical Enteropathogenic E.coli (EPEC), commonly referred to serogroups of E.coli, historically were associated with outbreaks of diarrhoea in young children and infants. Facultatively Enteropathogenic E.coli (FEEC) were non-EPEC serogroups associated with sporadic diarrhoea, and included many serogroups associated with the normal intestinal flora. Enteroinvasive E.coli (EIEC) exerted their pathogenic effect through an invasive shigella like infection of the gastrointestinal tract, characterised by fever, diarrhoea and dysentry with toxaemia. Enterotoxigenic E.coli (ETEC) was commonly isolated from outbreaks of traveller's diarrhoea, infantile diarrhoea and sporadic nursery outbreaks and included those strains which produced a heat-stable enterotoxin (ST) only or a heat-labile

enterotoxin (LT) only and/or those producing both ST and LT. These organisms adhered and colonized the epithelial cell surface of the proximal small intestine. This colonization was mediated by specific types of fimbriae which are host-specific. Toxigenicity was plasmid-related (Sack, 1981; Gaastra and Graaf, 1982; Kornacki and Marth, 1982b).

(i) Enteropathogenic E.coli (EPEC)

Classical enteropathogenic E.coli (EPEC) commonly referred to serogroups of E.coli, historically, were associated with outbreak of diarrhoea in young children and infants (Evans Jr. et al., 1979). These serogroups included O:20, O:26, O:55, O:46, O:55, O:86, O:111, O:114, O:119, O:125, O:126, O:127, O:128, O:142, O:158 and O:159 (Orskov and Orskov, 1977; Evans Jr. et al., 1979; Mehlman and Romero, 1982). Several serotypes that were particularly common among enterotoxin producing strains were identified. They were O:128 and O:129 (Evans Jr. et al., 1979). Since infantile diarrhoea was a leading cause of death in children under five years of age in developing countries, it appeared possible that these organisms might contribute significantly to this mortality (Sack, 1980; Kornacki and Marth, 1982b).

The mechanism of action of EPEC was presumed to be of ETEC type, and Shigella toxin type. However, secretory type of response was commonly observed (Gangarosa, 1978; Sank, 1978).

(ii) Facultatively enteropathogenic E.coli (FEEC)

FEEC are non-EPEC serogroups, associated with sporadic diarrhoea, and included many serogroups associated with the normal intestinal flora (Evans Jr. et al., 1979). It was proposed that E.coli serogroups O:1, O:2, O:4, O:6, O:7, O:15, O:19, O:21, O:25, O:27, O:51, O:73, O:75, O:78, O:83, O:85 and O:117 be classified as FEEC when they were associated with diarrhoea or enteritis and exhibited a haemagglutination pattern which was referred to as type III (Evans Jr. et al., 1979).

(iii) Enteroinvasive E.coli (EIEC)

The serogroups of EIEC included O:28, O:112, O:115, O:12 O:136, O:143, O:144, O:147, O:152 and O:164 (Evans Jr. et al., 1979; Tullock et al., 1973). The invasion into colonic epithelium and multiplication within the mucosa were two virulence attributes of this type.

(iv) Enterotoxigenic E.coli (ETEC)

ETEC included those strains which produced either ST enterotoxin alone or LT enterotoxin alone or both ST and LT

(Evans Jr. et al., 1979; Raskova and Raska, 1980).

These strains were characterised by their ability to proliferate in the anterior part of the small intestine and by the production of one or both of two types of enterotoxins (Smith and Halls, 1967a,b., Raskova and Raska, 1980; Gaastra and Graaf, 1982). Colonization of mucosal epithelium was mediated by specific adhesions with which the cells could resist the flushing action of the peristalsis of the gut (Gaastra and Graaf, 1982).

The sero group of ETEC strains, mostly from sporadic cases, were O:6-H:16; O:8-H:9; O:15-H:11; O:25-H:42; O:78-H:11, O:78-H:12; O:128-H:7; O:149-H:10; O:159-H:4 (Orskov and Orskov, 1977, Sack, 1978). The other ETEC serotypes reported with colonizing factor antigen/I (CFA/I) and CFA/II were O:63, O:114, O:153, O:80, O:85 and O:115 (Craviato et al., 1982, Gaastra and Graaf, 1982).

(B) Other coliforms of importance:

Non-E.coli coliforms also appeared to be capable of colonizing the human gut and producing potent enterotoxins of high yield (Twedt and Boutin, 1979). During the last few years strains of Klebsiella, Enterobacter and Citrobacter of which some were shown to be enterotoxigenic had been isolated from stools or the intestinal tract of

children and adults in several epidemilogical studies of acute and chronic diarrhoeal diseases (Klipstein et al., 1973; Klipstein and Engert, 1975; Twedt and Boutin, 1979; Back et al., 1980; Sadruddin et al., 1981). The heat stable toxins produced by these strains were purified and the properties studied (Klipstein and Engert, 1976a,b).

It was postulated that a family of enterotoxins might exist among the various members of Enterobacteriaceae (Twedt and Boutin, 1979). Recent findings had suggested that plasmids encoding for enterotoxin might spread between related species. In fact, the intergeneric transfer of plasmids among members of Enterobacteriaceae was one of the reasons for the difficulties encountered when studying the genetic relatedness of the family (Sanderson, 1976). The non E.coli bacteria were generally more labile enterotoxin producers, than E.coli strains (Back et al., 1980).

In a study, the enterotoxigenecity of 12 strains of coliforms comprising Enterobacter cloacae, Klebsiella pneumoniae and E.coli, isolated from the gastro intestinal tract of persons with diarrhoea, were compared with that of the 13 strains of coliforms from urine culture (Klipstein et al., 1977). The heat stable toxins from these isolates were studied in rat jejunal perfusion model. All 12 gastro intestinal strains and only 6 of the 13 urinary

strains elaborated one or both forms of enterotoxin. The toxin was weak from the isolates of urine, but had a minimal effective concentration of toxin as low as 0.1 to 10 ng/ml from the gastro-intestinal isolates (Klipstein et al., 1977)

The heat stable enterotoxins produced by Klebsiella pneumoniae and Enterobacter cloacae had been assayed in rabbit ileal loop for fluid secretion (Klipstein et al. 1973, Klipstein and Schenk, 1975). The toxins produced by these strains were reported to be heat stable at 100°C for 30 minutes and of low molecular weight and hence they were not antigenic (Klipstein and Engert, 1976a,b).

In a comparative study, it was indicated that the close immunological relationship of cholera toxin and E.coli heat-labile toxin existed with the heat labile toxins of Klebsiella and E.cloacae and to a lesser extent to the heat stable toxins of E.coli and Klebsiella pneumoniae. This similarity between enterotoxins of E.coli and other coliforms supported the hypothesis that these toxins were mediated by plasmids transmissible between species and genera resident in the human intestine (Twedt and Boutin, 1979). In an epidemiological investigation of recent nursery outbreak of diarrhoeal disease, nine different serotypes of three different

species of enterotoxigenic organisms viz. E.coli Klebsiella and Citrobacter were identified (Guerrant et al., 1976). The authors opined that the outbreak could have been related to the intergeneric spread of toxigenicity by a plasmid (Guerrant et al., 1976).

Food, medical and public health microbiologists should be aware that non E.coli coliforms might also develop pathogenecity as a result of acquiring plasmids while being maintained in the environment or in the human host. Strains that were routinely dismissed on taxonomic grounds during microbiological examination of suspect food, might actually pose a potential public health hazard. Recognition of these organisms should therefore rely on tests for enteropathogenic capabilities (Twedt and Boutin, 1979).

(C) Historical food and water borne outbreaks:

During the last 95 years E.coli had been considered to be a potential pathogen for men and domestic animals (Mehlman et al., 1976). Infections caused by this bacterium had been studied since the early 1900s when a relationship between E.coli and white scours in calves was noted (Gangarosa, 1978). In the 1920s and 1930s studies suggested that strains of E.coli caused diarrhoea

among infants (Gangarosa, 1978). In the 1940s, it was demonstrated that these strains were of global importance in producing serious outbreaks of diarrhoeal disease in nurseries. At this time mortality rates approached 50% (Mehlman et al., 1976). Since then the epidemic potential of enteropathogenic E.coli had diminished notably in the developed nations because of improved sanitation, efficient management of nurseries, increased awareness of the potential illness, wiser use of antibiotics and administration of fluids (Mehlman et al., 1976). Since a large inoculum of enteropathogenic Escherichia coli (10^6 - 10^9) was required, E.coli diarrhoea like cholera, required a very insanitary environment for transmission of the illness. This was attributed as a reason why enteropathogenic E.coli was still an important cause of diarrhoea in developing nations (Gangarosa, 1978).

The first recognized outbreak of gastroenteritis caused by E.coli in adults from the western hemisphere occurred at a conference center near Washington DC in 1967, and was traced to a contaminated non-chlorinated well system. The wells providing water to the conference center were improperly constructed. The water of the wells had high coliform counts on several occasions and E.coli O:111-B:4, a known pathogen, was isolated from it

and from the stools of some of those who had been ill as well as a few of those who had not become ill but had been present at the centre during the diarrhoeal episodes. While it was not concluded that E.coli O:111-B:4 caused the illness, it seemed likely that it did it (Schoreder et al., 1968).

The first well-documented outbreak of dairy foodborne illness due to imported cheese in the United States occurred in 1971, and involved atleast 387 persons in 107 episodes was E.coli. E.coli O:124 and O:125 were isolated from faecal specimens obtained from patients involved in the outbreak. Epidemiologically this outbreak was traced back to the cheese plant and E.coli serotypes O:124 and O:125 were isolated. Several of these samples contained Enterobacter hafniae strains which cross reacted with E.coli O:112-B:11 antisera. Counts revealed 10^5 to 10^7 E.coli O:124 per gram of cheese. Counts on cheese in the ripening process showed 10^2 and 10^3 coliforms per gram. E.coli O:124 isolated from the patients and cheese, were shown to be invasive and not enterotoxigenic (Marrier et al., 1973).

Between 1969 and 1972 E.coli accounted for less than two percent of the outbreaks of foodborne illness in the

United States and less than six percent of total cases of bacterial gastroenteritis (Mehlman *et al.*, 1976). In 1971 when the afore mentioned outbreak involving soft ripened cheese occurred, *E.coli* ranked sixth among recognized etiological agents of bacterial gastro-enteritis (Kornacki and Marth, 1982b).

In another waterborne outbreak *E.coli* O:6-H:16 was isolated from the residents and from their water supply. This represented about four percent of the total outbreaks of water borne illness for that year and about nine percent of the total cases. In 1976 a number of people developed a diarrhoeal illness during a four day voyage on a cruise-ship. Nonmotile ETEC serotype O:25 producing LT enterotoxin was isolated from the ailing people. This was the first reported outbreak caused by *E.coli* producing only LT enterotoxin (Anon, 1976). Several unidentified etiological factors were still there in 60% of food borne illness, declaring them as "etiology unknown" (Busta, 1979).

(D) Incidence of Enteropathogenic coliforms and serotypes of *E.coli*

In the last decade increasing attention had been focussed on the role of enteric bacteria other than

Salmonellae and Shigella in diarrhoeal diseases. Enterotoxin producing E.coli had been implicated in many outbreaks. An increasing number of reports in recent years had also implicated enterotoxigenic Klebsiella, Enterobacter and Citrobacter (Sadruddin et al., 1981).

The majority of E.coli serotypes from foods were confined to O:6, O:8, O:15, O:78, O:124, O:125 and O:149 serogroups (Orskov et al., 1976; Marrier et al., 1976). Other serogroups as O:11, O:55, O:26, O:126, O:128 had also been reported in a different geographical location. (Ranganathan, 1973; Singh and Ranganathan, 1974).

This indicated that apparently wherever E.coli was found, enteropathogenic strains may also be present, but the proportion of non-pathogenic to enteropathogenic strains seemed to vary considerably in any given environment, whether one considers the environment or the type of food involved (Kornacki and Marth, 1982b).

(E) Established sources of contamination:

Water had been incriminated as a probable source of contamination in the first historical outbreak of E.coli diarrhoeal illness (Schorder et al., 1968). The possible role of personnel in contaminating the food was established

by examining the stools of 219 food handlers. About 6% of them carried EPEC strains (Hall et al., 1967). With the sanitary environment, marked reduction of the E.coli enteric diseases were reported due to improvement in sanitation in developed countries (Mehlmen et al., 1976; Gangarosa, 1978; Sack, 1980).

(F) Incidence of EPEC and coliforms in Dairy Products:

(i) An important epidemiological study:

The isolation of E.coli O:124 and O:125 from an outbreak and consequential conclusive epidemiological study tracing to a contaminated cheese in another continent had been narrated (Marrier et al., 1973).

(ii) Incidence in Milk:

Ten EPEC strains were reported in raw milk. E.coli virulent for mice were isolated from 45 out of 128 samples of raw milk. Majority of these strains belonged to O:55-B:5 and O:26-B:16 (Ranganathan, 1973; Singh and Ranganathan, 1974). A survey of five raw milk samples showed the presence of classical EPEC serotypes (Glatz and Brudvig, 1980a,b). Nine samples of pasteurized milk contained five mice virulent serotypes O:26-B:6, O:55-B:5, O:11-B:4, O:126-B:16 and O:128-B:12 (Ranganathan, 1973; Singh and Ranganathan, 1974).

In the study of role of milk and milk products in the etiology of diarrhoea in infants, it was pointed that out of 11 isolates of E.coli, from milk food 10 were common to the isolates in stool of infants (Misra and Rao, 1975). A report of enterotoxin production in milk by known ETEC cultures was made (Glatz and Brudvig, 1980a), wherein production of heat labile toxin by one of the three strains tested, at a pH above 6.5, was observed.

(iii) Incidence in Milk products:

Prevalence of EPEC and coliforms in milk products like ice cream, kulfi, cream, butter, cheese, yoghurt and butter milk were extensively reported Goel et al., 197 ; Ranganathan, 1973; Marrier et al., 1973; Singh and Ranganathan, 1974; Mehlman et al., 1976, Glatz and Brudvig, 1980b). The presence of these organisms in pasteurized products were incriminated to the failure in the sanitary manufacturing procedures or inadequate pasteurization or post pasteurization contamination (Kornacki and Marth, 1982b). The majority of serotypes of E.coli encountered from food borne diseases were 0:8, 0:15, 0:78, 0:124, 0:125 and 0:149 though there were other serotypes in existence (Orskov and Orskov, 1977). The serotypes of 0:20 and 0:44 had been reported as ETEC from cheese in India (Panhotra et al., 1981)

(iv) Survival of coliforms in milk products:

Long storage period, low temperature, low hydrogen - ion concentration had been reported to have a deleterious effect on the survival of coliforms in milk. The storage period over 24 hours at 19°C in fermented milk products reduced the E.coli and E.aerogenes count, with E.coli surviving longer than E.aerogenes (Goel et al., 1971; Prasad et al., 1980). Growth of E.coli was best controlled by the lower incubation temperature and higher salt concentration (Frank and Marth, 1978).

The factors which influenced the survival of coliforms in cheese had been extensively studied. They included temperature of storage, initial level of coliforms in milk and cheese, length of storage, moisture content, amount and type of starter culture, strain difference, hydrogen ion-concentration due to complete or partial failure of acid development, salting, washing, presence of antibiotics in cheese, location in cheese and type of cheese (Fantasia et al., 1975; Frank et al., 1978; Mikolajcik, 1980; Kornacki and Marth, 1982a,b).

4. Enterotoxigenic Coliforms:

As, essentially all enteric pathogens are proven or presumed to be acquired by the faecal-oral route, they could be considered to be potentially transmissible via contaminated food or water (Richard et al., 1981). Food microbiologists recently added the names of Yersinia, Campylobacter and enteropathogenic E.coli (EPEC) to their vocabulary (Foster, 1982). Research needs exist to recognize and control new unusual food-borne disease agents before they manifest themselves by massive and disastrous outbreaks of disease (Foster, 1982).

The global impact of the enterotoxigenic E.coli (ETEC) is huge. ETEC had been identified as the most common enteric pathogen infecting travellers recently arriving in developing countries and also in paediatric diarrhoea throughout the world (Shore et al., 1974; Sack et al., 1975; Echeverria et al., 1978b; Echeverria et al., 1979; Sack, 1980; Sack, 1981; Richard et al., 1981; Kornacki and Marth, 1982b).

ETEC was also recognized to cause cholera like syndrome in the native adults, living in the cholera endemic areas, an occasional cause of nursery outbreaks of diarrhoea in developed countries and an occasional

cause of common source of outbreaks of diarrhoea secondary to faecal contamination of water and food (Sack, 1980).

Several coliform species other than Escherichia coli were often associated and possibly responsible for acute and chronic diarrhoeal illness (Twedt and Boutin, 1979). Among Puerto Ricans with untreated tropical sprue, these bacteria consisted principally of Klebsiella pneumoniae and less commonly of Enterobacter cloacae or Escherichia coli (Klipstein and Engert, 1975). Enterotoxins had been demonstrated in still other enteric organisms viz. Enterobactor, Klebsiella, Citrobacter and Proteus that had not been proven to produce diarrhoeal disease (Sack, 1980). They had been reported from a new born special care unit out-break (Guerrant et al., 1976) and cruiseship outbreak (Wachsmuth et al., 1979).

(A) Enterotoxins of E.coli:

(i) Characteristics of heat labile toxin (LT)

The heat labile enterotoxin (LT) is antigenically related to the enterotoxin produced by Vibrio cholerae (Choleragen) and is nondialyzable. The molecular weight of LT is placed between 95,000 to 96,000 daltons. It is destroyed at 60°C when held for 30 minutes. LT enterotoxin

induces fluid secretion in the small intestine. The LT is antigenic (Mahlman et al., 1976; Burgess et al., 1978; Clements and Finkelstein, 1979). The secretory response of intestinal mucosa to LT is delayed in onset and of long duration, whereas, the intestinal response to stable toxin is quick in onset and of short duration (Sack, 1980).

(ii) Heat Stable toxin (ST)

The heat-stable enterotoxin (ST) produced by some ETEC strains had been found to be heat stable and dialyzable having a molecular weight between 1,000 - 10,000 daltons (Klipstein et al., 1976; Burgess et al., 1978; Clements and Finkelstein, 1979). The antigenecity of ST had been recentl established (Klipstein et al., 1983a). Human E.coli ST was synthetically produced and found to have all the biological properties of the ST produced by the bacterial growth. The synthetic toxin was found to be antigenic (Klipstein et al., 1983b).

(a) Classification of ST and its characters:

The ST was classified as STa and STb in a porcine strain (Burgess et al., 1978). STa is soluble in methanol with lower thermal stability, active in the infant mouse

and neonatal piglets, whilst STb is insoluble in methanol with higher thermal stability and active in the ligated loops of weaned pig and rabbit (Burgess et al., 1978). Thermal stability of 80°C for 20 minutes had been reported with a pig strain (Olsson, 1982). Heat stability at 100°C for 30 minutes had been reported with E.coli, K.pneumoniae and E.cloacae heat stable toxins (Klipstein and Engert, 1975; Klipstein and Engert, 1976). In human beings and pigs, the proportion of strains producing STb were more than STa (Robichaud et al., 1978). Stable toxin had been characterized by rapid onset and short duration (Whipp et al., 1975). Recent studies combining ST and LT had showed that the ST might also be antigenic and vaccine could be developed to protect against either toxin. This immunogen had protected rats against challenge (Gianella et al., 1981; Klipstein et al., 1983a,b).

The mode of action of ST had been known only recently. It was demonstrated that ST, purified to homogeneity stimulated a marked increase in cyclic guanosine 5'-monophosphate (GMP) concentration in the small intestine, and that this increase preceded fluid secretion. Furthermore they showed that when the animals (Suckling mice) were given cyclic 8-bromo-GMP

or cyclic B-bromo-adenosine 3', 5' monophosphate, fluid secretion mimicked that induced by ST (Gianella and Drake, 1979). After intragastric injection of ST into the mice, there was a marked increase in cyclic GMP after three minutes and this level peaked to 10 times greater than the control level. After this point it fell but remained above the control level at 120 minutes later. Fluid secretion was induced 13 to 39 minutes after injection. The increase in cyclic GMP before fluid secretion was consistent with the hypothesis that ST induced cyclic GMP production which mediated the secretary response (Gianella, 1978).

The minimal dose of ST required to give a positive response in suckling mice was 2.5 ng and in rabbits it was 10 mgm (Levine et al., 1980). Staples et al.(1980) described a highly purified ST produced by a strain of E.coli from human origin. The toxin was active in suckling mice at doses as low as 2.7 ng and contained 18 amino acid residues. The enterotoxin had been reported to occur intracellularly and extracellularly (Smith and Halls, 1967b and Olsson, 1982).

STa and STb activity in bioassay raised some doubt as to the usefulness of the infant mouse assay for ST toxin since it was only sensitive to STa.

However, it was suggested that in future it be used in conjunction with the rabbit ligated loop assay to determine if both STa and STb are present (Burgess et al., 1978). Recently it was demonstrated that STa can be fractionated into two distinct moieties (Burgess et al., 1980). There might be some human health significance to these findings since it had been reported that partially purified ST from E.coli H 10407 (a human strain of ETEC) could be separated into two distinct moieties by ion exchange chromatography (Starvic et al., 1978). Both components were active in infant mice, and appeared to have different molecular weights. Hence, they might correspond to the subfractions of STa since they are both activein infant mice (Kornacki and Marth, 1982b).

(B) Typical serotypes of ETEC:

The serotypes that are particularly common among enterotoxigenic strains, regardless of geographical location are O:6-H:16, O:8-H:9, O:15-H:11, O:25-H:42, O:26, O:78-H:11, 12, O:128-H:7, O:149-H:10 and O:159-H:4, 34 (Orskov et al., 1976)

(C) Disease manifestation in human:

One of the most common and perhaps the most versatile enteric pathogen would be *E.coli* (Richard *et al.*, 1981)

It was reported that three clinical syndromes could be induced by ETEC. The most severe of these was a cholera-like syndrome which was clinically indistinguishable from that caused by *V.cholerae*. This syndrome could be fatal although the mortality rate was ill-defined (Richard *et al.*, 1981). It was commonly seen in geographic locations where cholera was endemic (Sack, 1978). Secondly ETEC was the most common cause of traveller's diarrhoea. This illness was relatively mild. It had been suggested that traveller's from more developed countries were relatively non-immune and developed the illness with greater frequency (30% - 60%) within the first three weeks while visiting developing countries. It was not known why this disease was relatively mild considering the severity of the cholera-like illness (Sack, 1978). The third syndrome was infantile diarrhoea, also more common in developing than in developed countries and a leading cause of mortality among children less than five years of age. ETEC might be a significant contributor to this problem (Sack, 1978).

In the most severe form, volunteers experienced severe diarrhoea, with the passage of 5 to 10 watery stools per day, lasting upto 19 days. Toxigenic E.coli was recovered in large numbers from jejunal and ileal contents as well as from the stools. Travel history, watery diarrhoea, nausea and vomition would be the history with ETEC diarrhoea. The illness lasted 3 to 19 days (Richard et al., 1981). The mean incubation period was 26 hours and ranged from 8 to 44 hours after ingestion. A four-fold increase in serum antibody was detected in 5 out of 7 men studied with the illness. To induce diarrhoea with these two strains of ETEC, it was necessary for volunteers to ingest from 10^8 to 10^{10} organisms (Dupont et al., 1971). The inoculation necessary to initiate illness might be dependent upon existing conditions in the stomach at the time of ingestion (Dupont et al., 1971). This level (10^8 - 10^{10}) of ETEC necessary to initiate diarrhoeal illness was generally accepted, although smaller numbers of ETEC might be equally effective with freshly isolated strains (Mehlman et al., 1976).

(D) Host Specificity and Immunity:

ETEC appeared to be host-specific. When ETEC strain 263 which caused diarrhoea in piglets, was fed to human

volunteers at levels of 10^6, 10^8 and 10^{10} cells, only one out of 4 men ingesting 10^{10} cells became mildly ill. Although it was isolated from stools (10^8 per g) and was present in the ileum (1 x 10^6 cells per ml of intestinal fluid), it could not be found in jejunal samples. It was suggested that this might have resulted from failure of the organisms to multiply in the small bowel or from species insensitive to "Swine" enterotoxin (Dupont et al. 1971). However, when nonpathogenic E.coli K 12 was made the recipient of a plasmid coding for production of toxin and fed to animals, it was not consistently pathogenic in all hosts although toxin was produced as demonstrated by in vitro tests. When another plasmid coding for production of an antigen involved in the adhesion of these cells to the small intestine was inserted into these toxin production cells, the ability to produce diarrhoea was established (Mehlman et al., 1976). Contrary opinion of lack of strict host specificity also prevailed. The presence of K 99, K 88ab and K 88ac antigens in the E.coli isolates from both cow calf and human sources was an indication of lack of strict host specificity (Kumar et al., 1982).

The antigenicity of ST had recently been established (Gianella et al., 1981; Klipstein et al., 1983a,b). A complete understanding of the organisms in its immunological

perspective might eventually lead to the development of an effective vaccine of major value in diminishing the diarrhoeal problems (Sack, 1981).

(E) Correlation of Serotype and Toxigenicity:

The relationship between serotype and enterotoxin production in 109 ETEC strains isolated from 109 patients with severe cholera-like diarrhoea in Dacca, Bangladesh, was studied by Merson et al.(1979). They demonstrated that atleast in one geographical setting, E.coli strains producing both heat-labile and heat-stable toxins were more restricted in their O groups and O:K:H. serotypes than was E.coli that produced only heat-stable toxin. There were 69 strains which produced both toxins; of these 59 (86%) belonged to one of four serogroups (O:6, O:8, O:78, O:115) and 56 (81%) of these strains belonged to one of six O:K:H serotypes. However, the 34 strains which produced ST only, were distributed among fifteen O groups. Only eight (24%) of these strains were found in the four major LT-ST O groups. On a similar study on E.coli strains, 33% possessed O:6, O:8 or O:78; 93% of these were stable producers of LT and 86% produced both LT and ST. O:78 strains possessed CFA/I whereas O:6 and O:8 possessed CFA/II. The two most commonly isolated serogroups which produced ST only were O:78 and O:128

(Bark et al., 1980). Other serogroups had also been reported. In Japan in 38 outbreaks during a period of a decade, occurrence of O:6-H:16 (LT, ST) O:11-H:27 (ST) O:159-H:20 (ST) were reported (Mehlman and Romero, 1982). In India, O:1, O:11, O:17, O:36, O:38, O:44, O:59 and O:125 were isolated from the diarrhoeal cases of infants (Kumar et al., 1982).

This type of information would be of great importance to develop serological pools for use in slide agglutinatio tests that could be used for rapid simple diagnosis of ETE disease and for epidemiological investigations (Merson et al., 1979).

(F) Adhesins as virulence attributes:

A non flagellated host specific filamentous appendage and its significance was first described by Duguid et al. (1955). These non-flagellated filamentous appendages were called as fimbriae by Duguid et al.(1955) and later were called as pili by Brinton (1965). Now, since these appendages were primarily involved in adhesions these are termed as adhesins (Gaastra and Graaf, 1982). These appendages are thinner and more numerous than flagella and confer adhesive properties and haemagglutinating activity to the bacteria. These fimbriae had been

classified into several types based on their HA property (Duguid et al., 1955, Brinton, 1965).

The adhesive properties and haemagglutination property of type I fimbriae, produced by majority of E.coli, are inhibited by D.mannose and are called mannose sensitive haemagglutination (MSHA). These adhesive properties helped to colonize the epithelial surfaces and therefore contributed to the pathogenecity of EPEC strains (Brinton, 1965, Brinton, 1967, Duguid and Old, 1980).

In contrast to type I fimbriae, another class of E.coli fimbriae seemed to occur exclusively on ETEC. They adhere to the intestinal epithelia of very limited animal species and specifically agglutinate only certain species of erythrocytes. In contrast to the type I fimbriae, the HA activity of these adhesins was not inhibited by D.mannose and are called mannose resistant haemagglutination (MRHA).

These adhesins which occur exclusively on ETEC are surface antigens which conferred adhesive properties to the ETEC to colonize the intestinal epithelium of animals and man. The first surface antigen was recognized in the oedema disease and enteritis of swine in 1961 (Orskov et al., 1961). A similar porcine antigen 987 was also

recognized subsequently. Among calves, a similar antigen K 99 was reported in calf enteropathogenic strain of E.coli (Orskov et al., 1975).

A surface antigen with characteristics similar to K 88 and K 99 antigens of animals was first described in a human ETEC isolate made from a diarrhoeal outbreak in Bangladesh (Evans et al., 1975). This surface antigen was called CFA/I when a second immunologically distinct surface antigen from human ETEC was discovered. The second surface antigen was consequently termed CFA/II (Evans and Evans Jr., 1978). The human factor was also able to colonize rabbit small intestine (Evans and Evans Jr., 1978, Evans Jr. et al., 1979). These structures, were found necessary for the ETEC to express their full pathogenicity (Evans Jr. et al., 1979).

The haemagglutinating typing system for ETEC with CFA/I and CFA/II had been proposed (Evans Jr. et al., 1979). The association of CFA/I and MRHA of human and bovine or human erythrocytes and of CFA/II and MRHA of bovine erythrocytes had been established (Back et al., 1980; Levine et al., 1980; and Deneke et al., 1981).

The location of CFA/I or CFA/II and enterotoxin production on the same plasmid obviously explained the

plasmid borne characters and close association of both the properties (Evans and Evans Jr., 1978; Levine et al. 1980). Controversy had creeped over the prevalence of CFA/I and CFA/II in human ETEC strains and the necessity of the same to manifest virulence and cause intestinal colonization and immunological response (Levine et al., 1980).

One explanation for results of studies which indicated a low prevalence of CFA's had been the loss of CFA plasmids before testing (Orskov et al., 1976; Gross et al., 1978). Alternatively other adherence factors might be present in strains lacking CFA/I or CFA/II (Deneke et al., 1979, Levine et al., 1980). It was also demonstrated that six ETEC strains which caused disease in humans did not cause MRHA of human bovine or guinea pig erythrocytes and lacked CFA/I or CFA/II (Levine et al., 1980).

They concluded that there existed in human ETEC, other adhesion pili not associated with MRHA or other surface structure that served as colonization factors such as polysaccharide, slime layers or lectins. Some other attachment pili of ETEC differed in their HA pattern from those of CFA/I and CFA/II. They were physically and chemically distinct from previously

reported pili types (Deneke et al., 1979). Demonstration of a new adhesive factor antigen (E 8775) in ETEC of serogroup O:167 was recently reported (Thomas et al., 1982)

The existence of colonization factor antigens in specific serotypes of E.coli had been reported. CFA/I was found predominantly on strains of O:78 serogroup and CFA/II was found predominantly on strains of O:6 and O:8 (Evans and Evans Jr., 1978). Other serotypes reported with CFA/I were O:15, O:25 and O:63. Strains belonging to other O serogroups which had been found to possess one of the adhesions occasionally were also reported (Gaastra and Graaf, 1982).

The CFA of human ETEC had not been used to produce antisera that were used for vaccination. Immunity to ETEC was observed in human volunteers who were rechallenged nine weeks after first infection with ETEC (Levine et al., 1979). The pili antiserum against K 88 was found to have no productive effect against ileal loop dilatation. Hence modified method was required to make pili antigen immunogenic (Molendo et al., 1983).

(i) Klebsiella type I pili:

Klebsiella pneumonia type I pili was found to

agglutinate guinea pig red blood cells (Fader et al., 1979). The piliated phase of a urinary tract isolate was found to cause erosion of bladder epithelium and appeared to have invasive type of pathogen&city. With the type I pili the organisms were able to mediate and adhere to the epithelial cells (Fader et al., 1979 Fader and Davis, 1980). However, these authors also felt that the pili were not the sole virulence factors of these organism. Apart from pili, T3 and T1 coliphage receptors of the organisms also found to mediate the adhesions (Pruzzo et al., 1980)

The agglutination of guinea pig red blood cells by type I pili was inhibited by mannose (25 mgm/ml) in PBS. Hence these pili agglutination were mannose sensitive (MSHA). The MSHA, was a tool to detect the presence of type I pili (Fader et al., 1979, Fader and Davis, 1980, Pruzzo et al., 1980)

Intestinal isolate of K.pneumoniae was found to adhere to human intestine cells. However, the adhesion was least compared to buccal epithelial and bladder epithelial cells (Pruzzo et al., 1980)

(G) Plasmids as determinants of virulence:

Toxigenicity is mediated by a plasmid referred to as 'Ent' and is transmissible to other strains of E.coli and other members of the family Enterobacteriaceae also (Brill et al., 1979, Twedt and Boutin, 1979). Toxigenesis and other potentially virulent properties, like colonizatior factor and hemolysin production, are plasmid mediated in strains producing only ST, LT-ST and LT only strains (Wachsmuthu et al., 1979). Previous genetic analysis of ETEC strains producing LT and ST had shown a plasmid location for both toxin determinants (Wachsmuthu et al., 1979).

(H) Virulence and Antibiotic resistance:

Genes which code for antibiotic resistance and enterotoxin production were frequently transferred together (Echeverria et al., 1978a, Echeverria and Murphy, 1980, Echeverria et al., 1981). Resistance to one or more antibiotics was noted for 72% of the isolates; 44% were resistant to four or more antibiotics. ETEC producing LT and ST, LT only and ST only were frequently resistant to multiple antibiotics. When 25 of the 31 resistant isolates were tested in bacterial mating experiments, 80% transferred antibiotic resistance.

In 35% of the matings transferring antibiotic resistance the ability to produce enterotoxin was also conferred on the recipients. Among the antibiotics, tetracycline had no effect on patients who were infected with ST producing ETEC (Merson et al., 1980).

(I) Factors affecting production of Enterotoxin:

A number of environmental conditions affect enterotoxin production by E.coli (Sack et al., 1971; Evans et al., 1973b; Mundell et al., 1975; Greenberg et al., 1977; Lallier et al., 1980). Production of ST and LT is related to constituents of bacterial growth media, pH of media, incubation time or age of the culture agitation of the culture. Temperature and pH of culture filtrate had an effect on stability of LT (Mundell et al., 1975).

(i) Labile Toxin broth:

Several liquid media were compared for their ability to support LT production. Casamino acid yeast extract medium (CYE) was found to be suitable with pH at 8.2 (Mundell et al., 1975).

(ii) Stable Toxin broth:

Optimal ST production was obtained in 3% casamino

acid medium with added glucose (0.2%) and yeast extract (0.06%). The optimal pH for ST production was 7.2 to 7.8. At this level maximum toxin production occurred (Lallier et al., 1980). Milk as a medium for enterotoxin production had been experimentally studied. Toxin production was observed at optimal pH and temperature range (Lovett et al., 1979; Glatz and Brudvig, 1980a)

(iii) Age of the culture:

The age of the culture was an important factor in LT production and ST production (Mundell et al., 1975) and Lallier et al., 1980). The LT production was maximum at 18 hours. Maximal yield of ST was observed after seven hours of incubation under forced aeration and agitation (Lallier et al., 1980)

(iv) Agitation and Aeration of cultures:

Agitation and aeration of the culture had a significant effect on production of both LT and ST toxins (Evans et al., 1973b, Mundell et al., 1975; Greenberg et al., 1977; Klipstein et al., 1979; Lallier et al., 1980). Maximum aeration in stationary culture increased the production of ST and LT (Klipstein et al., 1976)

Reports of production of ST by E.coli F11 (P155) of porcine origin when cultured with agitation (150 rpm) and forced aeration (5ℓ per minutes) in a Casamino acids yeast extract medium containing 0.2% glucose were available (Lallier et al., 1980). Maximum aeration increased the production of LT (Klipstein et al., 1976). A simplified procedure for production of ST was described by Gomes et al.,(1979). Low volume of medium per volume in flat bottomed flasks were used in stationary cultures. In this method 1:10,000 merthiolate was used as a preservative.

(v) Effect of antibiotics:

Presence of certain antibiotics in growth media might affect production or release of LT by ETEC. Mitomycin-C was found to have variable effect on the production of LT (Evans et al., 1973b; Isaacson and Moon, 1975). This variability was attributed to the activation of temperate phage, which released the cell bound LT on lysis of cell (Kornacki and Marth, 1982b). The other antibiotics like Lincomycin 20/mg/ml and Clindamycin (5 µg/ml) in growth media influenced the production of LT (Brill et al., 1979).

(vi) Temperature range for Toxin Production:

The ability of an enterotoxigenic (LT+/ST+) E.coli strain to produce enterotoxins in the range of 15 to 50°C was studied (Lovett et al., 1979). The optimum temperature for toxin production was 35°C and 24 hours of incubation was necessary to detect toxin at this temperature. Departure from this optimum temperature in either direction delayed the appearance of enterotoxins. Also, no enterotoxin production occurred when ETEC strains were grown at 5 to 15°C in beef broth or milk (Lovett et al., 1979).

(vii) Factors affecting Stability of Toxin:

(a) Temperature:

When ETEC (H 10407) culture filtrates were heated at 60°C for five minutes, the activity in the adrenal cell assay was decreased by one-third. Greater than 90% of the steriodogenic activity of the culture filtrates was lost after heating at 60°C for 15 minutes. Culture filtrates from E.coli H 10407 was stored at -70°C for almost three weeks without an appreciable loss of LT

activity as measured by steriodogenesis (Mundell et al., 1975).

(b) Hydrogen ion concentration:

When the pH was adjusted below six there was a marked loss of LT activity (Mundell et al., 1975). An optimum pH of seven for activity of partially purified LT pathogenic for swine, was reported (Jacks et al., 1973).

(viii) Purification:

A simple ammonium sulphate concentration was shown to increase the activity of E.coli enterotoxins approximately 20 fold (Robins Browne et al., 1982). Nalin et al.(1974) had demonstrated that ammonium sulphate treatment might disclose hitherto unrecognized E.coli enterotoxins.

(J) Enterotoxigenic non-Escherichia coli coliforms:

Recent evidence had suggested that non-Escherichia coli coliforms might be capable of colonizing the human intestine and producing enterotoxins in high yield (Twedt and Boutin, 1979). Whether these organisms were

newly capable of causing disease because of infestation with extrachromasomal factors mediating pathogenicity or simply because of inherent pathogenic capabilities that had gone unrecognized, pose a public health hazard (Twedt and Boutin, 1979). The enterotoxigenic material elaborated by K.pneumoniae is heat stable and its molecular weight is less than 10,000 (Klipstein and Engert, 1975a).

Culture supernatants of strains of Enterobacter, Klebsiella, Citrobacter and Proteus had been found in some laboratories to produce enterotoxin like reactions, in tissue culture assays, although they had not been extensively studied in the standard intestinal assays (Sack, 1980). Enterotoxigenic E.coli and other Gram negative bacteria of infantile diarrhoea, were studied in detail with reference to surface antigens, haemagglutinins, colonization factor antigen and loss of enterotoxigenicity. In this study non-E.coli bacteria were found more often than was generally reported in other studies on enterotoxigenic bacteria in diarrhoeal disease. The non-E.coli strains were generally more labile enterotoxin producers than E.coli strains (Back et al., 1980).

5. Current Methods in the Identification of Enterotoxigenic coliforms:

Many techniques currently exist to determine the presence of adhesins in E.coli (Evans et al., 1978; Evans et al., 1979; Evans Jr. et al., 1979; Hadad and Gyles, 1978; Levine et al., 1980) and to determine whether or not enterotoxins are produced by a strain of coliform (De and Chatterjee 1953; Dean et al., 1972; Donta et al., 1974; Evans Jr. and Evans, 1977). The demonstration of adhesins or pili is by haemagglutination test (Evans Jr. et al., 1979), serological test (Guinee et al., 1976) and electron microscopy (Wadstrom et al., 1978). The traditional methods generally involved the use of test animals (De and Chatterjee, 1953; Dean et al., 1972; Gianella et al., 1976; Nalin et al., 1974; Klipstein et al., 1976). Newer simple methods by employing tissue cultures (Donta et al., 1974; Guerrant et al., 1974) and serological tests (Evans Jr. and Evans, 1977; Greenberg et al., 1977) are available for LT estimation.

While the earlier bioassay methods appeared to be specific and sensitive they were often difficult to do, expensive and results were not quantifiable (Brill et al., 1979). Further more with tissue culture assays viable

cell culture must be maintained and the results were often quite subjective (Brill et al., 1979). The aim of routine testing for LT, required a simpler, more sensitive and more direct method of testing stool specimens (Tenney et al., 1979).

Newer in vitro systems were simple to do and more sensitive than the older bioassay systems for LT estimation (Greenberg et al., 1977; Levine and Rennels, 1978; Tenney et al., 1979; Brill et al., 1979). The staphylococcal co-agglutination technique had potential for routine use in diagnostic laboratory, although it was not as sensitive as the radioimmunoassay (RIA); enzyme linked immunosorbent assay (ELISA) or passive immune haemolysis (PIH) (Kornacki and Marth, 1982b).

In the estimation of ST, the older conventional bio-assay system had not been replaced by the simpler newer, serological tests, as the toxin could not elucidate serum antibodies (Gianella, 1976; Burgess et al., 1978; Moon et al., 1978; Robichaud et al., 1978; Olsson, 1982). Modified bioassay using rabbit model for LT estimation had been a recent study probably because of its consistency (Moon et al., 1970, Burgess et al., 1980). The rabbit ileal loop method was so far the

preferred method for establishing enterotoxic activity of cell free filtrates of various microorganisms (Raskova and Raska, 1980).

(A) Demonstration methods of adhesins:

(i) Haemagglutination:

Colonization of ETEC was mediated by specific type of fimbriae K 88, K 99, CFA/I, CFA/II and a new fimbrial antigenic type (E 8775) common pili. These were antigenically distinct and each of these was haemagglutinin identifiable according to the species of erythrocyte agglutinated (Duguid et al., 1979; Evans Jr et al., 1979; Thomas et al., 1982).

(a) Red blood cells:

Haemagglutination typing pattern for ETEC possessing CFA/I and CFA/II had been proposed (Evans Jr. et al., 1979). CFA/I was found to agglutinate the Human A bovine and chicken red blood cells in MRHA pattern. CFA/II positive strains exhibited only MRHA with bovine and chicken red blood cells (Evans et al., 1979). Mannose sensitive haemagglutination of guinea pig cells were used to demonstrate type I somatic pili. Human A cells for CFA/I

and bovine cells for the CFA/II were only used in later study (Levine et al., 1980).

(b) Media for expression of adhesins:

Colonization factor Antigen (CFA) agar was preferred for expression of pili associated with MRHA while it was unfavourable for type I and somatic pili. Static Mueller Hinton Broth cultures offered optimal growth conditions for expression of type I pili (Evans et al., 1978; Evans Jr. et al., 1979; Levine et al., 1980).

(c) Temperature of the test:

MRHA was carried out at 24°C and 4°C with human and guinea pig cells and at 4°C with bovine cells (Evans et al., 1978; Evans Jr. et al., 1979 and Levine et al., 1980)

(d) Method of the test:

Various methods had been demonstrated in the haemagglutination test. A method using depression tiles and rocking it at 20°C with bacterial suspension and red blood cells was demonstrated (Duguid et al., 1979). Slide agglutination test was also made use of

in the demonstration of haemagglutination (Stirm et al., 1967; Evans et al., 1978; Levine et al., 1980).

Another method of micro haemagglutination test using microtitre plates was evolved by Jones and Rutter (1974).

(ii) Agglutination test:

Agglutination tests with antipili monospecific antiserum after absorption of the other non-specific antibodies had been widely used, to detect the pili antigens (Guinee et al., 1976; Evans et al., 1978). Plate agglutination tests and slide agglutination tests were used to determine this serological reaction (Guinee et al., 1976; Evans et al., 1978).

(iii) Indirect fluorescent antibody technique (IFAT)

The serological test using immunofluorescent procedure for the detection of eneteropathogenic E.coli in soft ripened cheese was attempted with E.coli O:128-B:12 (Yoger and Kershaw, 1974). The authors, however, concluded that in naturally occurring E.coli, this procedure was not successful in cheese samples. Detection of bovine EPEC from dung smears by indirect fluorescent

antibody technique had been standardized (Hadad and Gyles, 1978).

(iv) Electron microscopy:

The pili or adhesins are long and very thin structures having diameters between 3 and 25 nm, and hence electron microscope alone could be used to study this appendage. The pili associated with colonizing factors of human ETEC was studied under electron microscope and for their hydrophobic absorption property. [Wadstrom et al 1978].

(B) Assay of Escherichia coli enterotoxins:

(i) Heat labile enterotoxins:

Originally LT was tested by its capacity to cause fluid accumulation in the small intestine. Later suitable cell culture assays were developed. Since the labile toxin is antigenic and purified products are available serological methods had been introduced (Donta et al., 1974; Guerrant et al., 1974; Evans Jr. and Evans, 1977; Greenberg et al., 1977, Raskova and Raska, 1980).

(a) In-vivo assay:

Ligated intestinal loops:

These methods were derived from the biological assay of cholera toxin, the main criteria being, accumulation of fluids in the ligated loops of intestine. The rabbit model was used by the original authors to develop this system (De and Chatterjee, 1953; Burrows and Musteikis, 1966). Escherichia coli of human and animal origin caused fluid accumulation in the ligated loops. The interval of 18 hours between LT containing filtrate administration and fluid accumulation was found optimal for the assay (Moon et al., 1970, 1971; Evans et al., 1973a., Raskova and Raska, 1980; Richard et al., 1982; Pitarangsi et al., 1982). In the interpretation of results, volume to length ratio of fluid secreted to the length of the loop in RILT ranged from 0.5 to 3.1 mL/cm of gut in the LT assay in 18 hours (Sadruddin et al., 1981).

Vascular permeability assay:

Intradermal injection of filtrates into rabbits, followed by intravenous administration of Evans blue produced permeability changes of rabbit skin in the positive LT assay (Evans Jr. et al., 1973b).

Infant Rabbit assay:

Oral feeding of enterotoxic filtrates to infant rabbits (upto 14 days of age) as in the infant mouse test assay for ST was recommended by Burgess et al.(1978)

(b) In vitro assay:

Tissue culture:

Adrenal cell mono layers (Donta et al., 1974) Chinese Hamster Ovary cell culture (Guerrant et al., 1974), vero cell culture from African Green monkeys (Starvic et al., 1978) had been used in the LT assay and the cytotoxicity studied.

Serological methods:

Passive immune haemolysis (Evans Jr. and Evans, 1977) solid phase radioimmuno assay (Greenberg et al., 1977) and enzyme-linked immunosorbent assay (ELISA) (Yolken et al., 1977) had been elaborated. The passive immune haemolysis test was found unsuitable for LT identification produced by strains of porcine and food origin (Serafim et al., 1979).

Stapylococcal co-agglutination test:

The beneficial utilization of the immunological cross reactivity of the LT and cholerogen had resulted in a simple and cheap test called staphylococcal co-agglutination technique. Protein containing staphylocci (Cowan I strain) sensitized with anticholeragen was agglutinated by heat labile toxin of ETEC (Brill et al., 1979).

(ii) Heat stable toxin:

(a) Bioassay:

Originally ST was discovered using the ligated small intestine loop (Smith and Halls, 1967a). Since then, many bioassays in different models had been evolved (Dean et al., 1972; Evans et al., 1973a; Klipstein et al. 1976).

Infant mouse test (IMT):

With slight modification of the original procedure, this test had been used by most investigators (Dean et al., 1972). The culture supernatant or broth containing ST was injected intragastrically in 2-4 days old mice with

dye for easy visibility. After 3-4 hours the intestine was removed and the ratio of intestine to the remaining body weight was estimated and interpreted with the ST values (Gianella, 1976; Olsson, 1982).

Assay of the toxigenecity with diarrhoeal score index at 37°C had also been evolved (Moon et al., 1978). The limitations of the infant mouse test for estimation of E.coli STb (ST 2) was studied and the test was under scored as a mean of detecting ST in porcine isolates of E.coli (Gyles, 1979). This test was evaluated as 50% sensitive when compared to rat perfusion technique (Klipstein et al., 1979). However, this test had been used in detecting the E.coli strains of other species. A simplified ST preparation for the mouse test had been proposed by Gomes et al.(1979). This IMT had been used by many workers successfully (Whipp et al., 1975; Kapitany et al., 1979) and had been a preferred method (Burgess et al., 1978).

Rabbit ileal loop technique (RILT):

This method since its introduction by De and Chatterjee (1953) had been used by many workers for assay of enteropathogenicity of various organisms (Burrows and Musteiks, 1966; Smith and Halls, 1967a;

Sack et al., 1971; Lariviere et al., 1972; Pitrarangsi et al., 1982). The preparation of rabbit and the anaesthetic techniques using intravenous and other parental techniques had been reported (Kaplan and Timmons, 1979). The response of lapine ileal loop to enterotoxin, after six hours, was accumulation of clear, full of mucus and non-haemorrhagic fluid (Sack et al., 1971). The fluid accumulation at the end of sixth hour after administration of the toxin was measured and the volume of the fluid (ml) and the length of the intestinal loop (cm) was measured. If the ratio of fluid volume in mL to length in cm was 0.5 and greater, the test was considered positive (Moon et al., 1970; Evans et al., 1973a; Gyles, 1979). A fluid volume to the length ratio greater than 0.3 was also considered positive (Robichaud et al., 1978) The maximal response of fluid volume to length ratio had been upto 2.75 depending upon the size of the rabbit (Sack et al., 1971).

The rabbits were considered four times sensitive than pigs on the basis of dry weight of the intestines (Moon et al., 1970). For other toxins, the rabbit ileal loop method was so far the preferred method of establishing enterotoxic activity of various microorganisms (Raskova and Raska, 1980; Robins-Browne et al., 1982).

Generally 10 mgm of crude material had been found necessary to evoke intestinal response in ligated intestinal loop (Klipstein et al., 1977). The antipili antibodies to non toxigenic E.coli did not inhibit the fluid secretion, in the immunized rabbit ligated loops when EPEC live cultures were inoculated. But antibodies against EPEC was found to inhibit, the fluid secretion in the ligated loops when the live cultures were inoculated (Molenda et al., 1983).

The stable enterotoxins of Klebsiella pneumoniae Enterobacter cloacae, Aeromonas hydrophila and Plesiomonas shigelloides had been assayed in RILT (Klipstein et al., 1973, Klipstein et al., 1975 Klipstein and Engert, 1975; Klipstein et al., 1977 Klipstein et al., 1979; Pitarangsi et al., 1982).

Histopathological studies:

Tissue sections of rabbit ileal loop seven hours after challenge with Aeromnas hydrophila (Pitarangsi et al.,1982) tissue sections of the rat jejunal segments after perfusion with Klebsiella enterotoxin (Klipstein et al., 1975) and of intestines of calves after inoculation with ETEC and Rota virus (Tzipori et al., 1983) after fixing in 10% buffered

formalin and staining with haemotoxylin and eosin had been studied for histopathological changes.

Histopathologically, the enterotoxin (ST) had been reported to cause broadening and blunting of villus of the rabbit intestine along with other pathological changes (Klipstein et al., 1975; Pitarangsi et al., 1982).

Infant rat model:

To suit pathophysiological studies a slightly bigger animal model than infant mouse was developed for assay of ST. ST had been found to cause fast onset of diarrhoea in the infant rat in contrast to slower and longer acting LT. LT did not react in the infant rat model (Kutas and Kovats, 1979).

Rat jejunal perfusion technique:

Heat labile and heat stable toxins of E.coli, Klebsiella and Enterobacter cloacae were identified. The toxins induced net water secretion into the perfused intestine (Klipstein et al., 1976, 1979).

Dog loop assay:

The test used small intestine loops of dogs. Concentrated ST filtrates had to be used and net absorption measured. ST activity was observed within 20 minutes after administration, whereas LT activity appeared after a lag period of 4-6 hours (Nalin et al., 1974)

Human buccal adherence test:

Demonstration of adherence to buccal epithelia by ETEC of human origin had been described as an assay procedure for ST (Thorne et al., 1979; Panhotra and Agarwal, 1981).

(C) Antibiotic sensitivity assay:

The disc diffusion test method was used in 99 percent of the laboratories in Britain during 1970 (Cruickshank et al 1975). In this test, small absorbent paper discs impregnated with known amounts of antibiotics were placed on an agar culture plate that had been seeded uniformly with the bacteria of the isolate to be tested. When the test conditions are standardized, the zone of inhibition was inversely

related to the minimal inhibitory concentration (MIC) of the antibiotic. The clinical interpretation (resistant, sensitive or intermediate) of the zone sizes was based on its MIC susceptibility value as determined from tube tests (Bauer et al., 1966; Cruikshank et al., 1975)

The sensitivity of the members of coliforms and tox$^+$ E.coli had been assayed to various antibiotics (Echeverria et al., 1978a; McConnell et al., 1979; Echeverria et al., 1981).

CHAPTER III

CHAPTER III

EXAMINATION OF RAW MILK AND ITS ENVIRONMENT FOR COLIFORMS

(A) Introduction

Raw milk was defined as milk which had not been treated by heat (Harvey and Hills, 1951). The coliforms in raw milk produce product quality deterioration, and cause public health concern (Richter, 1981). Routine quality control examination of raw milk in the dairy industry is confined to the examination for coliform count per ml of milk without identifying the source of contamination and the members of the coliform group.

Heat treatment of raw milk after production at farm is done at the processing plant. The point at which the coliform standards applicable for raw milk had not been specified (Indian Standards No. 1479-Part III-1977). These coliforms were being considered as an indicator organism, of insanitary practice, in the production process and mainly concerned with E.coli and Aerobacter aerogenes (Foster et al., 1958, Indian Standards No.1479-1977). The latter group now is divided into a motile Enterobacter aerogenes and a non motile Klebsiella aerogenes which is currently given nomenclature as

Klebisella pneumoniae (Collins and Lyne, 1970; Edwards and Ewing, 1972, Buchanan et al., 1974). The two other members of the group which had not been studied in detail are Enterobacter cloacae and Citrobacter freundii.

This study is an attempt on different farm environmental factors that contribute for coliform contamination into the raw milk and the relative constitution of the individual members in the farm environment. An attempt has also been made, to correlate the existing suggested Indian Standards No. 1479-Part III-1977 to the stage of production and handling of raw milk, and also set an acceptable or tolerable standards of commingled raw chilled milk at the dairy dock.

(B) Materials and Methods

(i) Selection of farms:

The visits to the farm were for comprehensive studies including milk and its environment and also to a specific study related to the raw milk alone. Factors like easy approach, local facilities for preliminary bacteriological work, hygienic practices followed during milk production in farms of private and public sectors, were taken into consideration, while selecting the farms. No prescribed

standards were available to evaluate the hygienic practices in the farm. However, visual examination and other organoleptic judgements were used to grade the farms and milk to group A, B and C before sampling based on the guidelines suggested by Barber (1962). The group A consisted of commercial farms where sanitary practices were given second preference to the production and despatch of milk. The group B consisted of farms of organized sectors where technical supervision was available. The sanitary practices were moderate. Group C consisted of farms where sanitary practices were experimentally imposed during the sample collection, which included one of the Group B farm.

(ii) Choice of samples on random and non random basis:

Usually attempts were made to take samples on random basis as specified by Indian Standards No. 1479-Part I-1960. Choice of samples from specific cases like healthy animal with healthy udder, air sampling at a place where milk would be exposed to air borne dust contamination, water used for rinsing the equipments were taken on non random basis. The milk bearing utensils and the milking personnel were selected on

random basis to the extent necessary (ICMSF, 1974; Harrigan and McCance, 1976).

(iii) Environmental sampling:

(a) Air: (Anon, 1972)

Sampling for airborne microorganisms in dairy farms were taken from the milking yard, weighment room and milk pooling areas. Splashings and spillages were carefully avoided during sampling.

The slit sampler (CF Casella and Co., England) was used as an equipment of choice. The number of particles carrying coliform bacteria per litre of air on MacConkey's agar was enumerated on incubation for 24 hours. Wherever the slit samplers could not be transported the settle plate method by exposing MacConkey's agar for 15 minutes was adopted. After incubation of the plates the developed colonies were counted and the result expressed for 15 minutes exposure or number of colonies present in 150 litres of air.

(b) Farm water:

The sampling procedure for chlorinated and non chlorinated water were as per the procedure of

(Anon(1975). The multitube technique using brilliant green bile broth was employed for the presumptive coliform test, wherever few numbers of bacteria were suspected to be present due to chlorination. The most probable number (MPN) estimates using three tube technique table suggested by Collins and Lyne (1970) was followed. The presumptive coli test by plating out 3 ml and 2 ml of water samples, on ~~crystal~~ violet Red bile agar (VRBA) with overlay was also carried to pick out the isolates (Harrigan and McCance, 1976). After 24 hours of incubation the results were expressed as MPN of coliforms per 100 ml in the multitube technique.

(c) Farm utensils: (Indian Standards No. 5253 - 1969).

The surface samples of cans and milking pails were taken with cotton wool swabs moistened with 25 ml quarter strength Ringers' solution. The surface samples were approximately limited to 100 sq.cm. instead of 900 sq.cm. as prescribed by the Indian Standards No.5253-1969. For serial dilution to pour plates buffered dilution water suggested by Richardson et al.(1980) was used.

Stock phosphate buffered saline (PBS):

Thirty four grams of $KH_2 Po_4$ was dissolved in one

litre of pyrogen free water (pH 7.2). The stock solution was sterilized and preserved.

$Mgcl_2$ stock solution:-38 g of $Mgcl_2$ was dissolved in one litre of distilled water. After sterilization it was preserved.

Buffered dilution water:

Stock PBS	1.25 ml
Stock $Mgcl_2$	5.00 ml
Distilled water to	1000 ml

The buffered dilution water was dispensed and autoclaved in the required quantity for dilution purposes. Crystal Violet Red Bile Agar (VRBA) was used for plating. The colonies were counted and the results were computed and recorded as per the procedure suggested by Richardson et al.(1980). The results were reported as number per 100 sq. cm of the surface swabbed.

Rinse method with quarter strength Ringer's solution was also adopted as described by Indian Standards No. 5253-1969. This method was adopted wherever the farms were nearer to the laboratory. The counts were recorded as coliform counts per litre capacity of the vessel.

Coliforms not more than 10 per litre capacity were considered satisfactory. Coliform count over 10 to 100 per litre capacity were considered fairly satisfactory and coliform count over 100 were considered unsatisfactory.

(d) Personnel (Milkman's hand)

Roughly 100 sq. cm. area on the palm and forearm of the milkmen were swabbed just prior to hand milking as described for utensil swabbing. The swab was processed as already described and the counts were recorded as number of coliforms present per 100 sq. cm. area.

(e) Teat swab (Rendos et al., 1975)

The teat ends of the dairy cows were swabbed and the swab washings were processed in the pour plate method using VRBA. The number of organisms present per teat end was recorded.

The break up figures of environmental sampling are furnished in table 1.

Table 1

PARTICULARS OF ENVIRONMENTAL SAMPLING FOR COLIFORM ENUMERATIONS

Source	SAMPLES					
	Air	Water	Personnel	Teat swabs	Milking utensils	Total samples
A	5	4	5	7	4	25
B	6	4	4	6	4	24
C	6	4	5	5	4	24
Total	17	12	14	18	12	73

A Commercial farms with insanitary practices

B Public sector farms with moderate sanitary practices

C Experimental stations with improved sanitary practices

(iv) Sampling of milk (Indian Standards 1479 - Part I - 1960)

The milk was collected atleast from two quarters from a cow in a sterile container, after letting down four streaks from each teat. The pooled milk in cans and cammingled milk in road tankers were sampled after plunging to take a representative samples of the bulk. The particulars of milk sampled for the study are furnished in table 2.

(v) Transport and examination of samples:

After proper labelling with indelible ink on adhesive tapes the milk samples were packed in polyethelene water proof bags and preserved in ice during transport to the laboratory. The milk samples were immediately plated out by pour plate method for presumptive coliform count. The three tube MPN technique in brilliant green bile broth was also followed for fresh milk (Indian Standards No.1479-Part III, 1962; Collins and Lyne, 1976). The commingled milk samples were also plated out for standard plate count in plate count agar (Difco).

(vi) Statistical methods for selection and examination of Bacterial colonies: (Harrigan and McCance, 1976)

Every colony on the plate was selected when the

Table 2

PARTICULARS OF MILK SAMPLING IN DIFFERENT FARMS AND DAIRY DOCK AT MADHAVARAM

Source	Individual milk samples	Pooled milk samples	Commingled milk samples	Total samples tested
A	21	8	82	111
B	17	17	18	52
C	30	4	30	64
Total	68	29	130	227

A Commercial farms with insanitary practices

B Public sector farms with moderate sanitary practices

C Experimental stations with improved sanitary practices

numbers present were roughly equal to number required to be examined. Whenever the number of colonies required to be picked out were less the Harrison's disc (1938) was used. All colonies occurring in the area 1 of the disc were picked out for examination. Whenever this number was insufficient, the colonies occurring in areas 2, 3 and 4 were picked out for further study.

(vii) Preservation of the isolate for further study:

The colonies picked out from the pour plate cultures, were streaked on Eosin Methylene Blue Agar (EMB Agar). Atleast five typical E.coli colonies and non E.coli colonies were inoculated in Casamino acid yeast extract medium (CYE) with 15% glycerol and frozen at -10°C in partial modification of the procedure suggested by Evans et al.(1977). For routine use, stock cultures were kept in 2% peptone agar stabs after paraffin sealing of the cork.

(viii) Identification of cultures:

The stock cultures in the paraffin sealed stubs were plated out on a non inhibitory media, like Tryptic Soy Agar (Difco, Detroit) and subjected to conventional tests (Edwards and Ewing, 1972) primarily in the patterns

of tests suggested by API 20E system. The medium for the tests were prepared and primary colony characters on EMB agar, motility test with H_2S production (Difco, media) decarboxylase reactions, indole production, Simmons' citrate utilization, phenylanine deaminase test, urease test and inositol fermentation were studied for identification purposes.

(C) Results and Discussion

(i) Farm air:

The results of the air sampling is presented in table 3. The coliform counts were found varying from source between A and B, A and C and B and C. The high coliform count in source A indicated that there was much bodily movement of animals and/or other disturbances resulting in the presence of environmental dust (Anon, 1972).

Reports prescribing standards of coliform density in the farm air are scanty. The total coliform load in the air had been studied and it had been reported that the percentage varied from 2.5% to 5.6% of the total viable count (Benham and Eddell, 1970; Fiser and

Table 3

MEAN COLIFORM COUNT IN AIR SAMPLES DURING MILKING OPERATIONS

Source	No.of samples studied	Method of sampling		Grading
		Settle Plate- Organisms per 15 minute exposure	Air Sampler- Organisms per 150 litres of air	
A	5	75**	Not done	Unsatisfactory
B	6	Not done	50**	Unsatisfactory
C	6	10	Not done	Fair
Total	17	-	-	-

** Highly significant ($P<0.01$)

Grading 1. Air sampler: Organisms in 150 litres of air:-

i) Less than 3 .. Satisfactory
ii) Greater than 3 and less than 10 .. Fair
iii) Greater than 10 .. Unsatisfactory

2. Settle Plate: Settled in 15 minutes in 100 mm plate:-

i) Less than 3 .. Satisfactory
ii) Greater than 3 and less than 10 .. Fair
iii) Greater than 10 .. Unsatisfactory

Svitavsky, 1975, Rondinni, 1982). Since the coliform load represented around 5% of the aerobic viable counts in the earlier studies norms for air borne coliform load were now fixed at 5% of the aerobic viable count. As the APHA (Anon, 1972) suggested a wide range of 4.5 to 50 bacterial count per 15 minutes exposure or one cubic foot of air, the 5% of the higher value in the range was taken into consideration to set the norms. So a satisfactory norm would be the presence of less than three coliforms per 15 minutes exposure or 150 litres of air. Value over 3 and upto 10 was considered fair and above 10 warranted investigation as to the cause for high aeromicrobial load.

In this study, the source A was over stocked with milch animals and had mixed farming within the same premises. The dung was not removed frequently and the animals were let loose in the same area till the milking operations started resulting in much bodily movement of animals. In the study group B the animals and the floor were not washed before milking though the environment was comparatively clean. In the study group C the floor and animals were washed before milking and movements of animals were restricted. Otherwise the flooring of the milking yard and feeding schedules were similar in all the three study groups. Probably the dust free condition

in the group C had resulted in low count though there was no separate milking parlour. A clean ideally built milking parlour might further reduce the coliform fall out in these farms.

In this study, the fall out varied from 10 to 75 in 15 minutes exposure of 100 mm plates. Assuming that 20 to 150 coliforms settled in 200 mm diameter neck of the milk can or pail, it could be calculated that the aerial contamination of milk would be very negligible as compared to other sources of contamination as was also observed by Benham and Egdell (1970). However a study on the coliform bacteria in the air of a farm with calf septicaemia revealed high E.coli count (80%) among coliforms (Jacquet and Chiki, 1977). Such circumstances warrant a need for aerosal spray with sanitizers to kill the probable enteropathogenic E.coli serotypes.

(ii) Farm water:

The most probable number (MPN) coliforms per 100 ml of tap water and stored water is furnished in table 4.

The results in the study group AB and AC were statistically highly significant. Though the tap water had high coliform count the stored water had no coliforms

Table 4

MEAN PRESUMPTIVE COLIFORM COUNT IN WATER SAMPLES

Source	No.of samples	Multitube technique MPN/100 ml		Grading*	
		Tap water	Stored water	Tap water	Stored water
A	4	450**	0	Unsatisfactory	Satisfactory
B	4	72	141**	Unsatisfactory	Unsatisfactory
C	4	40	70**	Satisfactory	Unsatisfactory
Total	12	-	-	-	-

* Grading : Presumptive coli less than 50/100 ml - Satisfactory

ND : Normal deviate

** ND (AB): Highly significant ($P < 0.01$)

** ND (AC): Highly significant ($P < 0.01$)

in study group A. The optimal chlorination and cleanliness of tank without organic matter, aided in the reduction of coliforms in this group. Either insufficient chlorination, or the obvious presence of organic matter would have been the reason resulting in the increase in numbers in the group B and C. Much attention to chlorination of water was not paid in the experimental group C as the subsequent sanitation of water with iodophor at 75 ppm was planned for rinsing the milk pails, washing the milker's hand and teats. The addition of water into the milk, either from sources of tap water or from the stored water in groups (B and C) for the purposes of adulteration, would have resulted as an inoculum for coliforms and psychrotrophs (Thomas, 1958; Palmer, 1980; Cousins, 1982). Hence contaminated water might not be a major source of bacterial contamination to milk unless such water was added deliberately into milk as was also considered by Palmer (1980). The contaminated water if used for washing and rinsing of farm utensils without sanitization would be a source of contamination to the milk.

Chlorination of water to the optimal extent might destroy all the coliforms (Clark, 1962). However the high coliform count in the tap water sources in all

the farms should be investigated into as it might indicate manure pollution or damage to the walls of the bulk storage points permitting seepages (Cruikshank et al., 1975). The very recent study by Walsh and Brissonnette (1983) had thrown light on the effect of chlorination on the enterotoxigenic Escherichia coli (ETEC). Even insufficient chlorination at the rate of 0.5 to 0.75 ppm of available chlorine might damage and cause sublethal injury to ETEC. Such a sublethal injury damaged the surface adhesins leading to less adhesive property in ETEC. Their study which was oriented in the recovery of the injured ETEC from the chlorine treated water had opened a new path of research in water sanitation.

(iii) Farm utensils:

The results of the rinse test of milk pail and milk can in the study group A, B and C are presented in table 5. The statistical analysis had revealed that the results are highly significant in the milk hygiene aspect of the three groups. The milk contact surfaces of the equipment or utensils where the milk invariably got into contact either during storage or transportation were the major sources for the contamination of coliforms (Mabbit, 1980b). The importance had been emphasized by public health

Table 5

PRESENCE OF COLIFORMS IN DAIRY UTENSILS

Source	Milk pail Rinse Test-coliforms per litre capacity	Milk can Rinse Test-coliforms per litre capacity	Grading*	
			Milk pail	Milk can
A	17.5×10^4	2.75×10^7	Unsatis-factory	Unsatis-factory
B	450	164	Unsatis-factory	Unsatis-factory
C	0	110	Satis-factory	Unsatis-factory

ND (AB)	Highly significant	$P < 0.01$
ND (AC)		
ND (BC)		

* As per Indian Standards:5253 (1969)

authorities like American Public Health Association (Anon, 1972) and National Standards Institutions (Indian Standards No. 5253 - 1969).

In the present study the milk pail and milk can of source A had contributed millions of coliforms in the milk. This could be a major source of bacteria in ex-farm milk (Mabbit, 1980b; Gehriger, 1980; Fluckiger et al., 1980). Though the level of contribution was less in the source B, or comparatively very meagre, yet their multiplication in the milk could pose problems if the milk was not chilled immediately (Gehriger, 1980; Cousins, 1982). The absence of coliforms in the milk pail of group C indicated the cleaning and sanitization efficiency with iodine compounds. The unsatisfactory grading of milk can in group C was due to the cleaning and attempted sanitization by an another agency which was responsible for collection of milk. Though mechanized detergent cleaning and hot water sterilization were available in the can washers of the organised dairy industry which had collected milk, the result had indicated that the process was not satisfactory. So it could be suggested that all the milk collecting utensils should invariably be sanitized either with acidified chlorine in the concentration of 500 ppm of chlorine or 75 ppm of iodine or 100 ppm of hexachlorophene (Hansen, 1973; Palmer, 1980). When such practices were

adopted, bacteriological quality of farm bulk milk tanks showed much lower bacterial count (Palmer, 1980).

(iv) Milking personnel and teat of the cow:

The results of the swab test of the palm and the forearm of the milking personnel and swab-test results from four teats are presented in table 6. Statistically the results are highly significant between A and B, A and C and B and C. The standards furnished by Harrigan and McCance (1976) for the milk contact surfaces had been suitably modified to cover the test results as there were no other available standards. The surface area covered while swabing were extended to an area of approximately 100 sq. cms.

In the study in group A, no cleaning and subsequent sanitization of the hands of the milking personnel and teat were being followed, as this group was a commercial one. This commercial group showed urgency in all aspects including transportation of raw milk to the star hotels of the city by their transport vans. The study group B followed sanitization programme with commercial iodine preparation. However it was lacking in thorough cleaning of the hands of the personnel, animal and udder surfaces. In the group C attempt was made to clean and sanitize

Table 6

PRESENCE OF COLIFORM IN MILKERS' HAND AND TEAT OF THE COW

Source	No.of samples		Milkers' hand organisms per 100 sq.cms.	Teat swab of four teats	Grading*	
	Personnel	Teat			Personnel	Teat
A	5	7	3.6×10^3	32,000	Unsatisfactory	Unsatisfactory
B	4	6	113	360	Unsatisfactory	Unsatisfactory
C	5	5	10	50	Fair	Unsatisfactory
Total	14	18	-	-	-	-

* Grading: (Harrigan and McCance, 1976)

Hand swab: 10 coliform bacteria per 100 sq. cms. - fair
No coliform bacteria per 100 sq. cms. - satisfactory

Teat swab: 10 coliform bacteria per 4 teats - fair
No coliform bacteria per 4 teats - satisfactory

ND (AB)
ND (AC)
ND (BC) Highly significant ($p < 0.01$)

the hands of personnel and teat of cow with iodine at 75 ppm. However the drying of udder with a clean towel or disposable paper towels was not taken up.

Phosphoric acid and iodine combinations were in use in the dairy industry since a decade. Normal milk contains about 165 µgm. of iodine per litre of milk. A farm survey in Australia reported values upto 346 µgm. per litre where sanitization agents containing iodine were used in the farm (Hemken et al., 1981). Recent fear of the iodine hazard to human health had been proved to be unfounded since the daily intake of iodine to the extent of 100-300 µgm. is needed for a healthy adult. The peak concentration in the dairy environment after dipping or spraying the teat with 5 g. available iodine per litre of water caused 0.645 µgm. per litre of farm air. This level remained below the recommended maximum of 1 µgm per litre of atmospheric air in dairy establishments. Hence this also proved to be of no health hazard by the way of inhalation (Sheldrake and Hoare, 1982). The pre-milking and post-milking teat dipping or washing not only improved the milk quality but also reduced the intramammary infection rate by 50% (Cousins, 1972; Cousins, 1979; Sheldrake and Hoare, 1982).

Non-iodoform cleaning methods or teat dips were associated with low iodine concentration in milk. An acidified chlorine sanitizer (500 ppm) with an alkali (Hexachlorophene 100 ppm) may be used as was suggested by Hansen (1973) and Hubble and Mein (1981).

The absence of drying of the udder with sterile towel or paper towels was a shortfall in the complete sanitization programmes in the farms of group C. This could have been the reason for encountering the coliforms in the hands of the personnel as well as teat surfaces. A method suggested by Golton et al.(1982) for the pre-milking udder preparations would have helped in the total absence of coliforms in the udder surface and hands of personnel in this study. The authors had used prepstall (forcing of 19 litres of air per minute) and drying with paper towels. They concluded that clean and dry teat surfaces ensured high bacteriological quality of milk.

(v) Raw individual milk and raw pooled milk:

The coliform content of individual cow milk and pooled milk samples are furnished in the table 7. The low coliform count encountered, indicated the absence of subclinical mastitis in the cows examined which is in

Table 7

MEAN COLIFORM COUNT PER ML OF RAW INDIVIDUAL MILK AND POOLED MILK

Source	Individual cow milk		Pooled milk		Grading*	
	Number of samples	Mean per ml	Number of samples	Mean per ml	Individual cow milk	Pooled milk
A	21	10	17	39,000	Unsatisfactory	Unsatisfactory
B	17	10	8	27,000	Unsatisfactory	Unsatisfactory
C	30	4	3	30	Satisfactory	Unsatisfactory
Total	68	-	28	-	-	-

* Indian Standards : 1479 - Part III - (1977)

ND (AB)
ND (AC) Highly significant ($P < 0.01$)
ND (BC)

agreement with the finding of Rao and Nambudripad (1978). The statistical analysis revealed the highly significant data between A and B, A and C, and B and C. As far as compliance with the specifications of Indian Standards Institution No.1479 - Part III - 1977 only the individual milk samples of group C conformed to the standards, while none of the pooled milk samples complied. Similar data on the achieved levels with pooled milk were also reported under Indian conditions (Gahlot et al., 1975; Singh and Ranganathan, 1978), as well as from abroad (Rangasamy, 1980; Kaloyanov and Gogov, 1977). The Indian standard would however apply when the milk was drawn from the udder after letting down the first four or five streaks to flush out the bacteria in teat canal as it was obtained in the group C.

In this study coliforms were present at the teat ends in the range of 50 - 32000 per cow i.e., 12 - 8000 per teat end (Table 6). Tolle (1980) also observed that if milk was removed from the gland or teats of a healthy mammary gland by aseptic puncture of the wall or by surgically implanted catheter system, sterile samples could be obtained. The contaminating source at the animal point should be the teat ends. Rendos et al.(1975) reported that with different types of beddings, the

organism varied from 8 - 127 per teat and which included 1 - 11 Klebsiella also. Hence at milking level the primary sources of contamination could be the teat ends. The next source would be the milker's hands which carried a mean 3,600 coliforms in this study in the group A farms (Table 6), whereas in a similar study, 0.26 million of bacteria were added from a milker's hand when they were rinsed in 200 ml of sterile milk (Smythe, 1960).

So the pre-milking teat dips with solutions of chlorine, iodine or hexachlorphene would yield a quality milk of good bacteriological grade. A detailed study on the effect of using different udder washes on teat microflora was made by Hansen (1973).

As far as the milking techniques are concerned the ideal milking should include avoiding of moistening or wetting the hands with the first few drops of milk and touching the milk contact surfaces of milking buckets or pails. The milker should wash his hands and arms thoroughly with soap and sanitize with antiseptic solution prior to milking (Palmer, 1980).

The milking vessel and cans were the next points that should be given importance in producing milk of high bacteriological quality. The possible addition of

coliforms from utensils is presented in table 5. Mabbit (1980 b) and Palmer (1980) considered that the equipments were frequently the main source of bacteria and an effective cleaning and sterilizing routine would keep the bacterial density less than 1000 bacteria per ml of milk. Obviously all the bacteria harboured in the equipments should invariably get into the milk whereas if the coliforms are present in milker's hand or teat there could be only a chance entry of them into the milk. So the pooled milk quality could be very strongly influenced by the cleanliness of milking utensils (Gehriger, 1980).

The multiplication of bacteria during farm storage could be another constraint in producing milk of high bacteriological quality. It depended upon the effect of initial level of contamination, the time of storage and effect of temperature (Lagrange, 1979, Gehriger, 1980). The storage temperature and the antibacterial system in the milk had an influence on the growth of microorganisms (Bjorck et al., 1975; Bjorck, 1978, Lagrange, 1979; Ayres et al., 1980; Cousins, 1982). Swartling (1967) established the relationship between the temperature and the growth of bacteria in milk. The growth rate of bacteria decreased with decreasing storage temperature.

Hence in the farm conditions the minimal transfer and handling of milk in various equipments would invariably reduce the chances of initial contamination. The methods of minimising the multiplication of coliforms would include the holding the milk at the farm as short as possible and cooling should be rapid and as low to a temperature as possible. These practices would minimise the coliform growth in the pooled milk.

(vi) Bacteriological quality of commingled milk:

The results of the examination of chilled milk from three different sources are furnished in table 8 and 9. The first of the two tables indicate the standard plate count/ml (SPC/ml) of milk and the second table the coliform density/ml of milk. The frequency and the percentage of distribution in the class are presented in the respective tables. The percentage of coliforms in the total viable count ranged from 0.47% to 1.16%. The coliforms, though represented a lower percentage out of the total aerobic count, were considered to have an impact on the final product quality by producing metabolic intermediaries and gassiness (Muier et al., 1978; Kraft and Roy, 1979). These high SPC and coliform

Table 8

STANDARD PLATE COUNT (SPC) PER ML OF REFRIGERATED COMMINGLED MILK AT DAIRY DOCK

	MILK SHEDS		
	A	B	C
No.of samples*	82	18	30
Mean MBRT in hour	0.5	1.0	1.5
Range of the count:			
1 - 2 M	0	11.11 (2)	30.00 (9)
2 - 5 M	0	-	30.00 (9)
5 - 10 M	1.22 (1)	16.67 (3)	36.67 (11)
10 - 50 M	21.95 (18)	33.33 (6)	3.33 (1)
50 - 100 M	15.85 (13)	11.11 (2)	-
100 - 200 M	29.27 (24)	22.22 (4)	-
200 - 300 M	31.71 (26)	5.56 (1)	-
Mean SPC in million	146.41	68.67	5.87

* Sample size depended on the no.of supplies from each source.

M - Millions

MBRT Methylene Blue Reduction Time

Figures in parentheses indicate the number of samples tested.

A - Education on clean milk production absent, milk chilling around five hours after production (Poor).

B - Education to farmers on clean milk production and milk chilling around four hours from production (Fair)

C - Milk sheds wherein orientation on clean milk production and chilling of milk within four hours of production (Satisfactory)

Table 9

COLIFORM COUNT PER ML OF REFRIGERATED COMMINGLED MILK AT DAIRY DOCK

	MILK SHEDS (Values - in percentage)		
	A	B	C
No.of samples*	82	18	30
Mean MBRT in hour	0.5 and below	1.0	1.5
Range of the count:			
1000 - 5000	-	-	6.67 (2)
5000 - 10000	-	-	26.67 (8)
10000 - 20000	-	-	23.33 (7)
20000 - 30000	-	5.56 (1)	13.33 (4)
30000 - 50000	-	5.56 (1)	10.00 (3)
50000 - 0.1 M	-	22.22 (4)	20.00 (6)
0.1 M - 0.6 M	14.63 (12)	66.66 (12)	-
0.6 M - 1.0 M	2.44 (2)	-	-
1.0 M - 3.0 M	82.93 (68)	-	-

Conti.....

Table 9 Conti....

	MILK SHEDS		
	A	B	C
Mean	1.70 M	320,000	28,000
Conformance to IS:1479-Part III-(1977)	0	0	0
Percentage in total viable count (SPC/mL)	1.16	0.47	0.48

* Sample size depended on the no.of supplies from each source

M -Millions

MBRT Methylene Blue Reduction Time

Figures in parenthesis indicate the number of samples tested

A - Education on clean milk production absent, milk chilling around five hours after production (Poor)

B - Education to farmers on clean milk production and milk chilling around four hours from production (Fair)

C - Milk sheds wherein orientation on clean milk production and chilling of milk within four hours of production (Satisfactory)

count indicated that cooling could not serve as a substitute for improper cleaning and sanitization methods of production, as observed by Gehriger (1980). The antibacterial system present in fresh raw milk would be also of less significance in such a high contamination (Foster et al., 1958; Ayres et al., 1980). Further it was observed that a low count milk with 1990/ml had a keeping quality of 46 hours at 18°C, while a high count milk with 200 million/ml held at 22°C was found to be sour on clot on boiling test. The comparison between such grades of pooled milk at farm level (Table 7) chilled milk at dairy level (Table 9) reflected the growth of bacteria from the farm stage to the processing stage at the dairy dock.

Statistically in the group A study for coliforms 68 our of 82 samples fell into 1 million to 3 million modal class. In the group B, 12 out of the 18 samples were found to have counts ranging from 0.1 to 0.6 million. In the group C, the modal class were seen in the range of 5,000 to 10,000 coliforms per ml with 8 out of 30 samples falling in this class.

The low count of the group C wherein sanitary methods were carried out under the supervision of the

researcher obviously explained, the possibility to execute good sanitary practices that should be followed in the clean milk production. The failure to observe these sanitary practices would result in spoilage of milk, production of off flavour of the milk and allowing the toxigenic organisms to multiply (Mikolaj cik, 1979, 1980).

The modern trend in the transportation of milk demands hygienic quality of milk ex-farm so as to transport the chilled milk to the processing dairy, after a long haulage in the road tankers, avoiding frequent heat treatment and thus preserving its nutritional quality. The percentage of increase in coliforms in the refrigerated tankers was only 12 and it was as high as 357 when they were transported as unchilled in cans (Fluckiger et al., 1980). This obviously necessiated the production of milk with low coliform density and the need for immediate chilling for long transportation.

The quality of 130 commingled milk samples tested in this study from various sources inclusive of the group C revealed that none of the samples complied with the

suggested Indian Standards 1479 - Part III - 1977 which specified the absence of coliforms in 1/100 dilution of raw milk, i.e., not exceeding 10 coliforms per ml. The lowest number of bacteria in the best possible sanitary standards of tropical climate was 28,000/ml (mean). This is 2,800 fold higher than the suggested guideline for acceptance by Indian Standards No.1479 - Part III - 1977. Hence the suggested guideline could atmost be taken as desirable standard at the producing farm and not at the acceptance level at dairy docks. The bacteriological standards of raw chilled milk had been given less significance, because of the ensuing heat treatment and manufacture of various diversified milk products depending upon its quality (Fluckiger et al., 1980). But with the present knowledge on Enterotoxigenic E.coli and its known toxigenic potential, the dependence on heat treatment had to be reviewed with regard to coliforms which could produce heat stable toxins (Marrier et al., 1973; Klipstein and Engert, 1975; Mehlmen et al., 1976; Batish et al., 1981b; Glatz and Brudvig, 1980a,b). The type of the end products obtained would not have any influence on these toxins whether they were fluid milk or a solid heat processed product. The stable toxin might be carried over through the heat treatment so as to cause public health hazard.

These hazards were recently emerging and might assume a major role in the late eighties (Busta, 1979; Richter, 1981; Kornacki and Marth, 1982b; Winslow, 1982; Kraft, 1982). Hence the presence of coliform in milk products hitherto considered as an indicator organisms of faecal pollutions (Indian Standards No.1479 - Part III - 1977) proved to be a potential public health problem as they had been incriminated in the diarrhoeal diseases of infants and adults (Marrier et al., 1973; Twedt and Boutin, 1979; Glatz and Brudvig, 1980a,b).

(vii) Distribution of coliforms:

The isolates statistically picked out on the basis of Harrison (1938) were identified utilizing the conventional tests of API 20E series. The API 20E series of tests and additional battery of tests were interpreted with differential chart of API 20E and tables of Edwards and Ewing (1972). The colony characters in the eosin methylene blue agar were also observed to differentiate between the E.coli and Klebsiella, Enterobacter and Citrobacter groups. About 10.26% of the total organisms could not be identified and this group was classified as untypable and was not taken up for further study.

The distribution of coliforms are presented in table 10. In the farms where insanitary conditions prevailed during milking operations, E.coli (37.13%) dominated the environment followed by Klebsiella pneumoniae (30.54%) E.aerogenes (13.77%) and Citrobacter freundii (11.38%). E.cloacae was lowest (7.18%). In the group B and C Klebsiella dominated the prevalence followed by E.cloacae in the group B and E.coli in the group C. As a whole Klebsiella pneumoniae (39.54%) dominated the farm environment followed by E.coli (27.12%) E.aerogenes (11.44%), E.cloacae (11.11%) and Citrobacter freundii (10.79%).

Statistically the distribution of E.coli was highly significant between A and B and B and C. The K.pneumoniae also attained high significance between A and B and A and C. The E.cloacae was placed in the highly significant position between A and B and B and C (Table 11).

In a study in Russia Enterobacter was present in the milk at the rate of 47.7%. It was also considered as an indicator organism by these authors (Kartashova et al. 1974). The distribution of E.coli was (40%) followed by E.aerogenes (23%) in an environmental study made by Misra and Sinha (1978) in India. Contrarily Enterobacter aerogenes constituted 35.0% of the group followed by

Table 10

PERCENTAGE OF COLIFORMS IN DAIRY ENVIRONMENT BY STATISTICAL SELECTION* OF BACTERIAL COLONIES

Source	E.coli	K.pneumoniae	E.aerogenes	E.cloacae	C.freundii	Total identified
A	37.13 (62)	30.54 (51)	13.77 (23)	7.18 (12)	11.38 (19)	167
B	5.49 (5)	53.85 (49)	6.59 (6)	23.08 (21)	10.99 (10)	91
C	33.33 (16)	43.75 (21)	12.50 (6)	2.09 (1)	8.33 (4)	48
Total % of distribution	27.12 (83)	39.54 (121)	11.44 (35)	11.11 (34)	10.79 (33)	306

* Harrison (1938)

Figures in parenthesis indicate the number of isolates tested

Table 11

STATISTICAL ANALYSIS (ND) OF ENVIRONMENTAL DISTRIBUTION OF COLIFORMS

Comparison between groups	E.coli	K.pneumoniae	E.aerogenes	E.cloacae	C.freundii
A and B	6.000**	4.339**	1.227 NS	2.565**	0.140 NS
A and C	0.036 NS	3.771**	0.038 NS	0.830 NS	0.350 NS
B and C	6.036**	0.568 NS	1.315 NS	3.395**	0.210 NS

ND = Normal deviate

NS = Not significant

** = Highly significant ($P < 0.01$)

Citrobacter (29.8%) and E.cloacae (23.9%) (Kaloyanov and Gogov, 1977).

All these studies indicated, that the presence of individual members of coliform group depended on local environmental conditions, associated probably with the distribution of the intestinal flora of animals housed, the fodder fed, and prevalance of enteric diseases.

(D) Summary

The environmental niches of the dairy farms of three groups based on sanitary practises were sampled for the study of coliforms. These included farm air, water, farm utensils, personnel and teat of cows. The individual cow's milk, pooled milk at the farm level and commingled milk at the dairy dock were also sampled for this study.

It was concluded that farm utensils were considered to be the major source of the contamination of milk, followed by personnel and teat ends. The farm air and farm water played an insignificant role compared to other sources. It was evident from this study that possible

sanitary practices at the farm level could very much improve the milk quality at the dairy dock. In such milk the mean coliform density was 28,000 per ml compared to mean of 1.7 million per ml, wherever insanitary practices prevailed.

The suggested only available guideline of Indian Standards No. 1479 - Part III - 1977 (Bacteriological analysis of milk) was found to be too rigid and unobtainable with regard to pooled milk and commingled milk in this tropical climate. The individual cow milk produced at sanitary environmental conditions was alone found to comply to this standard. The need for high bacteriological quality ex-farm milk in the organised dairy sector was discussed.

The distribution of each member of coliforms in the farm environment were found to vary from group to group. In an overall study E.coli dominated wherever insanitary conditions prevailed. K.pneumoniae dominated wherever sanitary practices were adopted during production Probably the distribution was influenced by bacterial flora of the dung and its collection and disposal, feed lots and other disease conditions prevailing in the farm.

CHAPTER IV

CHAPTER IV

METHODS OF IDENTIFICATION OF TOXIGENIC COLIFORMS OF THE ISOLATES

(A) Introduction

A high initial dose of the organisms possessing virulence factors might be necessary to initiate the infection by enterotoxigenic coliforms (Dupont et al., 1971; Levine and Rennels, 1978). Method of identification of toxigenic coliform included the demonstration of the colonization attribute mediated by host specific fimbriae. These host specific fimbriae are all antigenically distinct and each is a haemagglutinin identifiable according to its sensitivity to mannose and to the kind of erythrocyte agglutinated (Isaacson, 1978; Evans Jr. et al., 1979). Many human isolates of ETEC which produce LT and ST also possess fimbrial CFA either CFA/I or CFA/II depending on its serotype (Evans et al., 1978).

The production of enterotoxin is the other virulence attribute which must also be conclusively demonstrated to show an organism, to be potentially enterotoxigenic (Evans et al., 1973a; Greenberg et al., 1977; Brill et al., 1979) in any of bioassay methods available to

demonstrate ST producing potential (Sack et al., 1971; Dean et al., 1972; Klipstein et al., 1976).

To determine the enterotoxigenecity of the coliforms isolated in the present study and maintained for further study the adhesion factors (CFA/I and CFA/II) and the enterotoxin (ST) production were demonstrated. As the labile toxin would be destroyed at pasteurization temperature or at further boiling of milk, it was considered that the demonstration of ST alone would be sufficient as far as milk was concerned (Burgess et al., 1978). The presence of CFA/I and CFA/II pattern of MRHA in E.coli and the possible presence of similar fimbriae in other coliforms are discussed.

(B) Materials and Methods

(i) Identification of adhesion attribute:

(a) Mannose resistant haemagglutination (MRHA):

The organisms isolated for further study from milk and its environment were grown in 5% sheep blood agar. They were subcultured on colonization factor

antigen agar (CFA Agar) and incubated for a period of 24 hours (Evans et al., 1977; Levine et al., 1980).

CFA Agar:

Casamino Acid (Difco)	1%
Yeast extract	0.15%
$MgSo_4$	0.005%
$MnCl_2$	0.0005%
Difco Agar	2%
pH 7.4	

(b) Microtitre procedure: (Jones and Rutter, 1974)

Bacterial suspension:

A homogenous bacterial suspension from the CFA agar was prepared. CFA/I and CFA/II were identified by MRHA of human type A (Evans et al., 1977) or bovine erythrocytes (Evans et al., 1978). Human type A and bovine erythrocytes were obtained fresh, washed twice in PBS at pH 7.2. The cells were suspended to prepare 3% suspension in PBS with mannose 0.5%. The bacterial suspension were made in doubling dilution in four wells of the microtitre trays previously cooled in refrigerator (8°C). All the E.coli and other coliforms were tested

with all the two species of erythrocytes. HA was carried out on microtitre plates at 24°C with human A red blood cells and at 4°C with bovine cells. The plates were incubated over night (12 hours). The agglutination in the first three wells were considered positive.

(c) Identification of type I pili of Klebsiella:

The MRHA human A and bovine red blood cells positive Klebsiella pneumoniae were tested for presence of type I pili (Fader et al., 1979). The organisms were grown in trypticase soy broth with 0.5% glucose. The guinea pig red blood cells (3% v/v) in PBS was used in the slide agglutination test. Positive results were observed within two minutes. All the positive strains were subjected to mannose sensitive haemagglutination test (MSHA) in the microtitre with 0.5 percent mannose (Jones and Rutter, 1974). The incubation was at room temperature. The inhibition of the haemagglutination in the last three wells was considered as (MSHA) positive isolate.

(d) Serological identification of CFA/I and CFA/II among MRHA positive E.coli and non E.coli coliforms:

(Evans et al., 1978; Guinee et al. 1976)

The MRHA positive E.coli cultures were serologically

tested by slide agglutination test for CFA/I and CFA/II with the sera kindly provided by Center for Vaccine Development, Baltimore, U.S.A. The non E.coli coliforms which were similarly positive for MRHA were also tested for the related antigenecity with CFA/I and CFA/II. The working dilutions of CFA/I serum was 1:1000 (E.coli 3456/17 purified pili). The CFA/II antisera was from E.coli on M 424 C purified pili. The working dilution of CFA/II was 1:200. The working dilutions of the CFA/I and CFA/II sera was prepared as suggested by the Center for vaccine development, Baltimore, U.S.A.

(ii) Identification of toxin attribute:

(a) Bacterial cultures:

All MRHA positive coliform bacteria were taken for this study.

(b) Culture media:

Casamino acid yeast extract medium (CAY) was prepared as modified by Lallier et al.(1980). The media contained casamino acid 3.0% yeast extract 0.6% and glucose 0.20%. The pH was adjusted to 7.2 .

(c) Culture conditions and preparation of crude enterotoxin samples:

From a overnight culture of blood agar (5% v/v bovine blood) a few colonies were inoculated into the test medium of 10 ml. in 110 ml milk dilution bottles. The bottles were incubated overnight (18 - 20 hours) on a shaking water bath at 37°C with 120 revolutions per minute. The culture suspension was preserved by addition 1:10,000 merthiolate and stored at -10°C in deep freeze cabinets till test, which was carried out within a week.

(d) Rabbit ileal loop technique: (Lariviere et al., 1972; Gyles, 1979).

Rabbits weighing 1.0 - 1.5 kg were used for this study. The rabbits were starved for 24 hours. Anaesthetic ether was used for induction and maintenance of anaesthesia during the experimental procedure. Preparation of animal for laporatomy and the operation procedure till the exposure of viscera were followed on the lines of Kaplan and Timmons (1979). The small bowel was flushed with warm 10 ml of 0.1 M phosphate buffered saline (pH 7.2) after ligating the intestine in the

part about 50 cm from the pyloric end of the stomach. The flushed intestinal contents were held in the caudal end of the ileum by applying a ligature. The loops were made from the caudal end 4 to 6 cm in length by applying a single tie of single surgical silk thread between segments. In between these segments of 4 to 6 cm, small interloops of 2 - 3 cm were also made. Ten to twelve such larger segments were made with inter loops. While applying ligation the mesenteric blood vessels were carefully avoided from being included in the ligation and also from the needle puncture.

In the longer segments, 2 ml of the preheated (75°C for 15 minutes) sterile test ST preparations were injected with 26 G needle carefully avoiding the blood vessels. In the interloops 1 ml of 0.1 M PBS (pH 7.2) were injected. In the last segment 2 ml of sterile CYE broth with the same concentration of merthiolate (1:10,000) was inoculated as a control. The abdomen was closed and warm gauze packings was applied to the area of operation. Six hours after the administration of the toxins the rabbits were killed with terminal anaesthesia and the volume of fluid per cm of ligated segment were recorded for each segment. Ratios of volume to length (ml:cm) of 0.5 and greater were

considered positive. In later studies one known positive and a known negative controls from the local isolates were used.

(e) Histopathological examination:

Small segments of positive loops were opened with fine scissors, taking care not to disturb the villi pattern. The opened segments were made to adhere on a thick piece of Whatman filter paper and the entire piece was fixed in Bouin's fluid (Saturated picric acid 75 parts; formalin (40%), 25 parts, glacial acetic acid 5 parts) and sections were carefully made to include all the three layers of the ileum for haemotoxylin and eosin stain and were examined under light microscope for morphological changes.

(C) Results and Discussion

(i) Identification of adhesion attribute:

Among 306 isolates of coliforms tested for MRHA of human A and bovine red blood cells 90 strains were found to be positive for MRHA with human A or human A and bovine red blood cells and 40 strains of the coliforms

were found to be positive for MRHA with bovine red blood cells only (Table 12). The agglutination upto third well of the double fold dilution in microtitre were taken as positive. The prevalence of MRHA positive strains (human A and bovine red blood cells) was found to be more than 38% in case of E.coli and 35.53% in case of K.pneumoniae whereas in cases of E.aerogenes, C.freundii and E.cloacae it was found to be less than 22% (Table 12). MRHA of bovine red blood cells only was found to be more than 11% in cases of E.coli, K.pneumoniae E.cloacae and C.freundii, whereas in cases of E.aerogenes the figure was less than 6% of the total coliforms tested. In all 29.4% of the strains were MRHA positive for human and bovine or human red blood cells and only 13.07% of the strains were MRHA positive for bovine red blood cells (human - , bovine +) (Table 12).

HA test with bacterial cells are rapid, simple and economical to perform and thus are convenient for prompt screening of large numbers of isolates for haemagglutinins. Haemagglutination was the first observed manifestation of the adhesive properties of the fimbriated enteric bacteria (Duguid et al., 1955). Colonization of ETEC is mediated by specific types of fimbriae. These fimbriae appeared

Table 12

PERCENTAGE OF MRHA POSITIVE STRAINS AMONG COLIFORM ISOLATES

Name of the organism	No.of isolates tested	Human and Bovine Red blood cells	Bovine Red blood cells	Total
E.coli	83	38.55 (32)	18.08 (15)	56.63 (47)
K.pneumoniae	121	35.53 (43)	12.40 (15)	47.93 (58)
E.aerogenes	35	17.14 (6)	5.72 (2)	22.86 (8)
E.cloacae	34	5.88 (2)	11.77 (4)	17.65 (6)
C.freundii	33	21.21 (7)	12.12 (4)	33.33 (11)
Total	306	29.41 (90)	13.07 (40)	130

Figures in parentheses indicate the number of isolates

to determine host specificity because the colonization factor CFA/I and CFA/II are found on ETEC of humans (Evans et al., 1975; Evans and Evans Jr. 1978; Orskov and Orskov, 1977; Gaastra and Graaf, 1982). CFA/I fimbriae mediate MRHA of human and bovine erythrocytes whereas CFA/II mediate MRHA of bovine erythrocytes only. The aim of the present work is to screen the prevalence of public health significant enterotoxigenic coliforms among the isolates from dairy farm environment. The main virulence attributes of E.coli are the adhesins and enterotoxins (Evans et al., 1975; Evans et al., 1978) Demonstration of the above two virulence factors in large numbers of isolates is rather a Herculian's task and tedious (Sack, 1980; Mehlman and Romero, 1982). Hence MRHA of human and bovine red blood cells was followed in the present study to screen the isolates for the prevalence of enterotoxigenic pathogens.

Among E.coli isolates, 38.55% of the isolates exhibited MRHA pattern of CFA/I and 18.08% of the isolate exhibited pattern of CFA/II. The sensitivity of the MRHA to the serological identification of the presence of CFA/I and CFA/II is very much questionable. On serological identification, there were only one CFA/I

positive and one CFA/II positive isolates out of 47 E.coli tested (Table 13). Similar results of HA positive strains not similarly reacting in serological tests had also been reported (Scotland et al., 1981; Cravioto et al., 1982)

In the present study, apart from E.coli isolates other coliforms also revealed MRHA pattern similar to CFA/I and CFA/II but none of the strains were positive for CFA/I or CFA/II. It is probable that the HA test could indicate the presence of certain fimbriae. Fimbriae of different antigenic types might be clustered together since they belonged to the same HA group (Czirok et al., 1982). It was also observed that fimbriae might not be the only adhesin factor to mediate the pathogenicity of Klebsiella. Other adhesive factors like coliphage receptors had also been demonstrated (Fader and Davis, 1980; Pruzzo et al., 1980). So the MRHA of these strains might be due to fimbriae or other factors which are not known.

In case of Klebsiella species, prevalence of type I pili and its significance in the urinary tract infections had been reported (Fader et al., 1979; Fader and Davis 1980; Pruzzo et al., 1980). The role of the type I pili in

Table 13

PRESENCE OF CFA/I AND CFA/II ANTIGENS IN MRHA POSITIVE *E.COLI* AND NON *E.COLI* COLIFORMS

Organisms	Number tested	CFA/I	CFA/II
E.coli	47	1	1
Non E.coli coliforms	83	0	0

enteric infection is uncertain (Evans Jr. et al., 1980). Intestinal isolates of Klebsiella pneumoniae was found to adhere with type I pili to the epithelial cells of buccal cavity, urinary tract and intestine. While the adhesion was high in buccal and urinary tract epithelial cells it was fewest in intestinal epithelium. So the role of type I pili in the intestinal adhesions for production of enterotoxin appeared to be remote (Pruzzo et al., 1980). The studies on type I pili among human A and bovine MRHA positive Klebsiella species indicated that only two isolates out of 58 were positive in guinea pig MSHA test (Table 14). Citrobacter strains also possessed common fimbriae antigen shared by type I pili of Salmonella (Duguid and Campbell, 1969). No report on pili antigen of Enterobacter species could be traced. Further work is warranted to find out the exact significance of MRHA of human A and/or bovine red blood cells positive strains among coliforms other than E.coli.

One phenomenon that was observed in this study was that the MRHA pattern to human and bovine erythrocytes (CFA/I) dominated the MRHA pattern to bovine red blood cells alone (CFA/II (Table 12). The presence of MRHA bovine and human type pili in E.coli was 38.55% and in K.pneumoniae it was 35.53%. The overall percentage of

Table 14

IDENTIFICATION OF TYPE I PILI IN MRHA POSITIVE KLEBSIELLA PNEUMONIAE

Number of Klebsiella tested	No. positive	Method of identification
58	2	MSHA of guinea pig erythrocytes

of this pattern among coliforms was 29.41%. In a similar study on global collections in the division of enteric pathogens, Central public health laboratory, London, it was observed that prevalence of CFA/I dominated the prevalence of CFA/II. It was reported that six out of 28 ETEC cultures tested possessed CFA/I and none possessed CFA/II (Scotland et al., 1981). The higher prevalence of CFA/I pattern of MRHA in other coliforms also indicated the possible presence of CFA type of antigenically different pili in them.

(ii) Demonstration of toxin attributes:

On terminal anaesthesia the skin sutures (Fig. 1) were removed and the small intestines exposed. Distension of large loops (E) with fluid accumulation were observed in comparison with normal ileum (N) (Fig. 2). The ileum was dissected out carefully keeping intact of all loops. There were distentions with fluid secretions in first, third and seventh loops (Fig. 3). These distentions were absent or comparatively less in the interloops (second, fourth, sixth, eighth and tenth) where 0.1 M PBS was administered and in the negative and control loops (nineth, eleventh and twelth) with CYE broth containing merthiolate.

Figure 1 Rabbit ileal loop test : Rabbit after laparotomy for toxin assay by rabbit ileal loop test.

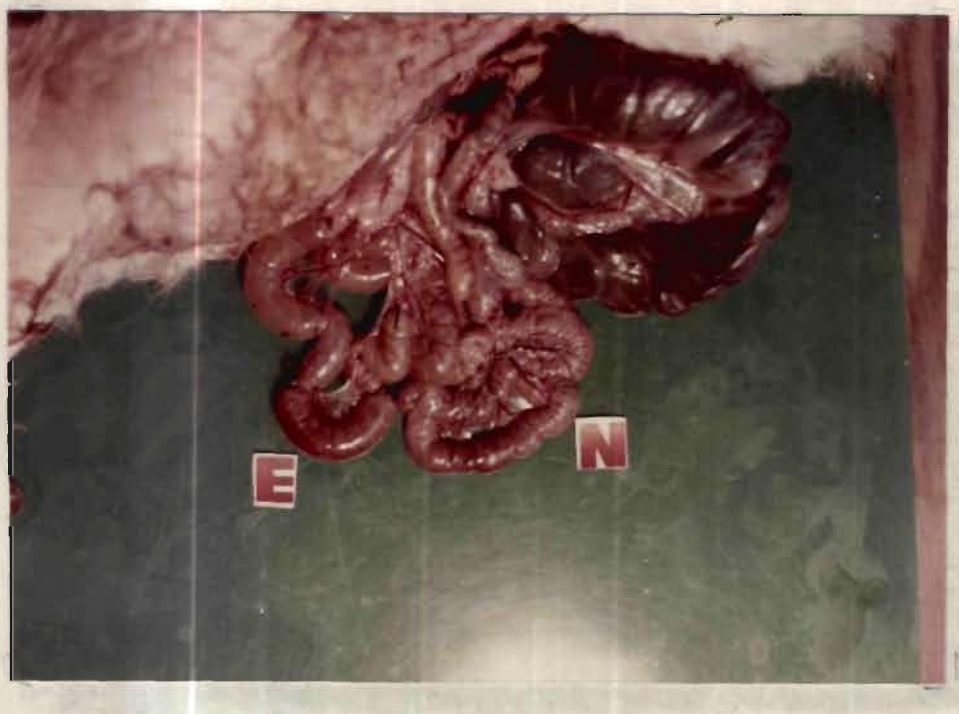

Figure 2 Rabbit ileal loop test: Distended toxin positive (E) ileal-loops compared to normal ileum (N).

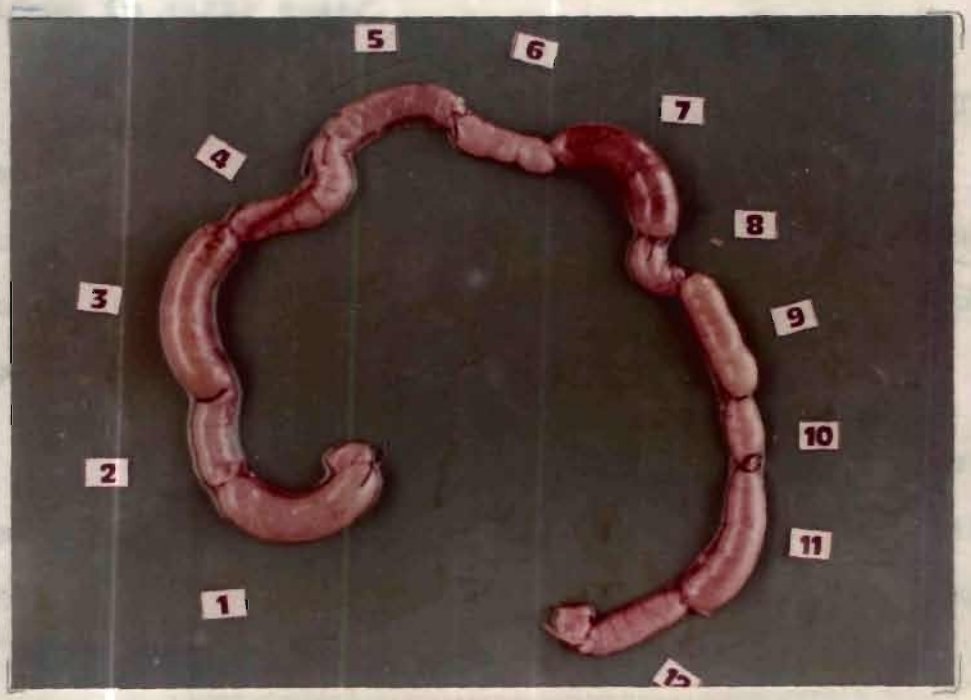

Figure 3 Rabbit ileal loop showing positive results (1,3 and 7) as compared with negative results (9, 11 and 12). The interloops (2,4,6,8 and 10) also showed the absence of distension.

The volume of the fluid was measured to the nearest 0.5 ml and the length was also measured to its nearest 0.5 cm. The ratio of fluid volume to length was calculated and the ratios of 0.5 and above were recorded as positive. The fluid secretory response was without acute inflammatory reaction though very mild congestions were noticed on the serosa in stray cases.

Out of 130 strains subjected to bioassay for ST by means of ligated gut loop technique in rabbits, 24 strains were positive for ST. The percentage of positive strains varied from 12.50% to 45.45% among different species of coliforms (Table 15).

In histopathological examination the control sections were observed with long finger or tongue shaped villi (Fig. 4 and 5). The goblet cell activity was minimal and a few histiocytes were present in the subepithelial area. All the layers of the intestines were seen in the section.

In the positive groups the villi showed structural abnormalities in the ileal loop. The activity was subdued and the villi had become shortened, broad and blunt. All the layers of the ileum were clearly seen (Fig. 6 and 7). Polymorphonuclear leucocytes were

Figure 4 Histopathological features of normal intestinal villi The sections of the villi showing three layers of intestine presented long tongue shaped normal villi · H and E x 120 ·

Figure 5 Histopathological features of normal intestinal villi pattern with minimal cellularity in the lamina propria. H and E x 320.

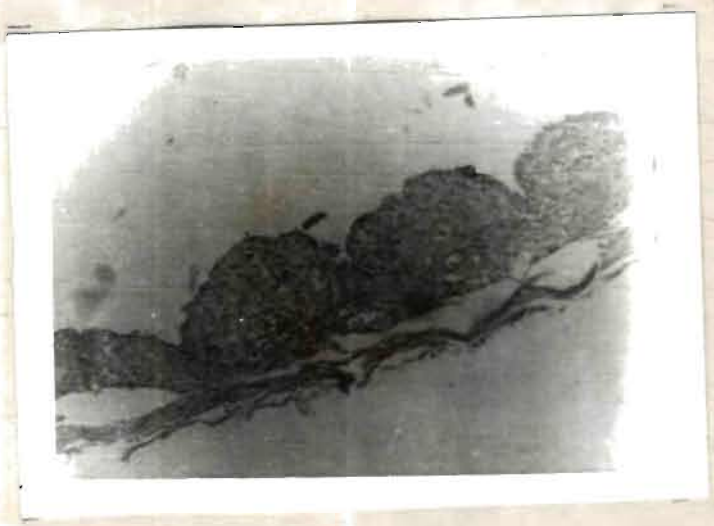

Figure 6 Histopathological features of villi of the toxin positive loops. The villi have become shortened and broadened with heavy cellular infilteration of lamina propria. All the intestinal layers are observed. H and E x 120.

Figure 7 Histopathological features of villi of toxin positive loops. Villi have become shortened and broadened with neutrophil infiltration . H and E x 320 .

Table 15

PREVALENCE OF TOXIGENIC COLIFORMS AMONG MRHA POSITIVE STRAINS (RABBIT ILEAL LOOP TECHNIQUE)

Organisms	Number assayed	Positive	Percentage
E.coli	47	7	14.89
K.pneumoniae	58	9	15.52
E.aerogenes	8	1	12.50
E.cloacae	6	2	33.33
C.freundii	11	5	45.45
Total	130	24	18.46

present in the laminapropria. The histopathological changes due to positive ST, were similar to the observations of Klipstein et al.(1975) and Pitrangsi et al.(1982).

The rabbit model was taken since STb which is more heat stable than STa is active in this model (Burgess et al., 1978). Further major role of STb in enteric infections was reported in the swine and human diarrheoal diseases (Robichaud et al., 1978). In the bioassay of stable toxin the response of the rabbit model was reported to be more sensitive as the response was secretary type (Raskova Raska, 1980; RobinsBrowne et al., 1982).

The correlation of MRHA positive coliforms with RILT is shown in table 16. The result indicates the correlation to be high with MRHA of bovine cells. This may be significant with screening tests as the association of CFA/II and enterotoxin production was marginally high than the CFA/I.

Among E.coli isolates tested, 14.89% of MRHA positive strains (47) were positive for stable toxin (Table 15). This finding is of significance in the light of other

Table 16

CORRELATION OF MRHA PATTERN WITH TOXIGENIC POTENTIAL (TOX^+)

Total MRHA positive isolates	Human and Bovine	Number positive	Bovine	Number positive
130	90	16.67 (15)	40	22.50 (9)

Tox^+ - Toxigenic strains (Echeverriah et al., 1981)

Figures in parentheses indicate the number of isolates

reports which indicated that the enterotoxigenic isolates among hospital environmental strains and of non-diarrheol origin were less than 1% (Scotland et al., 198i; Mehlman and Romero, 1982). Perhaps, these differences may explain the different sanitation levels, acquisition of enterotoxigenic potential with antibiotic resistance as they are single plasmic borne characters. Sensitivity of the RILT might also be a contributing factor for higher results. Different geographical setting might also be a factor for the high incidence in this region (Scotland et al., 1981). However, the identification of 8.43% of ETEC from total isolates (83) concurred with findings of Sack et al.(1977) (Table 17). These workers identified 8% from the total isolates of E.coli in food.

In addition to the E.coli ST production has also been demonstrated in other coliforms (Table 15). It was observed that toxigenic strains were high among C.freundii isolates (45.45%) followed by E.cloacae (33.33%), K.pneumoniae (15.52%) and E.aerogenes (12.50%) It appeared that non-E.coli coliforms are more significant than E.coli itself. Others had reported on the prevalance of enterotoxigenic coliforms in a survey of foods and

water by using rabbit model for assay of the stable toxin (Sadruddin et al., 1981).

The prevalence of all the toxigenic coliforms in relation to total isolates (306) is also presented in table 17. The highest prevalence was observed in C.freundii (15.15%) followed by E.coli (8.43%) and K.pneumoniae (7.43%). The toxigenic potential of C.freundii from the farm environment is established in this study.

In the rabbit model, the ratio of volume to length in this study varied from 0.5 to 0.83. The total toxigenic isolates were 18.46% of the MRHA positive isolates. The pathogenic isolates may probably be more if concentration of the toxin broth was made before the bioassay. Toxin purification and ammonium sulphate concentration of toxin had shown to increase the activity of the enterotoxins by 20 fold and by this way many new enterotoxigenic isolates could be detected(Nalin et al., 1974; Robins Browne et al., 1982). So the concentration of the toxin preparation would have disclosed hitherto unrecognised E.coli enterotoxins. However, it was not attempted in this study since the experimental conditions was set to simulate the real conditions in the possible production of toxins in milk.

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Table 17

NUMBER OF TOX^+ COLIFORMS OUT OF THE TOTAL ISOLATES

Organisms	Number of isolates	Number positive
E.coli	83	7 (8.43)
K.pneumoniae	121	9 (7.43)
E.cloacae	34	2 (5.88)
E.aerogenes	35	1 (2.86)
C.freundii	33	5 (15.15)
Total	306	24 (7.84)

Tox^+ - Toxigenic (Echeverriah et al., 1981)

Figures in parenthesis indicate the percentage

There could be chances for dilution as the toxin containing milk might be blended or used for recombination in the industry to increase the distribution volume.

The fluid response in the ligated loops to crude toxin preparation was the absolute reaction of the enterotoxin as the same sterile CYE with merthiolate did not give a similar response. This conclusion is supported by the recent report that rabbits immunized against pili antigen of non-ETEC did not inhibit the fluid secretion while a rabbit immunized against the ETEC cultures reduced or inhibited the fluid secretion (Molenda et al., 1983). The fluid secretion was non haemorrhagic, clear and secretary type (Sack et al., 1971). A minimum of 10 mgm of crude toxin might be essential to produce the secretory response (Klipstein et al., 1977). Two ml of the toxin broth used in this study would have had this as a minimum to elicit positive response. So in this study a complete toxin potential of coliforms had not been demonstrated since the other negative toxin preparations might have had some amount of toxin that would have been insufficient to elicit positive secretory response.

The other interesting feature was that the MRHA positive non-*E.coli* cultures responded equally or more to the bioassay of toxin. The hypothesis of intergeneic transfer of plasmids responsible for enterotoxin production (Guerrant et al., 1976; Klipstein and Engert, 1977) is supported by this study. This indicates that the plasmids responsible for enterotoxin production might associate with colonization factors in other non-*E.coli* coliforms also (Evans *et al.*, 1978; Evans *et al.*, 1979 Twedt and Boutin, 1979; Levine *et al.*, 1980). So future study necessarily warrant the study on the significance of MRHA pattern in other non-*E.coli* coliforms. More significance should also be given to non-*E.coli* coliforms, since they comparatively lack recognition as that of *E.coli*. The bioassay for detection of ST had been well developed since ST was considered to be non-antigenic. However, recent reports have proved that ST is antigenic and immunological tests are possible (Gianella *et al.*, 1981; Klipstein *et al.*, 1983a)

ST was also synthetically produced and found to have the antigenic characters of ST produced by bacteria (Klipstein *et al.*, 1983b). So the serological assay of ST as that of LT could be the forthcoming research problem for easy identification of ST.

(D) Summary

Three hundred and six isolates of coliforms were tested for MRHA of human A and bovine red blood cells. MRHA of human A and bovine red blood cells was observed in 29.41% isolates. MRHA of bovine cells only were observed only in 13.07% of isolates. Serological tests with imported CFA/I and CFA/II sera revealed two positive isolates out of 47 E.coli isolates. None of the non E.coli coliforms (83) was found antigenically related to CFA/I CFA/II. The significance of MRHA among non-E.coli coliforms was discussed. The dominance of human A and bovine (CFA/I pattern) over the other was reported and discussed. The presence of type I pili in K.pneumoniae was analysed and discussed. The bioassay of MRHA positive cultures revealed 14.89% of E.coli were positive for toxin production. C.freundii was positive at 45.45% level, followed by E.cloacae (33.33%) K.pneumoniae (15.52%) and E.aerogenes (12.50%). The results of the histopathological examination of the positive loops was furnished. The applicability of RILT in the bioassay was found to be sensitive. The future role of sero identification of ST was discussed.

CHAPTER V

CHAPTER V

STUDIES ON THE TOXIGENIC COLIFORMS IN THE ISOLATES FROM FARM ENVIRONMENT

(A) Introduction

A major problem in current efforts to identify pathogenic strain would be the identification of toxigenic coliforms. In specimens from patients, the pathogens frequently constituted only 10% of the E.coli isolates. In environmental specimens the incidence was less than 1%.(Mehlman and Romero, 1982).

The serological identification which was the easiest method of all the identification procedures was questioned because serological identification of the adhesins was incomplete since the cultures with few exceptions lacked enterotoxigenic potential (Scotland et al., 1981). However, the serology could be a tool for recognition of probable enterotoxigenic potential of ETEC strains in the absence of other practical tests (Scotland et al., 1981). But regrettably the antisera for determination of CFA, had not been standardized and only a limited sources are available commercially (Mehlman and Romero, 1982). Further additional colonization factors were being introduced and

and correlation of the presence of MRHA pili with toxigenicity did not always exist (Evans and Evans Jr., 1978; Levine et al., 1980; Gorbach et al., 1981; Thomas et al., 1982)

To find the correlation, serological studies of E.coli and other coliforms were attempted in this study with imported antisera. Demonstration of the presence of pili of toxigenic Escherichia coli strains and Klebsiella strains were also attempted under electron microscope. To attribute correlation of antibiotic resistance and enterotoxigenic potential, a study of the resistance pattern of toxigenic (tox^+) coliforms and nontoxigenic (tox^-) coliforms with common antibiotics for Gram negative bacteria was also made.

The probable environmental factors available for the toxigenic strains to produce toxins in milk were studied and discussed.

(B) Materials and Methods

(i) Serological Tests:

(a) Source of specific sera:

The antisera for CFA/I and CFA/II were kindly

provided by the Center for Vaccine Development, University of Maryland, Baltimore, U.S.A. In all 25 isolates were tested. K 99 specific antisera received from Rijks Institute, Netherlands were used for serological identification of K 99 pili among tox$^+$ E.coli.

(b) Bacterial isolates:

All the seven extra intestinal ETEC isolates which were proved to possess enterotoxigenic potential were used in this study as has been done by Scotland et al.(1981) The other non E.coli isolates which were positive in MRHA with human A, and bovine red blood cells, were also tested to find out the antigenic relationship if any with CFA/I and CFA/II. The seven E.coli strains were also tested against K 99 specific serum.

(c) Slide agglutination test: (Guinee et al., 1976; Levine and Rennels, 1978)

The over night growth on CFA agar at 37°C was used for slide agglutination test. The bacteria picked out from five colonies were suspended in 1:500 and 1:100 dilution of CFA/I and CFA/II serum, on a new dry glass slide. The working dilution of the sera were less than 1:1000 and 1:200 which was earlier used with MRHA positive strains. Control serum from a rabbit was also used.

Bacterial samples showing apparent agglutination with CFA/I or CFA/II serum and absence of such agglutination in the control rabbit serum were considered as CFA/I or CFA/II positives. Likewise all the non-E.coli cultures were tested with CFA/I and CFA/II serum. The ETEC strains grown on minca agar (Guinee et al., 1976) were also tested with 1:100 dilution of K 99 serum obtained from Rijks Institute, Netherlands.

(ii) Preparation of CFA specific serum:

Antisera to CFA/I (Evans et al., 1975) was prepared with local isolate that was positive for slide agglutination test. The ETEC CFA/I positive culture was cultivated in Roux flask on CFA agar. After overnight incubation, the growth was washed in 100 ml of PBS with sterile beads. The suspension was centrifuged and washed with 0.1% sodium azide PBS. Finally 0.1% sodium azide PBS was added to the original volume and the precipitate was suspended. The suspension was used to immunize two rabbits each weighing 2 kgs by repeated 4 inoculations (Strim et al., 1967). The dose was increased from 0.5 ml to 2 ml in the five day intervals between the inoculations. The rabbits were bled by the ear vein, eight days after the last injection. The sera were adsorbed with CFA/I positive culture grown at

18°C until the sera failed to agglutinate CFA/I culture grown at 18°C (Levine et al., 1980). However, CFA/I was still strongly agglutinated. Antiserum to CFA/II was likewise prepared.

These sera were compared with the imported sera by by testing 7 ETEC cultures 25 non ETEC cultures and 17 other non E.coli toxigenic coliforms.

(iii) Serotyping of ETEC cultures:

The ETEC cultures were serotyped for O serogroup at the Central Research Institute, Kasauli for epidemiological purposes.

(iv) Indirect Fluorescent antibody technique (IFA)

The CFA/I and CFA/II specific antisera were used at dilutions of 1:100 and 1:10 respectively. The fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG was obtained from Miles Yeda Ltd, Israel. The FITC was used at a concentration of 1:10.

(a) Bacterial isolates:

The two CFA/I and CFA/II positive strains by agglutination test were taken up for further study by IFAT.

(b) Test procedure: (Hadad and Gyles, 1978)

The CFA/I and CFA/II positive ETEC isolates were grown on CFA agar overnight. Thin smears were made on a microscopic slide for each culture. The smears were air dried and fixed in 95% ethanol for two minutes. They were placed in a moisture chamber and flooded with the respective antisera. After incubation at room temperature (25°C) for 30 minutes, the slides were washed and held for 30 minutes in 0.1 M PBS (pH 7.2), which was whirled in a beaker, with the help of a magnetic pellet and stirrer. Three changes of PBS were used. The conjugate was added and held at room temperature for 30 minutes. The slides were once again washed with 0.1 M PBS (pH 7.2) and allowed to dry. Using FA mounting fluid the coverslips were fixed The results of two CFA positive cultures identified by agglutination test were confirmed.

(v) Electron Microscopy:

The CFA/I positive E.coli was grown for six hours in CFA agar. The Klebsiella pneumoniae were grown on trypticase soy broth for expression of pili (Fader et al., 1979). The growth on solid media was suspended in the distilled water. Ammonium molybdate 0.16 M was mixed with 0.1 ml of

bacterial suspension in equal quantity. A drop of the mixture was applied to a carbon coated colloidion grid. The grids were examined under JEOL - transmission type electron microscope from 10,000 x onwards.

(vi) Stable toxin production in milk: (Glatz and Brudvig, 1980a)

Toxigenic coliforms isolated in this study were used, for toxin production. The study group consisted of two ETEC, one toxigenic Klebsiella and another Citrobacter sp. About 10 - 20 ml of sterilized standard milk (4.5% fat and 8.5% solids non-fat) in one litre Roux flask was inoculated with a loop full of bacterial colonies picked out from overnight growth on 5% bovine blood agar. The inoculated milk was incubated at 37°C for 16 hours, with periodical vigorous shaking for aeration. The bacterial density at the 16th hour was determined in violet red bile agar (VRBA). The whey was separated and preserved by addition of 1:10,000 merthiolate and tested in the bioassay within a week. Before testing, the whey was held at 90°C for 10 minutes and centrifuged at 5000 rpm for 10 minutes. The supernatant was taken up for the bioassay. The rabbit ileal loop technique (Robichaud et al., 1978) and infant mouse diarrhoeal score test (Moon et al., 1978) were adopted in this procedure. The results of ST production

were recorded. Control test with non ETEC was carried out.

(vii) Antibacterial drug sensitivity test: (Bauer et al., 1966)

(a) Susceptibility discs:

The "Biodisc" '212' for Gram negative bacteria containing known amounts of 12 antibiotics or chemotherapeutic agents were obtained from Pasteur Biological laboratories (India).

(b) Media:

Mueller-Hinton agar was poured in glass petri dishes to a depth of 4 mm. The sterile moisture free plates after 24 hours of incubation were used.

(c) Preparation of inoculum:

The 24 toxigenic coliforms and randomly picked 26 nontoxigenic coliforms were used in this study. The overnight growth of organisms were inoculated into trypticase soy broth by transferring four or five colonies. On incubation for six hours at 37°C, broth cultures with moderate turbidity were used as inoculum.

(d) Streaking of plates:

The Mueller Hinton agar plates were streaked with the aid of sterile cotton swab, dipped and squeezed with the inoculum streaking successively on three different directions to obtain a sheet of growth. The plates were incubated for about 3-5 minutes for the inoculum to dry. The susceptibility discs were placed and gently pressed over the inoculated surface. On overnight incubation the results were read.

(e) Reading of the plates:

The diameter of the each zone including the disc diameter were measured at the obvious breakpoints with a vernier calliper to the nearest point. The readings were interpreted with the zone size interpretative table provided by the manufacturer. The results were recorded, as resistant which included both resistant and intermediate column and absolutely sensitive.

(C) Results and Discussion

(i) Serological Test for Colonization Factor Antigens:

The seven isolates on agglutination test with CFA/I and CFA/II specific antiserum revealed that one strain was

positive for CFA/I and another for CFA/II (Table 18). The other five strains were negative either for CFA/I or CFA/II. The above two strains are of significance since they possess both the virulence attributes i.e. enterotoxin production and adhesins.

Perhaps this is the first report of isolation and demonstration of potential enterotoxigenic strain of human significance from dairy environment in the part of the country.

The pathogenic potential of the other five isolates which showed prevalence of STb, but lacking in CFA/I or CFA/II is difficult to ascertain. The possibility is that these strains may harbour other colonization factor antigen apart from CFA/I or CFA/II. Recent reports suggested that human enterotoxigenic strain may harbour a third colonization factor antigen (E 8775) (Thomas et al., 1982). So, a battery of such new antigens may be discovered in due course within which the other five E.coli may fit in. The other possibilities should also be considered in view of the recent report of the human enterotoxigenic strains that in addition to pili other factors such polysaccharides, slime-layer or lectin may mediate adhesion (Deneke et al., 1979; Levine et al., 1980)

Table 18

SLIDE AGGLUTINATION TEST OF TOX$^+$ COLIFORMS WITH SPECIFIC SERA

Organisms	Number tested	CFA/I		CFA/II		K 99	
		Positive	Negative	Positive	Negative	Positive	Negativ
E.coli	7	1	6	1	6	1	6
K.pneumoniae	9	0	9	0	9	0	9
E.cloacae	2	0	2	0	2	0	2
E.aerogenes	1	0	1	0	1	0	1
C.freundii	5	0	5	0	5	0	5
Total	24	1	23	1	23	1	23

Tox$^+$ - Toxigenic coliforms (Echeverriah et al., 1981)

(ii) Preparation of CFA sera:

CFA/I and CFA/II sera are not fully standardized and are not commercially available (Mehlman and Romero, 1982). An attempt was made to prepare CFA/I and CFA/II antisera for routine diagnostic purposes. The 24 entero-toxigenic cultures, 25 non-ETEC cultures were tested with imported CFA/I and CFA/II antisera and with the antisera prepared locally. There was 100% correlation between the tests. This indicate that local antisera can be prepared for large scale screening purposes.

(iii) Serotypes of toxigenic E.coli:

The results of O serotyping revealed that five were O:11 and the other two were untypable. The source of the five typical strains were two from soil one each from personnel, water and teat (Table 19). This showed that the same O sero group which was found in the milk was also seen in the farm environment. So the possibility of contamination of milk exist in the farm environment. In these circumstances, inspite of the absence of the adhesin/pili as observed, the five strains of the E.coli may still prove to be potentially pathogenic if stable toxin is produced in the milk.

Table 19

SOURCES OF TOX$^+$ COLIFORMS FROM THE FARM ENVIRONMENT

Organisms	Air	Water	Personnel	Utensils	Teat	Milk	Total
E.coli	-	1	1	-	1	4	7
K.pneumoniae	1	2	-	4	1	1	9
E.aerogenes	1	-	-	-	-	-	1
E.cloacae	-	1	-	-	-	1	2
C.freundii	1	1	-	1	-	2	5
Total	3	5	1	5	2	8	24

Tox$^+$ - Toxigenic

The classical O groups associated with CFA/I are O:15, O:25, O:63 and O:78. The above classical O groups does not include strains belonging to other O groups which were reported occasionally (Gaastra and Graaf, 1982). It was thought earlier that CFA/II occurs exclusively in O group 6 and 8 (Evans and Evans Jr., 1978). But later reports confirmed the prevalence of CFA/II in other groups apart from O:6 and O:8 (i.e.) O:80, O:85 and O:115 (Craviato et al., 1982). Further the production of ST, LT, CFA/I and CFA/II are controlled by plasmids. Hence the prevalence may be independant of serotypes in E.coli (Evans Jr., et al., 1977). However, it has been proved that there is clustering of ETEC strains in certain O groups perhaps due to unusual stability of virulence plasmids in certain serotypes (Evans Jr. et al., 1977).

The O group isolated in the present study is a known enteropathogenic O group of infants and adults (Ranganathan, 1973; Singh and Ranganathan, 1974; Orskov and Orskov, 1977; Mehlman and Romero, 1982; Kumar et al., 1982). The presence of serogroup O:11 in the dairy environment of Indian farms were also reported by Tripathi and Soni (1982). In the present study the E.coli strains of O:11 was found to possess both virulence attributes of enterotoxin and adhesin. It was postulated that the serotypes of ETEC

may vary from region to region (Sack, 1980). The isolates that produced ST only were found distributed among 15 O groups while the isolates that produced both the toxins were restricted to only four groups (Back et al., 1980). This indicated that if the isolates are only ST producers, they are likely to be distributed in various non-classical serogroups. Hence, the present findings confirmed the possibility of enterotoxigenic strains in other O groups apart from classical sero groups.

Out of the total isolates tested from the environment the prevalence of toxigenic non-E.coli coliforms is presented in table 20. The water and equipments rinsed with such water yielded high number than the other environmental niches. Hence the contaminated water would be a potential source if used untreated for rinsing purposes.

(iv) Application of serological tests:

As per the recent reports it is postulated that the virulence attributes of human E.coli (ie.) adhesin and enterotoxin are borne on the same plasmid and there is a possibility of clustering of human ETEC in certain well defined serogroups (Gaastra and Graaf, 1982). This hypothesis recommends the use of serotyping for routine

Table 20

TOXIGENIC ISOLATES IN THE FARM ENVIRONMENT

Source	Number of isolates	Number positive
Air	38	3 (7.89)
Water	38	5 (13.16)
Personnel	35	1 (2.86)
Teat	65	2 (3.08)
Utensils	56	5 (8.93)
Milk	74	8 (10.81)
Total	306	24 (7.84)

Figures in parenthesis indicate the percentage

diagnosis of ETEC mediated diarrhoeal infections of human and for epidemiological study of E.coli contamination in the milk foods. While the serogrouping may be a screening test for EPEC and ETEC sero groups in dairy products, the mere presence of it does not necessarily warrant the rejection of the milk foods possessing such serogroups. So, the reports stating that mere O grouping is not a satisfactory tool and identification of ETEC for enterotoxin assay has also to be borne in mind (Glatz and Brudvig, 1980b Orskov and Orskov, 1980).

(v) Demonstration of K 99 pili:

Out of the seven ETEC tested with K 99 specific serum, one isolate was positive for K 99 antigen. The K 99 was serologically demonstrated from the ETEC isolated from the milk of a cow in group A farms. The isolate might have had its origin from the interior of the udder or from teat tip. There was also a chance of contribution by the milker.

This colonizing factor of E.coli mediate adhesin in calves, lambs and pigs (Isaacson, 1978). The farm was a mixed type having swine population also. The reported classical serotypes were O:8, O:9, O:20 and O:101 in calves and lambs and O:64 and O:101 in swines (Gaastra an

Graaf, 1982). A random study of toxigenic and non enteropathogenic E.coli with K 99 specific antiserum did not reveal the prevalence of K 99 positive O:11 serogroup among cows and calves in Pantnagar, India (Kumar et al., 1982). Hence this reporting of K 99 positive O:11 serogroup in the dairy environment might also probably be a first report in this country.

(vi) Indirect fluorescent antibody technique (IFAT)

The CFA/I and CFA/II positive strains stained by indirect immunofluorescence methods revealed peribacterial fluorescence (Figure 8 and 9). The indirect fluorescent antibody technique results confirmed slide agglutination test results.

Rapid identification of ETEC is considered essential for epidemiological studies of food poisoning (Marrier et al., 1973). However, conventional procedure such as isolation, identification and demonstration of virulence attributes are time consuming and labour oriented techniques. The efficiency of IFAT for identification of ETEC had been reported by several workers (Hadad and Gyles, 1978; Isaacson, 1978; Moon et al., 1978; Benfield and Francis, 1980). In this study the demonstration of CFA/I and CFA/II

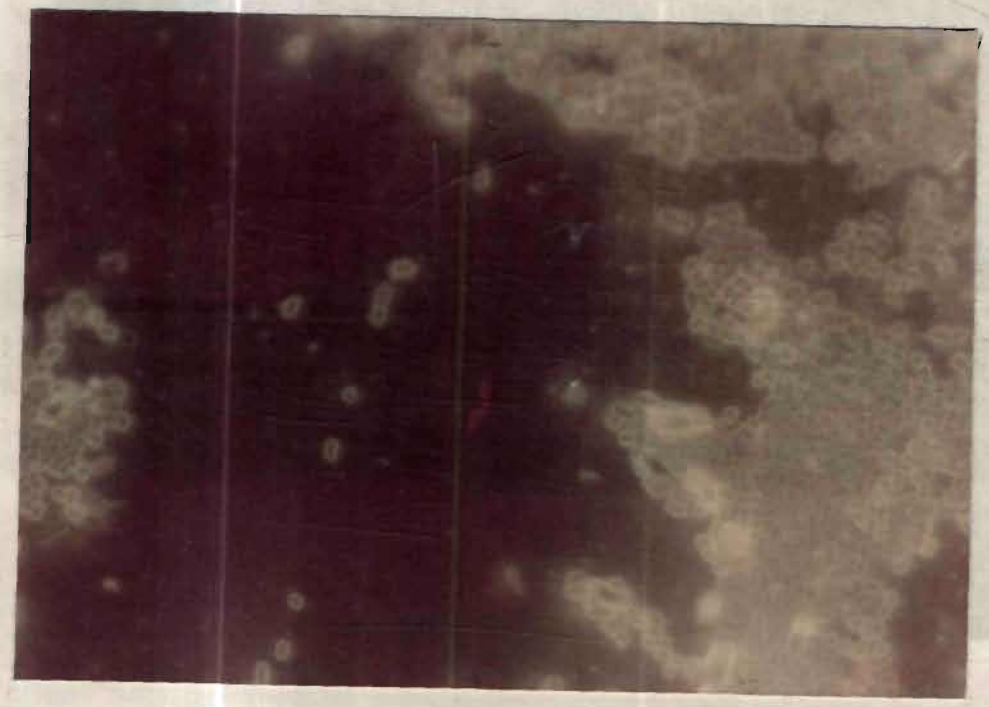


Figure 8 Colonization factor antigen I in indirect fluorescent antibody technique with CFA/I specific sera. The peri bacterial fluoresence is observed.

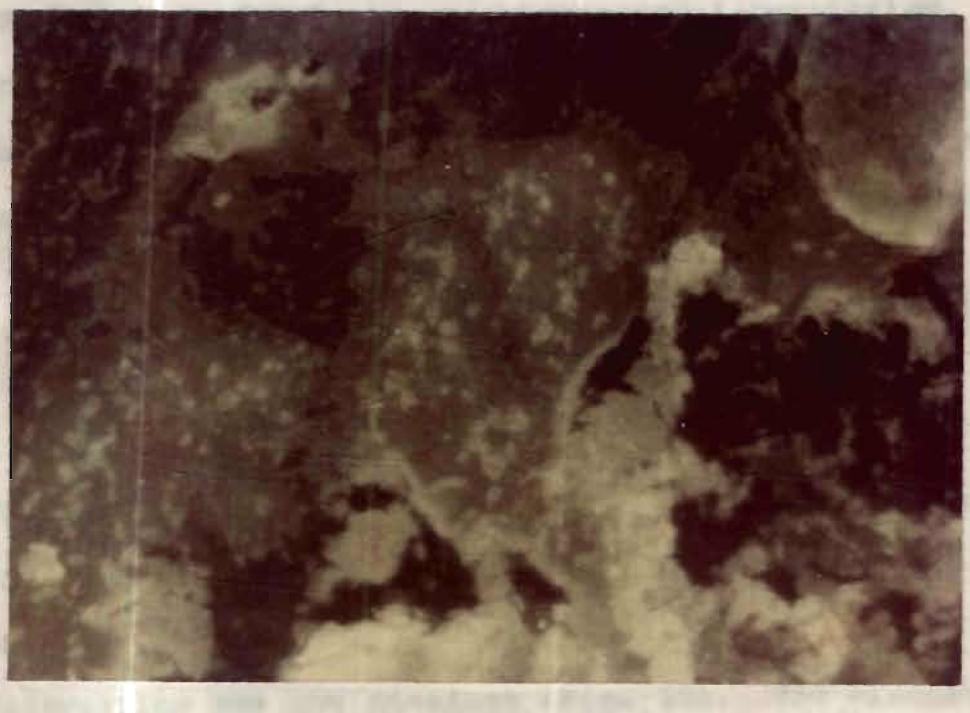


Figure 9 Colonization factor antigen II in indirect fluorescent antibody technique with CFA/II specific sera. The peribacterial fluorescence is seen.

antigen in two ETEC cultures by agglutination test had been confirmed. Since agglutination tests are easy and can be performed in ordinary laboratories, the IFAT, may be used as a tool for confirmation of the presence of CFA/I and CFA/II in the sophisticated laboratories. However, this test could not be applied in detecting a particular serotypes of E.coli in cheese samples (Yoger and Kershaw, 1974).

(vii) Electron microscopic study:

The toxigenic cultures with CFA/I and CFA/II were further examined under electron microscope. The moderate piliation were visualised as a fuzzycoat around the organism. In case of piliated bacteria cell wall demarcation was not distinct (Fig. 10). The electron microscopy is one of the methods used for the classification and the confirmation of the pili.

A non-piliated Klebsiella pneumoniae with capsule is seen in Fig. 11 . This toxigenic Klebsiella may probably be in the non-piliated phase of type 1 pili (Fader et al., 1979). The capsule is clearly seen in the Klebsiella. The fuzzy coat is absent and the cell wall is clearly distinct.

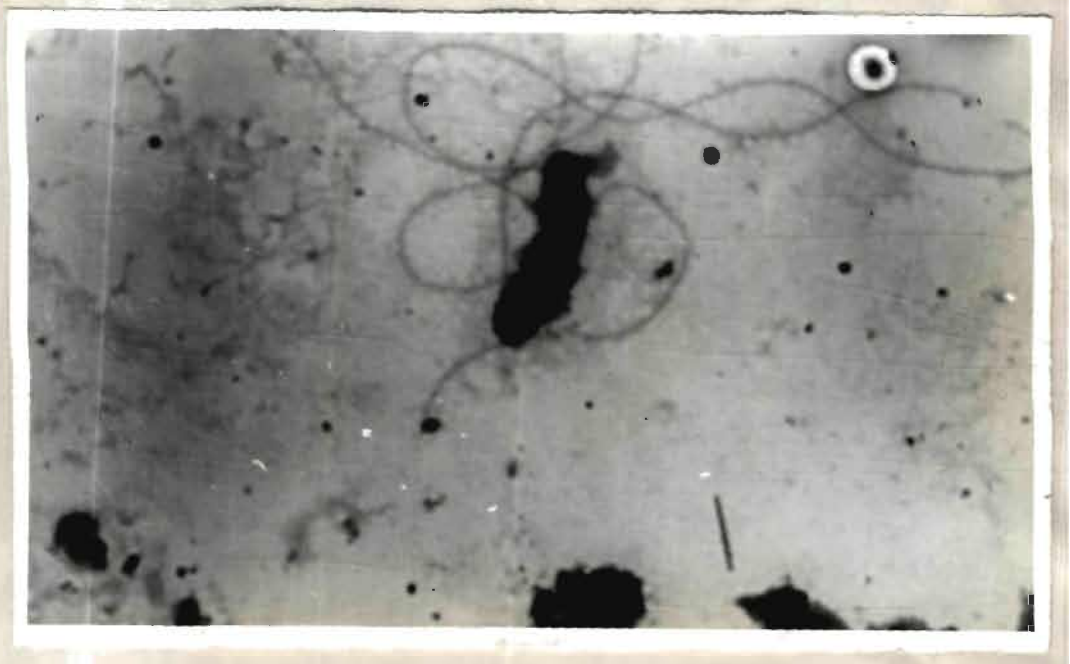


Figure 10 Electron microscopic picture of Escherichia coli with moderate piliation. The pili had shown tendency to aggregate twisting around each other. The cell wall is not clearly demarcated. Long peritrichous flagella also seen, x 10,000.

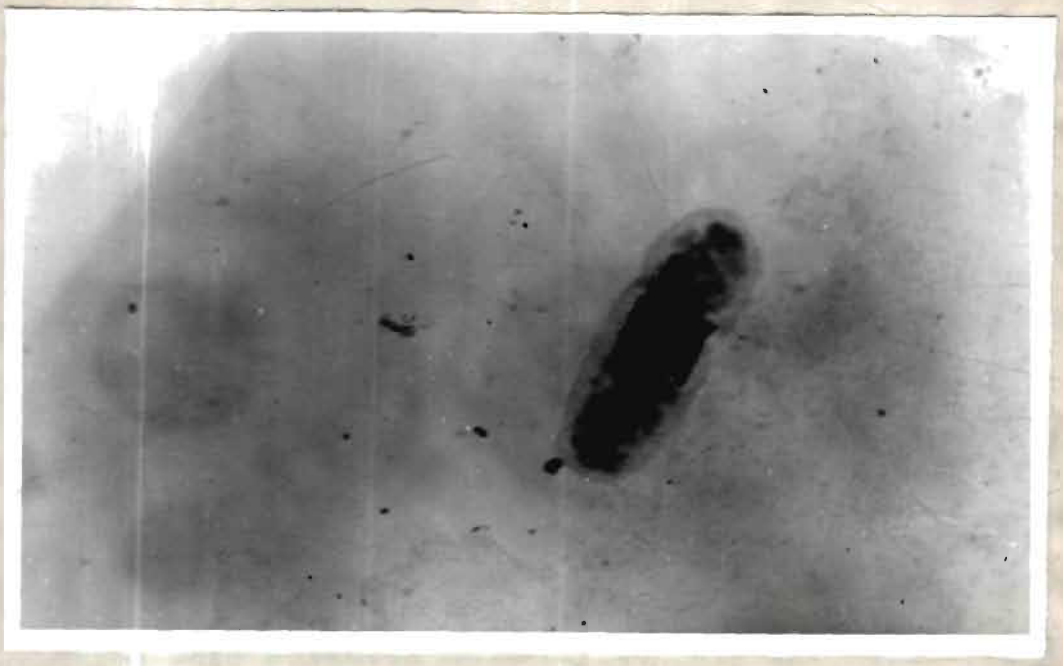


Figure 11 Electron microscopic picture of non-piliated Klebsiella pneumoniae with clear and distinct cell wall x 13,000

The CFA/I pili appeared to be non tubular. The fibres also showed a tendency to aggregate twisting around each other (Wadstrom et al., 1978).

(viii) Production of stable toxin in milk:

In the present study for stable toxin production stationary cultures having low volume of milk per volume of flask was used (Gomes et al., 1979). It was considered necessary that for production of enterotoxin sophisticated equipments such as incubator shaker were essential as the agitation and aeration had a significant effect on production of both ST and LT. Agitated cultures contained twice the number of bacteria than of stationery cultures (Mundell et al., 1975). In this study, the stationary cultures were agitated manually for aeration simulating a condition of transporting milk at ambient temperature in cans. In the field, low volume of milk per volume of can had been observed by this researcher during transport. This could provide optimal conditions for toxin production.

Out of four cultures inoculated in the milk media, two proved to be positive for enterotoxin production as assayed in rabbit ileal loop technique (RILT) and infant mouse test (IMT). In the IMT diarrhoeal scores were taken into consideration for interpreting result

(Moon et al., 1978). The test was conclusively positive as there was death of one mouse with the diarrhoeal scores of more than three (Fig. 16). One out of the two E.coli cultures was positive in the RILT and one out of one Citrobacter was positive in IMT and RILT. The bacterial density at 16 hours after inoculation for toxigenic cultures were six and nine logs (Table 21). The control with non-ETEC was negative in RILT and IMT.

Maximum yield of stable toxin was observed after seven hours of incubation under forced aeration and agitation in BHI broth (Lallier et al., 1980). The optimum pH would be 7.2 with variations upto 7.8. At this level of 7.2 - 7.8 maximal ST production occurred (Lallier et al., 1980)

The positive results in two of the four cultures indicate the potentiality of the enterotoxigenic coliforms to produce the toxin in the milk. The milk media could probably contribute the required nutrients as that present in ST broth (Lallier et al., 1980). The failure of the other two organisms to produce toxin might probably be due to adverse pH due to lactic acid production by these coliforms (Lallier et al., 1980). Alternatively the quantum of toxin might be lesser than

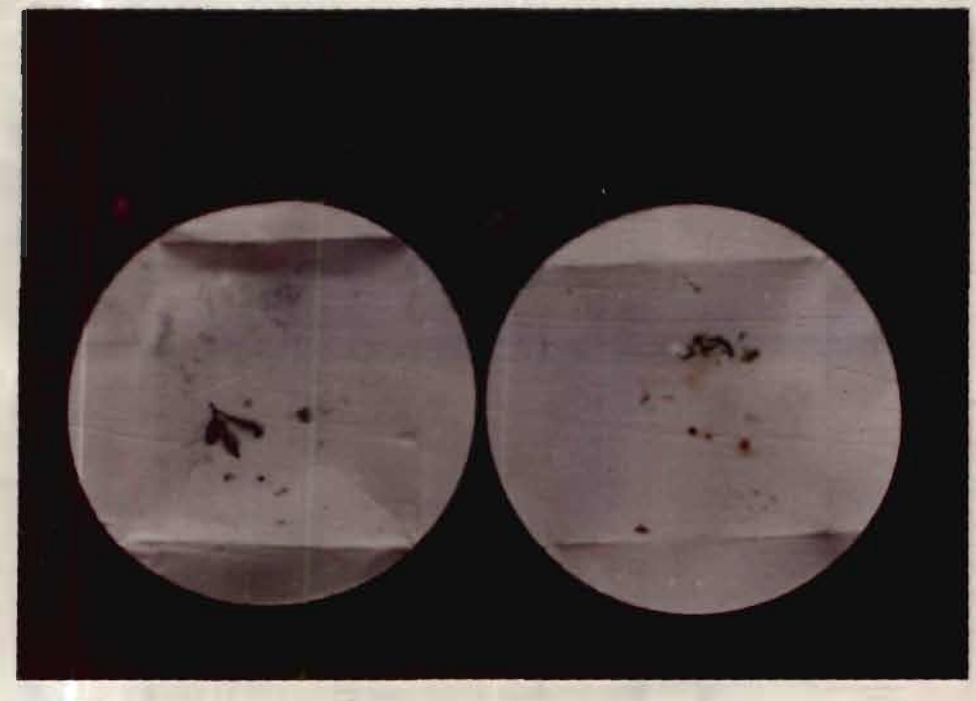


Figure 16 Infant mouse diarrhoeal score test in toxin assay of milk. The left filter paper shows the control while the right shows the positive result with reddish brown diarrhoeal matter.

Table 21

IN VITRO HEAT STABLE TOXIN PRODUCTION IN STERILE MILK AND ITS BIOASSAY

Tox$^+$ isolates	Number tested	Number positive	Mode of assay	Bacterial density/ml at 16 hours
E.coli	2	1	RILT	1.5×10^6
K.pneumoniae	1	-	-	-
C.freundii	1	1	IMT RILT	1.2×10^9

RILT - Rabbit ileal loop technique

IMT - Infant mouse test

10 mg per ml so that the toxin level was insufficient to elicit reaction in the bio-assay model (Levine et al., 1980). Earlier study in milk, also indicated that only one out of three known ETEC cultures produced only heat labile toxin (LT) (Glatz and Brudwig, 1980a). No ST was produced in the milk as reported by these authors.

So this experiment has conclusively proved that milk may substitute the CYE toxin broth. With ideal volume of milk for a stationary culture, the milk may support the growth of enterotoxigenic coliforms and provide optimal conditions for production of toxin. As the toxin is preformed without the need for adhesin factors, the milk may be potentially hazardous to produce diarrhoeal illness to the consumers especially in infants. Since these toxins being heat stable at 100°C for 30 minutes (Klipstein and Engert, 1975; Klipstein and Engert, 1976a,b) they could also be potent and active within the usual heat treatment.

The optimum conditions for the production of stable toxin are explicitly available when the milk is transported in the half-filled or quarter filled cans providing sufficient aeration for the toxigenic cultures which might have entered the milk from the farm environment. The requisite amount of agitation is provided when these milk

cans are transported in lorries in the village milk routes. The stable toxins thus produced may be heat stable and may be available in the fluid milk given for babies. Probably the infant is not able to tolerate even the diluted toxin which causes gastro intestinal disorders. The adult may be able to withstand as the adult mouse were found to be unsuitable for the estimation of diarrhoeal score (Moon et al., 1978). Reports are available that many fold increase of coliforms occurs when the milk is transported in cans (Fluckiger et al., 1980).

This hypothesis of availability of preformed toxin in the milk exposed to near 37°C with aeration due to agitation and growth of coliforms within about five hours, may have to be further studied in detail. The consumer attitudes of feeding the fresh milk to the babies may support the view that the heat processed dairy milk may be a cause for the infantile diarrhoea due to the stable toxins.

(ix) Antibiotic sensitivity:

The results of the antimicrobial disc diffusion technique for enterotoxigenic coliforms and non-enterotoxigenic coliforms are presented in table 22.

Table 22

COMPARISON OF DRUG RESISTANCE IN TOX$^+$ AND TOX$^-$ COLIFORMS

Antibacterial agent	Tox$^+$ coliforms 24	Tox$^-$ coliforms 26	
Ampicillin	100 (24)	69.23 (18)	**
Cephaloridine	100 (24)	46.15 (12)	**
Gentamicin	0	0	
Chloramphenicol	25 (6)	7.69 (2)	**
Carbenicillin	100 (24)	100 (26)	
Polymyxin-B	100 (24)	100 (26)	
Kanamycin	62.50 (15)	7.69 (2)	**
Co-trimoxazole	12.50 (3)	0	*
Streptomycin	58.33 (14)	15.38 (4)	**
Sulphatriad	95.83 (23)	84.62 (22)	
Tetracycline	100 (24)	46.15 (12)	**
Colistin	100 (24)	100 (26)	

Figures in parenthesis indicate the number of isolates

* Significant ($P < 0.05$), Tox$^+$ - Toxigenic coliforms

** Highly significant ($P < 0.01$) Tox$^-$ - Nontoxigenic coliforms

(Echeverriah et al.,19

It may be seen from the table that on statistical analysis tox^+ coliforms significantly differed from tox^- coliforms in seven out of twelve antibiotics. The resistance was noticed in ampicillin, cephaloridine, kanamycin, streptomycin and tetracycline. In co-trimoxazole the resistance was moderate.

The drug resistance pattern among tox^+ and tox^- coliforms are presented in table 23. Among tox^+ coliforms, for seven antibiotics five isolates (20%) were resistant. For eight antibiotics five isolates (20%) were resistant. Twelve isolates (50%) were found resistant to nine to ten antibiotics out of twelve employed. Among tox^- coliforms as much as 84.61% were found resistant from four to six antibiotics.

In a genetic study of ETEC isolated from pig with diarrhoea it was found that the genes controlling the antibiotic resistance and enterotoxin production were located in the same plasmid (Gyles et al., 1977). Ampicillin resistance had also been found to transpose into an ent plasmid in E.coli strain of human origin (McConnell et al., 1979). In this study also there were highly significant development of resistance for seven

Table 23

DRUG RESISTANCE PATTERN AMONG TOX^+ AND TOX^- COLIFORMS

	Number of antibiotics employed									Total tested
	4	5	6	7	8	9	10	11	12	
Tox^+ coliforms	-	-	-	20.83 (5)	20.83 (5)	33.33 (8)	16.67 (4)	4.17 (1)	4.17 (1)	24
Tox^- coliforms	38.46 (10)	26.92 (7)	19.23 (5)	7.69 (2)	3.85 (1)	3.85 (1)	-	-	-	26

Figures in parentheses indicate number of isolates

Tox^+ - Toxigenic coliforms

Tox^- - Non toxigenic coliforms

antibiotics among tox$^+$ coliforms which subscribed to the view of Gyles et al.(1977) and McConnell et al.(1979). This confirmed the early view with ETEC strains that genes which coded for antibiotic resistance and enterotoxin production are frequently transferred together (Echeverria et al., 1978a). This study illustrates that these plasmid borne characters are not only prevalent among ETEC, but also prevail in other coliforms also.

The tox$^+$ coliforms were hundred percent resistant to ampicillin and tetracycline (Merson et al., 1980). This is in contrary to the reports of Bishop et al.(1980) and Sack (1981) where the mastitic coliforms and ETEC were found to be susceptible to tetracycline, cephaloridine and ampicillin. However, the findings with tox$^-$ coliforms correlates with the study of Bishop et al.(1980). This once again confirms that the genes which code for antibiotic resistance and enterotoxin production are frequently transferred together. If genes encoding for antibiotic resistance and enterotoxin production are frequently carried on the same plasmids, antibiotic selective pressure should increase the prevalence of tox$^+$ coliforms in the dairy environment where antibiotics may be carelessly used in the treatment and as feed supplement.

This type of study was taken up by Echeverria et al. (1981) with the E.coli in human population with judicial administration of antibiotics. A controlled study of this sort in a dairy farm may enlighten the transposing of enterotoxin production in the plasmids for antibiotic resistance. The interspecies transfer of R factors along with the transfer of characters for enterotoxin production may also be a further study of interest.

(D) Summary

Out of seven ETEC isolates one strain was positive for CFA/I and another was positive for CFA/II by slide agglutination test. The presence of both the virulence attributes from the dairy farm environment may be first report in this country. The other five toxigenic culture may probably have new colonization factors or other adhesion factors. The other toxigenic coliforms did not possess any pili antigenically related to CFA/I or CFA/II as demonstrated by agglutination test. CFA specific sera were locally prepared and found to compare with the imported sera. The serogroups of the ETEC were identified as O:11 which were rare amongETEC possessing CFA/I and CFA/II. The distribution of serotype O:11 of E.coli in

farm environment was discussed. The farm water and farm utensils contributed highest tox^+ coliforms, than other farm sources. K 99 was demonstrated in one ETEC which also possessed CFA/II. The presence of CFA/I and CFA/II were confirmed by IFAT. Demonstration of piliated phase of E.coli and non piliated phase (type I) of Klebsiella was done under electron microscope. It was also found, that the milk could support the growth of toxigenic cultures and the production of toxin was demonstrated in RILT and infant mouse diarrhoeal score test. The drug resistance pattern of toxigenic coliforms were studied and compared with that of non-toxigenic coliforms. Association of antibiotic resistance and enterotoxin production was observed. The toxigenic coliforms were absolutely resistent to tetracyclines and ampicillin.

CHAPTER VI

CHAPTER VI

RAPID SCREENING TESTS TO DETECT MILK QUALITY

(A) Introduction

The milk is a perishable item where the bacterial flora is in a dynamic state of multiplication, and if uncontrolled, would lead to spoilage, and consequent salvage operations (Cousins, 1982). The bacterial flora of raw milk depended upon the care in its production (Mabbit, 1980a). The antibacterial lactenin system available in fresh clean raw milk might keep the bacterial multiplication especially that of Gram negative bacteria (GNB) under control for few hours from the production because of its bactericidal property (Bjorck, 1978), and chilling of milk within this period might keep the existing bacterial flora in its minimal metabolic status by lengthening the generation time (Ayres et al., 1980). But these antibacterial system might play a minor role in the defence mechanisms and probably did little to inhibit the dynamic multiplication of high initial contaminants, when the raw milk was exposed to tropical temperature before chilling (Ayres et al., 1980).

The estimation of the bacterial quality of raw milk would help in its grading (Indian Standards No.1479 -

Part I-1960). Standard tests such as dye reduction tests to measure the metabolic activity of micro-organisms could not be interpreted in terms of actual numbers of bacteria since they were dependant on the metabolic rates of microbes. Hence these tests could only serve as an index of microbial loads. Further they are not reliable for GNB (Luck, 1972, Ayres et al., 1980, Cousins, 1982). The enzymes of the GNB, could break down the milk fat and protein (Mabbit, 1980a). The role of GNB in the keeping quality of refrigerated raw milk had been comprehensively reviewed (Cousins, 1982) the estimation of which would be necessary as they were dominant in refrigerated milk (Ayres et al., 1980). The routine plate counts for general viable count or coliform count would consume a minimum of 18 hours being the incubation period (Indian Standards No.1479-Part III-1962, Indian Specifications 5402 - 1969).

Since the time involved for investigation is very limited the microbiologists should evolve new tests or apply available suitable tests for rapid screening of milk for enzyme producing GNB from a new unknown source. A fully automated systems was developed for one hour identification of the Enterobacteriaceae utilizing

fluorogenic substracts and luminescent tests and reactions (Hartman and Minnich, 1981), Other systems for rapid estimation were also reported (Gnan and Luedecke, 1982, Waes and Bossuyt, 1982).

The Limulus amoebolysate test is a reliable test for the presence of GNB. Here the clottable blood protein of Limulus (horse shoe crab) when allowed to react with the endotoxin of the GNB, would form gelation thus indirectly revealing their presence in the sample of milk, in one hour. This is considered as a rapid test to evaluate the number of GNB by appropriate calculation (Jay, 1977; Jay et al., 1979; Jay, 1981). Chromogenic LAL method had been recently developed in the diagnosis of gonococcal and nongonococcal urethritis in man. The test results had been observed within ten minutes (Prier and Spagna, 1983). The LAL had been applied to detect Gram negative bacterial endotoxins in normal as well as in mastitis milk (Hartman et al., 1976)

This preliminary study is an attempt in the application of Limulus amoebolysate test as a rapid screening test under Indian conditions.

(B) Materials and Methods

(i) Glassware and distilled water preparation:

All glassware and utensils employed in the LAL procedures were rendered pyrogen free. The glasswares were completely immersed for an hour after thorough cleaning and washing in fresh pyrogen free distilled water. The glasswares were packed with fresh aluminium foils and sterilized at 190°C for two hours on two subsequent days.

The fresh pyrogen free triple glass distilled water collected in a sterile container just prior to test was distributed aseptically in presterilized milk dilution bottles in 99 ml quantity and in test tubes in nine ml quantity using measuring jars and pipettes specially prepared for LAL test. The pyrogen free water thus prepared was utilized within two days of preparation. The water samples were twice tested for their pyrogenic contents in rabbits as described by Kaplan and Timmons (1979).

(ii) Milk samples:

Individual cow's milk samples and pooled milk samples collected in the pyrogen free glass container

were frozen till the test. A minimum quantity of 20 ml was collected for each test.

(iii) Test procedure (Jay, 1977)

The LAL test was performed by adding 11 ml of quickly thawed milk into 99 ml of pyrogen free water. The earlier attempt to extract the inhibitors by addition of equal volume of chloroform as suggested in endotoxin detection of blood plasma (Anon, 1973) and followed by Hartman et al.(1976) was discontinued in these studies for simplification as applied in meat studies by Jay (1971), Jay et al.(1979). To extract GNB and endotoxins in this study, the milk dilution bottle was beaten in the palm of the hand, 25 times within 10 seconds, with five minutes interval for three times. One ml of particle free homogenate taken below the upper layer of fat was placed into a sterile pyrogen free test tube. Serial dilutions were made up to 10^{10} for just drawn milk and fresh raw milk and upto 10^{18} for pasteurized milk, pooled aged milk and curdled milk samples. The LAL test was completed by inoculating 0.1 ml of each dilution in the specially prepared narrow sugar tubes to which 0.1 ml of lyophilized standard E.toxate® dry concentrate of Sigma Chemical, Saint Louis, Missouri, USA, diluted with pyrogen free water as

suggested by the manufacturer, was added. A positive control of reference endotoxin was simultaneously added with Limulus amoebocytelysate. The tubes were recapped and kept in a water bath at 37°C for one hour. Totally 25 standardized tests were performed limiting to five samples each, because of limited availability of the E.toxate ®. Gelation was recorded at the appropriate highest dilution for which the results were scored in modification of the manner suggested by Anon,(1973).

Positive results:

+++ = A solid gel formed at the end of one hour

++ = A soft gel formed at the end of one hour

\+ = Only an increase in viscosity. No gel formation after one hour.

Negative results:

In the absence of any of the above changes the results were recorded as negative.

With the same milk, serial dilutions were made upto 10^6 with buffered dilution water (PBS+$Mgcl_2$) (Richardson et al., 1980) for plating out, to enumerate coliform count in violet red bile agar (VRBA). Results were recorded as the number of coliforms per ml of milk.

(C) Results and Discussion

The water for dilution tested in rabbits did not show any rise in temperature and hence considered as pyrogen free. The ability of the Limulus test to detect endotoxins of GNB by gelation, of various samples of milk is presented in the table 24.

Incidences of false negatives in the lower titres such as 10^1 and 10^2 were encountered while gelation was observed in higher titres. Likewise, stray false positive: were also recorded in highest titres, while the corresponding lower titres, showed negative reactions.

The gelation with soft gel formation in the lower titres with increase in viscosity in the subsequent titres are shown in the figure 12. The last tube had shown the absence of gel and viscosity. The endotoxin titre and the corresponding coliform density are also shown in the table 24.

The present study indicated that LAL test was a specific and sensitive assay in the rapid detection of GNB in milk as was observed by Hartman et al.(1976). The endotoxin titre value for freshly drawn milk from

Figure 12 Limulus amoebolysate test showing gelation (From right to left). In the first two tubes solid gelatin is observed. From the third to sixth tubes soft gelation is observed. The seventh tube shows an increase in viscosity. The eighth tube shows absence of viscosity and thus is negative.

Table 24

MEAN COLIFORM NUMBERS AND THEIR CORRELATION TO THE ENDOTOXIN IN MILK

Type of sample	Hours from milking	Number of samples	Total aerobic count mean SPC/ml x 10^6	Coliform count mean coli/ml x 10^3	Total microbial endotoxin titre by LAL test
Fresh raw milk	0	5	0.01	0	$10^{1.6}$
Raw milk	2.0	5	2.20	3	10^6
Raw chilled (pooled) milk	24 hrs (8°C)	5	8.10	14	10^{10}
Pasteurized milk	Not known	5	1.10	5	$10^{10.6}$
Curdled milk	7.00 (35°C)	5	Not done	7,400	$10^{16.4}$

healthy udder was nil, which confirmed the non-availability of the bacterial endotoxin in the freshly drawn milk. Likewise, the high titre value in the curdled gassy milk, indicated the presence of endotoxin which required, higher dilution to avoid gelation. However the coliform count of different samples of milk did not vary significantly in accordance with the titre value, as the endotoxin assayed might be from the other members of the enlarged GNB spectrum (Cousins, 1982). The inhibitory effect of the test in the lower dilution might probably due to the presence of inhibitors, as the chloroform extraction of inhibitors was not done for the sake of simplification to suit ordinary laboratories (Jay, 1977). However the inhibitory effect did not carry on beyond 10^2 dilution within which no positive results were obtained. As there was gelation beyond 10^2 in all these tests the inhibitors were probably diluted and hence it was not a matter of concern. The stray false positive results in higher dilutions, might indicate the possible presence of pyrogenic material (Jay et al., 1979).

The study revealed that fresh raw milk should not have gelation at any dilution. A good quality raw milk, might have gelation upto 10^{6-8} dilution, a pooled milk of inferior quality upto 10^{10-12} dilution, and any gelation beyond 10^{13} might indicate the chances for curdling. The endotoxin content of pasteurized milk might represent the

endotoxin of non-viable heat killed cells as well as viable cells and hence no true picture could be arrived at. No attempt was made to report the actual concentration of endotoxin in a given sample as suggested by Anon (1973) and Hartman et al.(1976). However future studies might be attempted to estimate the endotoxin content per ml of milk and correlate it with the titre value as was done for meat samples (Jay et al., 1977; Jay and Margitic, 1979; Jay et al., 1979).

The minimal presence of atleast 10^4 to 10^5 GNB was found essential for giving a positive result (Jay et al., 1977; Nachum and Shambrom, 1981), whereas the present study revealed gelation even at 10^3 which was in accordance with the study of Reinhart et al.(1981) who on operating nebulizers observed sensitivity with 10^3 CFU per ml. This test might be more applicable at the dairy dock to the refrigerated raw milk held for a few days, during which the psychrotrophic and psychrophilic GNB might be dominating (Ayres et al., 1980; Cousins, 1982).

With regard to merits of the LAL test, the endotoxin content of viable and dead GNB could be estimated with relative easiness within an less an hour. The LAL test

results could be read without previous knowledge of microbiological findings (Prier and Spagna, 1983). The demerits would be that high titre could be obtained even in the absence of viable cells, as the test indicated the endotoxin of total GNB present, and not of the viable GNB alone. The routine dye reduction screening test, which is indicative of total bacterial metabolic activity rather than GNB alone would be comparatively cheaper, by hundred folds. As the dye reduction test might be routinely used to screen the known raw milk sources, unknown new sources of raw chilled milk might be examined by the LAL for the GNB consisting of mesophilic psychrotrophs and psychrophiles. Hence, though the LAL test was expensive, it still could be the best as observed by Reinhart et al. (1981).

2. PRESUMPTIVE RAPID IDENTIFICATION OF COLIFORMS. (API 20E SYSTEM)

(A) Introduction

The Appareils et Procedes identification (API) based on the work of Bussiere and Narden (1968) standardized miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other Gram

negative bacteria (GNB). It was a ready to use microtube system designed for the performance of 23 standard biochemical tests from a single colony of bacteria. The members of Enterobacteriaceae had been presumptively identified with API 20E system (Anon, 1979). There had been positive correlation with this system to conventional system although there was disagreement with few members of Enterobacteriaceae (Smith et al., 1972; Hayek and Willis, 1976, Holmes et al., 1978; Fung and Cox, 1981; Griffith and Phillips, 1982). The variability among biochemical tests had been reported (Holmes et al., 1978). The results obtained might be compared with differential chart and API profile Register for identification of the bacteria. To identify the bacteria quickly and precisely computer services were also utilized (Robertson and Maclowry, 1974, Anon, 1979). There were certain limitations like carryover of the substrate from microtube to microtube, false positive or negative results and insufficient incubation period (Anon, 1979, MacFaddin, 1980). However rapidity and easiness to perform made the test system advantageous (Fung and Cox, 1981). In this study the API 20E galleries were used for rapid identification of coliforms. The limitations and advantages are discussed.

(B) Materials and Methods

(i) Isolates studied:

Six strains of four species of coliforms (E.coli, E.aerogenes, E.cloacae and K.pneumoniae) already identified by macro methods (Edwards and Ewing, 1972) were used for this study. Citrobacter freundii was not taken up for this study as wide variability and disagreements in the percentage of positive reactions were observed (Edwards and Ewing, 1972, Anon, 1979, Blackall, 1980).

(ii) Procedure of testing:

The API 20E galleries were stored at 2 - 8°C till its use. The preparation of the galleries, preparation of bacterial suspension and inoculation of the galleries were as per the methods suggested by Anon (1979). Simultaneously motility agar (Difco) was also inoculated and the cultures were also streaked on MacConkey's agar. In all, the total test performed was 28 with the galleries of API 20E system inclusive of 24 for four species of organisms and four to check the reproducibility of the results.

(C) Results and Discussion

After 18 hours of incubation at 37°C the reactions of the tests were recorded, wherever reagents were not required.O nitrophenyl - β -D galactosidase (ONPG) and glucose were positive in all the tests. On addition of reagents for tryptophane deaminase (TDA), indole and Voges Proskauer (VP) tests the results were recorded. The motility test was recorded from the motility media. The results of E.coli and Klebsiella pneumoniae were seen correlated with conventional tests. The correlation and variation with the conventional system of the E.aerogenes and E.cloacae are presented in table 25 and 26.

The variable reaction of E.cloacae in citrate utilization and slight colour change in the inositol and sorbitol fermentation tests are shown in Figure 13. The E.aerogenes which showed variable reactions in ornithine decarboxylase are also shown in this figure 13.

The variable reaction of K.pneumoniae in the urease reaction is compared in figure 14. An untypable contaminant is shown in figure 15 along with organisms of figure 13 and 14, in a comparison. The test in API 20E system were seen reproducible in the four species retested.

PRELIMINARY IDENTIFICATION OF COLIFORMS IN API 20E SYSTEM

COMPARISON WITH CONVENTIONAL TEST

Organisms	Number tested	Identical results	Variable results	Percentage of correlation	Statistical analysis
E.coli	6	6	0	100.00%	Percent agreement in the case of E.aerogenes and E.cloacae though very high was found to be significantly lower than cent percent agreement in the cases of E.coli and K.pneumoniae (Normal Deviate was found to be 2.001*; (P 0.05)
E.aerogenes	6	5	1	83.33%	
E. cloacae	6	5	1	83.33%	
K.pneumoniae	6	6	0	100.00%	
C.freundii	ND	ND	ND	-	
Total	24	22	2	-	

ND : Not done Mean agreement : 91.66%

Table 26

VARIABLE RESULTS OF API 20E COMPARED WITH CONVENTIONAL TESTS

Name of the organism	Name of the test	Percentage positive in conventional test	Percentage positive in API 20E test	Deviation from conventional test	Remarks
E.aerogenes	Ornithine decarboxylase	98.73	83.33	15.40	** Highly significant ($P < 0.01$) (Normal Deviate = 9.333)
E.cloacae	Simmon's citrate utilization	93.80	83.33	10.47	Not significant ($P < 0.05$) (Normal Deviate = 1.662)

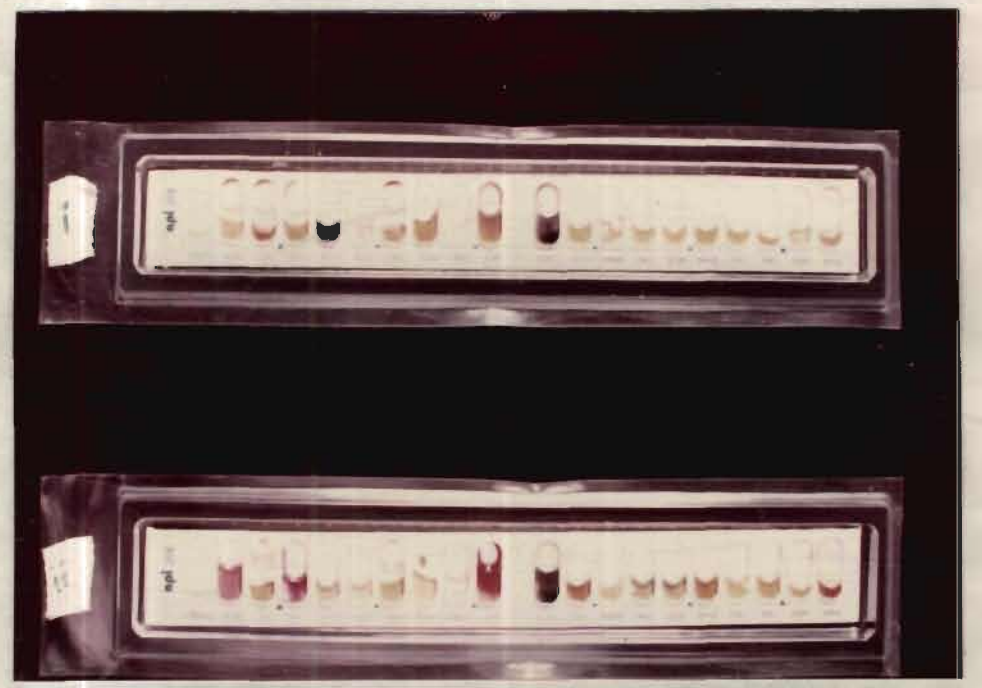


Figure 13 (i) *Enterobacter aerogenes* and *Enterobacter cloacae* in API 20E system

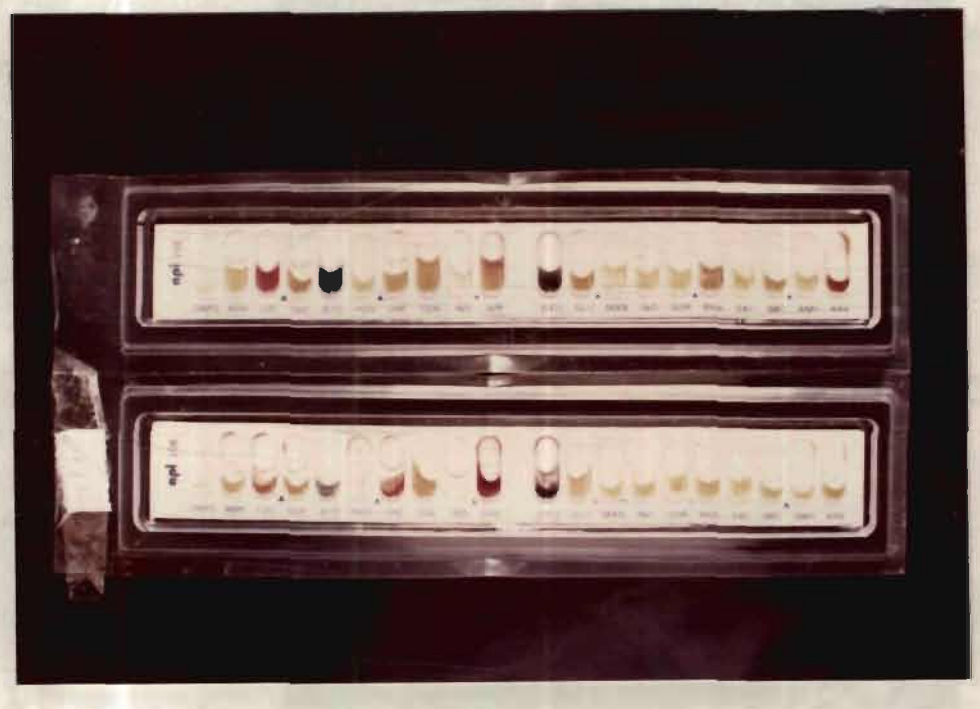


Figure 14 *Klebsiella pneumoniae* in API 20E system. The bottom gallery shows positive urease activity.

Figure 15 API 20E system - a comparative picture

i) Klebsiella pneumoniae

ii) Klebsiella pneumoniae with positive urease activity

iii) Enterobacter aerogenes

iv) Enterobacter cloacae

v) A contaminant with negative sugar reactions

The API 20E system is easy to perform when compared to the conventional system, since the system was a ready to use one, dispensing with preparation, sterilization and incubation of media before inoculation. The system was easy to store and handle. Thus lot of laboratory space and man power were saved, as observed by Fung and Cox (1981).

The system however appeared to be difficult to identify the bacteria with the aid of differential chart provided by the manufacturer. The aggregate reactions of the strain had to be considered as a whole to determine the identity. A possible combination of 2^{21} or 2097152 results could be possible as observed by Robertson and MacLowry (1974). Hence for an unfamiliar bacteria identification through this process, might give rise to problem without API index system viz. API Profile recognition system (PRS) and PRS computer services (Anon, 1979). These sophisticated services are not available as the system is yet to obtain popularity in this part of the world. The automation is yet to arrive in clinical and food bacteriology. However, for the familiar organisms, a competent microbiologist can interpret the results using his judgements, knowledge and other morphological characters. If necessary confirmative

results like serology may be used before finally deciding on the identity of the bacterium (Anon, 1979; MacFaddin, 1980). The diagnostic kits now available in the market are made for medical laboratory work and suitable modifications are required to fit them in the food microbiology. The emerging food poisoning organisms like Yersinia enterocolitica, Vibrio parahaemolyticus and Campylobacter jejuni should also find a place in the mini kit that may be evolved for exclusive purposes of food microbiology (Fund and Cox, 1979; Richard et al., 1981; Griffith and Phillips, 1982)

But with the present kit system available for Enterobacteriacae the identification of the members of coliforms was a simple process, with familiarity of reactions of these organisms. The accuracy of the results of the minitube system ranged from 83.33% to 100% in this study. Similar reports were made in the diagnostic bacteriology (Smith et al., 1972; Hayek and Willis, 1976; Holmes et al., 1976) and 82% to 99% of accuracy with conventional systems were also observed in food bacteriology (Poelma et al., 1977; Poelma et al., 1978; Cox and Mercuri, 1978; Mercuri and Cox, 1979; Cox and Mercuri, 1979). The organisms examined were E.coli, E.cloacae, E.aerogenes and K.pneumoniae the

common members of the coliform group, in which the results of API 20E system were correlated with the conventional test results of Edwards and Ewing (1972). The diagreeements in this study with conventional system were with E.aerogenes and E.cloacae where the percentage was 16.67% in both the cases. However, no disagreement with E.coli and K.pneumoniae was observed as similarly reported by Holmes et al.(1982). The mean agreement with the conventional system for these coliforms was 91.66%. The results correlated with the accuracy of 82% to 99% reported by other workers (Poelma et al., 1977; Poelma et al., 1978; Griffith and Phillips, 1982).

In the individual tests, the variations were observed in citrate utilization and ornithine decarboxylase to the extent of 10.47% and 15.40% respectively. Similar results with citrate utilization, arginine dihydrolase+ornithine decarboxylase were also noticed by Holmes et al.(1978) and Blackall (1980). The possible reasons could be that the reactions recorded as delayed positive with macromethods generally were shown negative on the API chart, since this chart is based on the results obtained in 18-24 hours of incubation. A rare phenomenon of dubious results were encountered with inositol and sorbitol fermentation of E.cloacae in this study. The explanation offered by the manufacturer for

this sort of reaction were reversion of reactions by the organism concerned (Anon, 1979). The other reasons, might also be the insufficient incubation for the organisms to exhibit the reaction.

However these limitations of identification, variation in test reactions and false positive or negative results were small and negligible constraints when more than 83% of the familiar members of coliforms in milk and its environment were identified with ease, rapidity and with limited laboratory facilities. However any of the identification system, had to be followed by with serological confirmation or by demonstration of atleast one of the virulence attributes of ETEC or other members of coliforms.

(D) Summary

The Gram negative bacteria (GNB) are enzyme producers which may affect the quality of milk food. The GNB include mesophilic and psychrophilic bacteria. The LAL test was used as a rapid screening test to detect the endotoxin level produced by the GNB, which included psychrotrophic coliforms also. The results were obtained within an hour

and reflected the endotoxin of both viable and non viable cells of GNB. The mean value of total microbial endotoxin titre for raw milk, chilled pooled milk, pasteurized milk and curdled milk were 10^{6}, 10^{10}, $10^{10.6}$ and $10^{16.4}$ respectively.

For preliminary identification purposes a rapid minitube identification system (API 20E) was used. The percentage of agreement between this test and conventional test results was acceptable for assignment of the familiar isolates to the species level. There were correlations upto 83.33% in cases of E.cloacae and E.aerogenes while the correlation was 100% in E.coli and Klebsiella pneumoniae. Individual tests like ornithine dlcarboxylase test and Simmon's citrate utilization reactions were found to deviate from conventional system by 15.4% and 10.47% respectively.

The constraints with these two systems were non availability of indigenous Limulus amoebocylsate and mini test strips and high cost of their import. These impediments could be warded off for the easiness and rapidity in the test procedures. With experience, the reading and interpretation of the results of minitube identification of familiar coliform isolates could be easier.

COMPOSITE PICTURE OF THE STUDIES

FIGURE 17

COMPOSITE PICTURE OF THE ECOLOGICAL STUDY ON COLIFORMS ISOLATED FROM FARM ENVIRONMENT

ENVIRONMENTAL NICHES

Number of samples	Air (17)	Water (12)	Equipments (12)	Personnel (14)	Teat (18)	Milk (227)

Coliform isolates obtained and taken for identification and characterization (306)

	E.coli	K.pneumoniae	E.aerogenes	E.cloacae	C.freundii
Isolates (306)	(27.12%) 83	(39.54%) 121	(11.44%) 35	(11.11%) 34	(10.79%) 23
MRHA positive (130)	(56.63%) 47	(47.93%) 58	(22.86%) 8	(17.65%) 6	(33.33%) 11
Presence of CFA/I	1/47	0/58	0/8	0/6	0/11
Presence of CFA/II	1/47	0/58	0/8	0/6.	0/11

Contd....

	E.coli	K.pneumoniae	E.aerogenes	E.cloacae	C.freundii
Piliated phase of type I pili (58)	ND	2/58	ND	ND	ND
ST (24)	(14.89%) 7	(15.52%) 9	(12.50%) 1	(33.33%) 2	(45.45%) 5
K 99 pili (7)	1	ND	ND	ND	ND
Serotyping (E.coli - 7)	5	ND	ND	ND	ND

Experimental Studies:

1) Study for piliated phase of CFAs					
a) Electron Microscopy (2)	1	1	ND	ND	ND
b) Indirect fluorescent Antibody Technique (2)	2	ND	ND	ND	ND
2) Toxin production in milk (4)	1	0	ND	ND	1

Other studies on coliforms in raw milk

1) Achieved mean coliform density from an experimental milk shed : 28,000/ml

2) Antibiotic sensitivity test : Comparison to tox^+ and tox^- coliforms, in their resistance pattern.

3) Limulus amoebolysate test (LAL) for estimation of endotoxin of Gram negative bacteria (GNB)

4) API (20 E) microtube system for rapid identification of coliforms

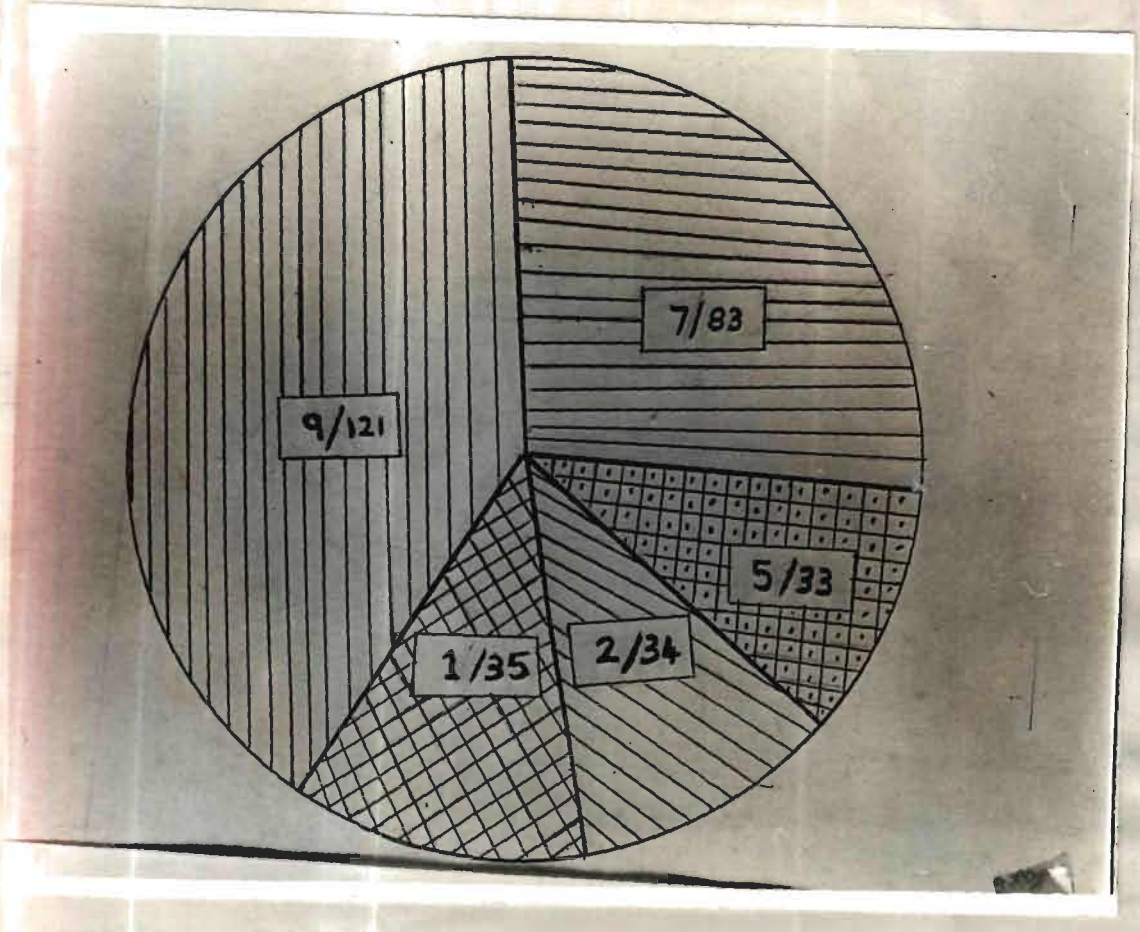
ND : Not done

tox^+ : Toxigenic coliforms

tox^- : Non toxigenic coliforms

FIGURE 18

COLIFORMS AND THEIR TOXIGENIC STRAINS IN DAIRY ENVIRONMENT



K.pneumoniae

E.coli

E.aerogenes

E.cloacae

C.freundii

Figure in numerator indicate number of toxigenic isolates

Figure in denominator indicate number of total isolates

CHAPTER VII

COMPOSITE PICTURE ON THE STUDIES OF COLIFORMS OF RAW MILK AND THEIR TOXIGENIC POTENTIAL IN DAIRY ENVIRONMENT

(1) Introduction

Social attitudes have recently made a greater impact on food protection than earlier days and will continue to do so. Increase in urban influx bring the change in the social attitude of the population. This urban population necessarily depend on supply of milk by organised dairies. These organised dairies enhance the procurement operations year after year from the remote villages of the milk sheds. The raw milk thus procured reaches the consumer after processing, a few days later after the procurement. This period exposes the milk to microbial threats of spoilage off flavour, intoxications and degradation of the nutritional quality. Consumer attitudes remain very positive in obtaining fresh milk if it is made available without adulteration. The non availability of fresh milk in metropolis brings about changing consumption pattern of switching over to processed milk which is evident in the last few years. The milk borne microbial threats are still unrecognised though infantile diarrhoea are popularly

attributed to feeding of packed milk. This awareness towards food safety and quality necessiates changes in food protection activity.

This study is broadly based on this concept that milk borne microbial threats are especially of those coliforms which were hitherto considered as indicators of pathogens. An interesting feature of this old concept, was that the so called indicator organisms by themselves produced heat stable enterotoxins that could be carried through all the stages of processing whereas the pathogens themselves are destroyed at pasteurization temperature (Kaplen et al., 1962).

The exposure of milk in the farm environment to the contaminating sources like air, water, unclean equipments personnel and cow itself are more when compared to closed pipeline circuits in the dairy plant. After contamination, the milk is exposed to conducive temperature of microbial multiplication at the farm, whereas in the plant the milk after heat processing is chilled. So the milk borne microbial threats at farm level are greater.

The earlier reports on the study of dairy environmental sanitation were scanty. Available studies indicated that enterotoxigenic coliforms were not commonly found in the

dairy farm environment (Glatz and Brudvig, 1980a). However, a greater understanding and appreciation of the importance of bacteriological quality of raw milk had developed in the last decade (Mabbit, 1980a,b) Gram negative bacterial (GNB) threats were greatly implicated due to their enzyme production and enterotoxin production (Cousins, 1982). The coliforms are mesophiles and could also multiply in refrigerated condition (Foster et al., 1958; Cousins, 1982). The factors influencing the bacteriological quality of raw milk had been well documented (Fluckiger et al., 1980; Gehriger, 1980; Mabbit, 1980a,b, Palmer, 1980). Milk food borne out-breaks due to E.coli causing diarrhoeal illness was an emerging problem from the late seventies (Mehlman et al., 1976; Sack, 1980; Mehlman and Romero, 1982). This ecological study has attempted to identify the contaminating sources of the enterotoxigenic coliforms in the farm environment, their toxigenic potential, their ability to grow in milk and related study on toxigenic coliforms{ Figure 17 and 18].

2. ENVIRONMENTAL COLIFORMS

Different farms were chosen and grouped into A, B and C depending on the commercial orientation and sanitary practices adopted. Environmental sampling were

done (Table 1) at the farm as suggested by International Dairy Federation (Mabbit, 1980a,b; Palmer, 1980). Insufficiently cleaned and sanitized surfaces of the farm utensils were frequently found to be the major source of coliforms in ex-farm milk as also reported by Mabbit, 1980b (Table 5). Effective washing and cleaning with iodophor at 75 ppm prevented this contamination. The milking personnel and teat (exterior) were other significant sources of contamination (Table 6). Farm air and water were of lesser significance (Table 3 and 4). However, farm water had more percentage toxigenic coliforms (13.16%) followed by the surfaces of the utensils rinsed with these water (8.99%) The other niches had comparatively less strains with toxigenic potential (Table 20). Sampling of milk were also taken as raw, pooled and commingled milk at various sources which included dairy dock also. The coliform density which included E.coli, Klebsiella, Enterobacter and Citrobacter species was identified and was found that the representation of each member of the coliform group varied from group to group of the farms (Table 10).

E.coli dominated wherever insanitary conditions prevailed. K.pneumoniae dominated in the farms where sanitary practices were adopted. Though Citrobacter freundii had the lowest distribution (10.79%), they had

the highest number of strains (15.15%) with toxigenic potential (Table 17). Probably the variations in the distribution of members of coliforms were due due to the presence of each member of the coliform group in the dung of the cattle population and its disposal.

3. COLIFORM DENSITY IN RAW MILK

A detailed study at the level of coliforms at various stages of production and handling was also made. A total of 227 samples were tested to assess the coliform level (Table 2). The only guideline of absence of coliforms in the dilutions of 1:100 for grading of raw milk supplies had been suggested in Indian Standards No.1479-Part III-1977. When this standard was applied none of the pooled milk samples and commingled milk samples complied to the satisfactory standards and the conformance level was zero. The raw fresh milk produced in the group C farm only was found to comply with this standard. So, as far as these standards are concerned, the applicability might be remote as similarly reported in other studies (Gahlot et al., 1975; Singh and Ranganathan, 1978). With the absence of milking machines and farm refrigerated bulk storage tanks and transport of raw milk in tropical climate this standard would be

too rigid and almost impossible to attain at the acceptance level. So, realistic standards to suit individual regions at various levels of raw milk handling had to be suggested by the national body. In this study with enforcement of economically possible hygienic milk production, the researcher did obtain 28,000 coliforms per ml of milk at the acceptance dairy dock while it was 1.70 million per ml from the uncontrolled milk sheds (Table 9). So the coliform count of 28,000 per ml may be explicitely imposed as an acceptable level at the dairy dock though a desired level may be set around 10,000 per ml. The existing Indian Standards No.1479-Part III-1977 might be made applicable for the individual raw fresh milk at the farm level.

4. RAPID TESTS OF THE FUTURE.

For identification purposes a rapid minitube identification system (API 20E) was adopted (Fig. 13, 14, 15). A screening test that was already available to detect the endotoxin level of Gram negative bacteria (GNB) in plasma, hospital equipments and urine was also used to detect the endotoxin due to Gram negative bacteria in milk (Fig. 12).

The test was based on gelling property of the lysate of the amoebocytes of Limulus polyphemus in the presence of endotoxins of the Gram negative bacteria (Hartman et al., 1976; Jay, 1977). These two simple rapid systems might find a place in the dairy industry for the screening and identifying coliforms as they were acceptably correlated with microbial quality of milk and conventional systems of enumeration of coliforms and identification (Tables 24, 25, 26). These tests are easy to perform without involving much labour. However, the constraints are high cost due to import, lack of availability of experienced worker to interpret these tests and certain drawbacks in the true reflection of the microbial quality of milk. Ofcourse these impediments could be warded off in the near future when their usefulness and importance are made known.

5. DEMONSTRATION OF VIRULENCE ATTRIBUTES

On identification of 306 coliform isolates, they were further processed for identification of toxigenic potential present in them. The identification system of the toxigenic E.coli were considered as an Herculian's

task as many isolates were to be examined either by bioassay or by other systems (Sack, 1981). To ease the tedious process, screening tests identifying the presence of adhesin or pili which was considered as associated with the toxin attribute had been attempted (Evans et al., 1975 and Evans and Evans Jr. 1978; Gaastra and Graaf, 1982).

(A) Detection of adhesins:

In human enterotoxigenic E.coli antigenically different two adhesive factors termed as CFA/I and CFA/II had been identified. The presence of these fimbrial antigen could be identified by expressing the manifestation of their adhesive properties. Haemagglutination was the first observed manifestation (Duguid et al., 1955).

A haemagglutinating typing system had been proposed to identify the CFA/I or CFA/II (Evans et al., 1977; Evans Jr. et al., 1979). The presence of this pili in E.coli and probable presence of such type of pili or adhesion factors in non-E.coli coliforms were detected by using MRHA of human A and bovine erythrocytes. All the 306 cultures were tested by this way and the MRHA pattern observed. The MRHA of human A and/or bovine erythrocytes were present in the E.coli and also in non-E.coli coliforms.

The percentage of distribution was nearly similar in some of the non-E.coli coliforms compared to the E.coli (Table 12) It is suggested that there had been some agglutinating factors like that of E.coli in the Klebsiella and other coliforms. The hypothesis of intergeneric transfer of plasmids, between related species might be correlated with these findings (Guerrant et al., 1976; Klipstein and Engert, 1977). Type I fimbriae were not present in all but two of the 58 MRHA positive Klebsiella, when tested for their presence by MSHA of guinea pig erythrocytes (Table 14) (Fader et al., 1979). Future study may probably reveal the relationship if any between the CFAs and type I pili of Klebsiella.

Totally 130 cultures were identified to possess the haemagglutinating activity for human and or bovine (CFA/I) and bovine erythrocytes alone (CFA/II) (Table 12). These strains were serologically tested by slide agglutination test for CFA/I and CFA/II with specific antisera. Only two E.coli isolates were found positive in this test (Table 13). None of the non-E.coli coliforms possessed any antigenic relationship with CFAs.

(B) Detection of toxigenic potential:

The MRHA positive cultures (130) were subjected to bioassay in rabbit ileal loop test for assay viz. the

other virulence attribute of the enterotoxigenic bacteria. The assay of labile toxin was not taken up since the organism capable of producing the labile toxin and the labile toxin produced by them are heat labile at pasteurization temperature or subsequent boiling of milk at home (Burgess et al., 1978), whereas the stable toxin was found to withstand even the 100°C for 30 minutes (Klipstein and Engert, 1975; Klipstein and Engert, 1976a,b) This bioassay detected presence of 24 toxigenic organisms in the coliform group (Table 15). There could be presence of undetectable level of toxin also. The rabbit model responded to the enterotoxin assay with an appreciable distention of ligated ileal loops (Fig. 2 and 3). These toxigenic organisms constituted 18.46% of the total MRHA positive strains and 7.84 (Table 17) of the total isolates. These findings coincided with the study made by Sack et al. (1977) in which 8% of the E.coli isolates from the food produced enterotoxin. However, the recent report of presence of less than 1% of ETEC among hospital environmental isolates (Mehlman and Romero, 1982) probably suggested the higher sensitivity of the bioassay model employed in this study. It is now pertinent that other coliforms also produced the enterotoxins as that of the E.coli (Table 15). Perhaps in the light of the study

made by Glatz and Brudvig (1980a) the isolation and identification of the toxigenic E.coli from dairy environment reported in this study might be a first report.

Though the other coliforms had been attributed as putative causative organisms in addition to E.coli in paediatric diarrhoea (Sadruddin et al., 1981), their demonstration as toxigenic organisms in farm environment had not been reported. So this identification of other toxigenic coliforms in the farm environment might also probably be the first report of this kind. The 24 enterotoxigenic coliforms were tested for serological identification of the presence of CFA/I, CFA/II and K 99 with type specific sera by slide agglutination (Table 18).

All the coliforms other than E.coli were found to be negative to possess related antigens of CFA/I and CFA/II. Out of the seven E.coli, one was positive for CFA/I and another was positive for CFA/II. Among E.coli these two isolates were conclusively proved to possess the two virulence attributes by indirect fluorescent antibody technique and electron microscopy (Fig. 8, 9 and 10). All the seven E.coli was also tested with K 99 specific sera and out of this, one which was serologically positive

for CFA/II was also found to possess K 99 pili. It indicated that a particular strain might be able to produce more than one type of fimbriae or adhesin (Gaastra and Graaf, 1982). The CFAs positive and enterotoxin positive isolates indicated the close plasmid borne association between the presence of CFAs and production of ST. Probably in other isolates there may be presence of unidentified new pili as (E 8775 or other adhesin factors (Levine et al., 1980 Thomas et al., 1982). However, the absence of colonization factors or adhesins are not of much significance as these E.coli are supposed to be destroyed during heat treatment and hence unlikely to adhere in the human gut. So even the presence of both the virulence attributes for colonization of the anterior part of the intestine and then enterotoxin production are not essential in raw milk. The presence of single attribute of producing potential exterotoxin in any of the coliforms would be sufficient to preform the heat stable toxin before the heat destruction of these organisms(Craviote et al., 1982).

(6) SEROTYPING OF E.COLI

The sero grouping of the seven E.coli revealed that they belong to O:11. Though this is not a classical O group possessing CFA/I and CFA/II as reported by

Gaastra and Graaf (1982) the association of this sero group in diarrhoeal diseases of human and calves had been reported from India and abroad (Singh and Ranganathan 1974; Tiripathi and Soni, 1982; Mehlman and Romero, 1982; Kumar et al., 1982). This sero group was distributed in a farm environment in water, personnel and the teat of the animal and ultimately in milk. Other coliforms were also like wise distributed in air, water, utensils, teat and milk (Table 19). This indicated the potential presence of toxigenic coliforms in the dairy environment which might subsequently get into the milk.

The agglutination testing of ETEC with K 99 antiserum revealed presence of K 99 pili in one of the seven ETEC cultures. This isolate was made from the milk of a individual cow. The O:11 serogroup with K 99 was not in the classical serogroup already reported (Gaastra and Graaf, 1982). Thus the extra intestinal source of K 99 positive O:11 ETEC is of significance.

(7) DRUG RESISTANCE PATTERN OF TOX$^+$ COLIFORMS

The antimicrobial sensitivity pattern of entero-toxigenic coliforms were significantly different from that of non-toxigenic coliforms isolated (Tables 22, 23).

Multiple drug resistance and resistance to ampicillin, tetracyclines & cephaloridines were of significance. Evidence for plasmid borne characters and association of characters of drug resistance and enterotoxin production were strongly implicated. The study indicated the possibility of inter species of transfer of R factors and ent$^+$ plasmids and warranted further study in the light of careless use of antibiotics in the treatment and as feed supplement.

(8) ENTEROTOXIN PRODUCTION IN MILK

The possibility of production of enterotoxin in the laboratory substituting the toxin broth in milk was also studied. The constituents of the milk would support the growth of toxigenic coliforms, as the constituents in toxin broth (Lallier et al., 1980). Out of the four toxigenic cultures tested, two could evidently produce toxins at detectable level in milk. Probably, the other two would have also produced the toxin which could have been elucidated, had the culture suspension was concentrated, thus throwing the light on possible production of the toxins by 7.84% of the coliforms, identified as toxigenic isolates in the milk during transport. The milk gets aerated and agitated

at the ambient temperature during transport in the uneven village milk routes. The time of exposure of raw milk to the ambient temperature, pH of milk and subsequent chilling to 4°C were also found near optimal to the ideal factors, conducive for the enterotoxin production and its stability (Lovett et al., 1979; Lallier et al., 1980). Even the antibacterial system would not retard the multiplication of coliforms if the initial level of contamination was heavy. Such a preformed heat stable toxin which could withstand 100°C for 30 minutes (Klipstein and Engert, 1975; Klipstein and Engert, 1976a,b) might potentially be hazardous and the production of infantile diarrhoea might occur when such milk was fed to unexposed infants. The exposure of these infants to the preformed toxin was similar to travellers' diarrhoea among travellers, from developed countries to the developing countries (Sack, 1980; Sack, 1981). Infant mice also responded to the diarrhoeal score assay in three hours at 37°C when such toxin containing milk was orally fed (Fig. 16) and (Table 21). The infancy and the diarrhoea in three hours at tropical climate might very well correlate the experimental mouse study with the reality in the infantile diarrhoea after milk feeding.

(9) BACTERIOLOGICAL ANALYSIS OF MILK FOR ENTEROTOXINS

In the examination of milk, a simple estimation of quantitative bacterial load had been found to be very much insufficient. In this study the presence of two virulence attributes had been established beyond doubt in atleast two isolates. The presence of pili had been further confirmed by indirect fluorescent microscopy and electron microscopy So, mere enumeration of the coliforms as it had been prescribed would only roughly indicate the milk quality. As 7.84% of the coliforms in milk were found to be potentially toxin producers, some other qualitative assay should invariably be incorporated. In these circumstances easy assay procedures should be evolved to detect the heat stable enterotoxin or the organism producing such toxins. The detection of pili antigen either by MRHA test or serological tests could be very much erroneous as the correlation between the main virulence attributes (i.e.) toxin production and adhesin factor (pili) were found to be as smaller as two out of seven in E.coli. Again suitable serological tests or established fool proof haemagglutination test were absent at present as far as non E.coli coliforms are concerned. As the adhesins

attribute was not considered essential to produce toxin in milk, the identification of enterotoxin attributes would be the only needed method in the non clinical and extra-intestinal isolates.

In the present study it was found that it is easy to detect the enterotoxin level by using rabbit ileal loop technique (RILT). More than 10 samples could be tested in one animal and this animal model was more suitable for STb assay. The milk samples may be concentrated to elicit detectable secretory response in the intestine of the rabbits. With an experienced worker, the laparotomy operations could be completed in 25 minutes and the results could be seen in another six hours after terminal anaesthesia. The interpretation of the results could be completed in fifteen minutes. A quantitative enumeration by plate count technique coupled with qualitative assay of ST in rabbit model would suit the present needs. As the antigenicity of the stable toxins were also currently assayed (Gianella et al., 1981) and synthesis of stable toxin had been attempted (Klipstein, 1983a,b), the serological test might in due course replace the bioassay of ST as in labile toxin. In such circumstances the simple

staphylococcal coagglutinin test might conveniently replace the bioassay.

In the farm side it was proved that compaign on clean milk production would bring down the total coliform level from nearly two million to 28,000 per ml at the dairy dock. The optimal chlorination of water, judicial use of iodine compounds in cleaning and sanitization, sanitary milking, handling and rapid chilling are practicable possibility in clean milk production. In this study it had been observed that farm water possessed highest percentage of toxigenic isolates (Table 19). The recent findings that the sub-lethal chlorination might injure the toxigenic coliforms had thrown a new light (Walsh and Bissonnette, 1983). So such a low level presence of chlorine and possibly iodine might injure the coliforms and affect the toxigenic potential though not totally be lethal to these organisms.

SUMMARY

CHAPTER VIII

SUMMARY

An ecological line study of coliforms from the origin in the dairy farm to the recent implication in the diarrhoeal disease in human was made. The ecological niches in the dairy farm environments of three different groups of farms based on their sanitation level and commercial involvement were identified and environmental samples were subjected to the study. The coliform enumeration and identification were done as per standard methods.

In the environment, dairy utensils, milkers hand and teat played an important role as a major source of contamination. Contaminations from air and water were negligible. The distribution of members of coliform group varied from group to group of the farms. Domination of E.coli was observed wherever insanitary conditions prevailed. K.pneumoniae dominated in the farms where sanitary practices were adopted. The commercially oriented farms were unsatisfactory in the sanitary standards. In an overall picture K.pneumoniae dominated all the other members of coliform groups in the farm niches. The percentage of distribution was 39.54, followed by

E.coli (27.12), E.aerogenes (11.44), E.cloacae (11.11) and C.freundii (10.79)

In the 227 milk samples tested for coliform density from three different farm sources, and milk sheds only the fresh milk had coliforms less than 10/ml. The added contamination and dynamic multiplication started right from the milk let down. At the acceptance dock the coliform density of commingled milk varied from a mean of 28,000/ml to a mean of 1.79 million per ml depending on the sanitation practices prevailed at the milk sheds. None of the pooled or commingled milk complied with Indian Standards No.1479-Part III-1977. As the conformance level was absolutely absent in these cases, there needed a rethinking on the highly rigid unrealistic standards at acceptance level in tropical countries.

The E.coli and other coliform isolates were screened for the adhesion attributes of enterotoxigenic organisms by mannose resistant haemagglutination test (MRHA) of human A and/or bovine erythrocytes.

The MRHA positive E.coli constituted 56.63% of the positive group; K.pneumoniae formed the next with 47.93% followed by C.freundii (33.33%), E.aerogenes (22.86%)

and E.cloacae (17.65%). The MRHA positive isolates were serologically tested with CFA/I and CFA/II specific sera by slide agglutination test. E.coli strains producing haemagglutination of human and/or bovine erythrocytes but lacking colonization factor antigen/I (CFA/I) or CFA/II were encountered in 45 out of 47 strains. None of the non-E.coli coliforms were serologically positive with CFA/I and CFA/II sera The probability of the presence of certain fimbriae of different antigenic type was indicated with other non-E.coli coliforms, since all were antigenically heterogenous to CFA/I or CFA/II. Type I pili in Klebsiella could be demonstrated by mannose sensitive haemagglutination (MSHA) of guinea pig erythrocytes in two out of 58 MRHA positive isolates indicating that the MRHA of human and/or bovine erythrocytes by this species was probably due to other factors.

All the MRHA positive strains were inoculated in the Casamino acid yeast extract (CYE) broth to test their potentiality in enterotoxin production. In the bioassay using rabbit ileal loop technique (RILT) 24 isolates, out of 130 MRHA positive coliforms (18.46%) were found to be potentially enterotoxigenic. C.freundii topped the percentage (45.45%) followed by E.cloacae

(33.33%), K.pneumoniae (15.52%), E.coli (14.89%) and E.aerogenes (12.50%). Citrobacter freundii though represent only 10.79% of the total coliform isolates had more strains with toxigenic potential. Out of the total isolates, species wise C.freundii had toxigenic strains of 15.15% followed by E.coli (8.43%), K.pneumoniae (7.43%); E.cloacae (5.88%) and E.aerogenes (2.86%). Among enterobacter, E.cloacae was found to be more pathogenic than E.aerogenes. In the farm environment water source had more toxigenic coliforms (13.16%) followed by equipments or utensils washed with such water (8.93%).

The toxigenic coliforms were once again tested with lower working dilution of CFA/I, CFA/II specific antisera. One ETEC was positive with CFA/I and another with CFA/II. The demonstration of CFA/I and CFA/II in two ETEC isolates by agglutination test was confirmed by IFAT and electron microscopy. One of the ETEC with CFA/II was found to possess K 99 pili by specific serum agglutination test using K 99 specific serum. The ETEC were serotyped. In this five out of seven belonged to O:11 serogroup. This serogroup was found distributed in the environmental niches of the farm and the milk.

The demonstration of both the virulence attributes in the E.coli of O:11 group in dairy environment might probably be the first report in India. The demonstration of K 99 pili in an ETEC of O:11 serogroup is also significant since the organism was isolated from the milk of the healthy cow. There could have been abundant chances for the organism to have its origin from the milker's hand or from the teat canal. The enterotoxigenic Citrobacter, Klebsiella & Enterobacter species from the dairy farm environment were also reported now though their pathogenicity had already been demonstrated in infantile diarrhoea.

In an experimental study the enterotoxin could be produced in milk by inoculating four proven toxigenic isolates. Two organisms could produce detectable level of enterotoxin by stationary culture method. This result could conceivably explain the probable production of enterotoxin in milk handled in low volume per volume of cans during transport and hold ups. In that case the diarrhoeal syndrome in infants after feeding such milk requires further study to attribute this reason. These toxins produced in milk could elicit diarrhoeal response in young mice.

The drug resistance pattern of toxigenic coliforms revealed that this group was significantly different from non-toxigenic coliforms. The hypothesis of genes which code for antibiotic resistance and enterotoxin production are frequently transferred together was confirmed in this study. This type of study which was hitherto limited to E.coli alone was extended to other members of coliforms in this study. The toxigenic coliforms were significantly resistant to ampicillin, tetracycline and cephaloridine. Multiple drug resistance pattern was also observed in toxigenic coliforms.

A simple screening test for endotoxin of Gram negative bacteria (GNB) was applied. The test called Limulus amoebolysate test [LAL] was sensitive enough to detect presence of endotoxins in raw milk, pooled milk and pasteurized milk. Coliform density as low as 1600 per ml was detected by this test though the minimum number was variable in other reports. The other Gram negative bacterial load of the milk could not be estimated. In the pasteurized milk, presence of viable and non-viable GNB tilted, the correlation of viable coliforms and endotoxin content. The limitations and advantages of this test were discussed.

The rapid minikit identification system (API 20E) was applied for the identification of different species of coliform group. With a limited study it was concluded that atleast 83.33% of the results of API 20E system, correlated with conventional system. Certain disagreements with ornithine decarboxylase and citrate utilization tests were encountered. This system could be utilized by the microbiologists as it is rapid, easy to perform without much labour force involved.

It is clear from the attempted ecological study of coliforms that the presence of these organisms in milk should not be taken only as indicator/index organisms for pathogens. Apart from the quantitative study, their toxigenic potential should also be identified, in simple assay methods like rabbit ileal loop technique. In such cases, the cases which were hitherto considered as "etiology unknown" may get themselves identified as a known cause.

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BIBLIOGRAPHY

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Adams,P. and Spendlove,J.C. (1970). Coliform aerosols emitted by sewage treatment plants. Science, 169: 1218-1220.

Anon. (1972). Standard methods for examination of dairy products, 13th Ed. New York. American Public Health Association.

Anon. (1973). Sigma Technical Bulletin No.210. Missouri Sigma Chemical Company.

Anon. (1975). Standard methods for the examination of the water and waste water. 14th Ed. Washington DC. American Public Health Association.

Anon. (1976). Food borne and water borne disease outbreaks, Annual summary report for 1975. Cited by Kornacki and Marth (1982b).

Anon. (1979). Appareils et Procedes d' Identification system 2010 API 20 Enterobacteriaceae. France

Awan,A.H. and Barya,M.A. (1972). A study of the surface microflora of the udder of buffaloes with emphasis on identification, antibiotics sensitivity and effect of selective disinfectants. Dairy Sci. Abst. 38:405

Ayres,J.C., Mundt,J.O. and Sandine,W.E. (1980). Microbiology of foods. SanFransisco, W.H.Freeman and Company.

Babel,F.J. (1977). Antibiosis by lactic culture bacteria. J.Dairy Sci. 60:815-821.

Back,E., Mollby,R., Kaijset,B., Stintzing,G., Wadstrom,T., and Habte,D.(1980). Enterotoxigenic Escherichia coli and other Gram negative bacteria of infantile diarrhoea, surface antigens, haemagglutinins, colonization factor antigen, and loss of enterotoxigenecity. J.Infect. Dis. 142:318-327.

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The codens and title abbreviations are followed as per the Biosis Lists of Serials (1977), Bio-Sciences Information Service, 2100 Arch Street, Philadelphia, Pennsylvania 19103 U.S.A.

Barber,F.W. (1962). Hygienic control of fluid milk. In Milk Hygiene, World Health Organisation Monograph series, 48:303-320.

Batish,V.K., Gang,S.K., HarishChander and Ranganathan,B. (1981a). Public Health significance of milk borne intoxications. I.Staphylococcus and Clostridium Indian Dairyman, XXXIII:435-438.

Batish,V.K., Gang,S.K., Harishchander and Ranganathan,B., (1981b) Public Health significance of milk borne intoxications II. E.coli, B.cereus, Streptococcus and Aspergillus. Indian Dairyman XXXIII:501-503.

Bauer,A.E., Kirby,W.M.M., Shessis,J.C. and Turck,M.(1966) Antibiotic susceptibility testing by a standardized single disc method. Am.J.Clin.Pathol. 45:493-496.

Beer,K. and Melhom,G. (1974). Bacterial contamination of air in cow sheds. Cited by Palmer, J.(1980).

Beer,K., Melhom,G. and Arnold,H. (1976). Bacterial contamination of indoor air of dairy cattle sheds. Technological factors and their influence of quantitative bacterial contamination. Microbiol.Absts. 12B:1018.

Benfield,D.A. and Francis,D.H. (1980). Diagnosis of naturally occurring calf diarrhoea associated with Coronavirus, Rotovirus and Escherichia coli. Proceedings of Third international symposium on neonatal diarrhoea, University of Saskatchewan, VIDO, Saskatoon, Saskatchewan, Canada.

Benham,C.L. and Egdell,J.W.(1970). Levels of air borne bacteria in milking premises. J.Soc.Dairy Technol. 23:91-94.

Bigalke,D.L. (1978). Effect of low temperature cleaning on microbiological quality of raw milk and cleanliness of milking equipment on the farm. J.Food.Prot. 41:902-905.

Bishop,J.R., Bodine,A.B. and Jenzen,J.J. (1980). Sensitivities to antibiotics and seasonal occurrence of mastitis pathogens. J.Dairy Sci. 63:1134-1137.

Bjorck,L.(1978). Antibacterial effect of the lactoperoxidase system on psychrophilic bacteria in milk. J.Dairy Res. 45:109-118.

Bjorck,L., Rosen,C.G., Marshall,V. and Reiter,B.(1975). Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other Gram-negative bacteria. Appl.Microbiol., 30:199-204.

Blackall,P.J. (1980). Evaluation of a multitest microtube system for the identification of veterinary isolates of Enterobacteriaceae. Vet.Microbiol. 5:229-237.

Brill,B.M., Wasilauskas,B.L. and Richardson,S.H.(1979). Adaptation of the staphylococcal coagglutination technique for detection of heat-labile enterotoxin of Escherichia coli. J.Clin.Microbiol. 9:49-55.

Brinton,C.C.Jr. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram-negative bacteria Trans.N.Y. Acad. Sci. 27:1003-1053.

Brinton,C.C.Jr. (1967). Contributions of pili to the specificity of the bacterial surface, and a unitary hypothesis of conjugal infections heredity P. 37-70 In Davis,B.D. and Warren,L.(ed). The specificity of cell surfaces. Prentice Hall Inc., Englewood Cliffs, N.J.

Buchanan,R.E., Gibbons,N.E., Cowan,S.T., Holt,J.G., Liston,J., Murray,R.G.E., Niven,C.F., Ravin,A.W. and Stainer,R.Y.(1974). Bergey's Manual of Determinative Bacteriology. 8th Ed. Baltimore, Williams and Willkins Co.

Buissiers,J. and Nardon,P. (1968). Micromethode d' identification des bacteries I.Interet de la quantification des characteres biochimiques. cited by Smith et al (1972)

Burgess,M.N., Bywater,R.J., Cowley,C.M., Mullan,N.A. and Newsome,P.M. (1978). Biological evaluation of a methanol soluble, heat-stable Escherichia coli enterotoxin in infant mice, pigs, rabbits and calves Infect. Immun. 21:526-531.

Burgess,M.N., Mullan,N.A. and Newsome,P.M. (1980). Heat stable enterotoxins from Escherichia coli P.16. Infect.Immun. 28:1038-1040.

Burrows,W. and Musteikis,G.M. (1966). Cholera infection and toxin in the rabbit ileal loop. J.Infect.Dis. 116:183-190.

Busta,F.F. (1979). Food protection for the 80's. J.Food Prot. 42:596-598.

Clark,R.N. (1962). Water supply and waste disposal for milk processing plants. In Milk Hygiene, World Health Organization monograph series, 48:499-529.

Clements,J.D. and Finkelstein,R.A. (1979). Isolation and characterization of homogenous heat-labile enterotoxins with high specific activity from Escherichia coli cultures. Infect. Immun. 29:91-97.

Collins,C.H. and Lyne, P.M. (1970). Microbiological methods 3rd ed. Baltimore, Butterworth and Co.

Cooke,E.M., Edmondson,A.S. and Starkey,W.(1981). The ability of strains of Klebsiella aerogenes to survive on the hands J.Med.Microbiol. 14:443-450.

Cousins,M.A.(1972). Sources of bacteria in farm bulk tank Milk. J.Dairy Technol. 25:200-205.

Cousins,M.A.(1979). Milking techniques and the microbial flora of milk. The Milk Industry, 81:24.

Cousins,M.A.(1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J.Food.Prot. 45(2):172-207.

Cowan,S.T.(1974). "Cowan and Steel's Manual for the identification of medical Bacteria" 2nd Ed. Cambridge The Cambridge University Press.

Cox,N.A. and Mercuri,A.J. (1978). Comparison of two minikits (API and R.B.) for identification of Enterobacteriaceae isolated from poultry and meat products.J.Food Prot. 41:107-110.

Cox,N.A. and Mercuri,A.J. (1979). Accuracy of Micro ID for identification of Salmonella and other Enterobacteriaceae from clinical and food sources. J.Food Prot. 42:942-945.

Cravioto,A., Scotland,S.M. and Rowe,B.(1982). Haemagglutinating activity and colonization factor Antigens I and II in Enterotoxigenic and non enterotoxigenic strains of Escherichia coli isolated from humans. Infect. Immun. 36:189-197.

Cruickshank,R., Duguid,J.P., Marmion,B.P. and Swain,R.H.A. (1975). Medical microbiology Vol.II. Edinburgh, London Churchill, Livingstone.

Culbertson,Jr.R. and Osburn,B.I. (1980). The biological effects of bacterial enterotoxin: A short review. Vet.Sci. Commun. 4:3-14.

Cullen,G.A. and Hebert,C.N.(1967). Some ecological observations on microorganisms in habiting bovine skin, teat canals and milk. Br.Vet.J. 123:14-24.

Cuthbert,W.A.(1960). Bacteriological problems arising from circulation cleaning. J.Soc.Dairy Technol. 13:142-144.

Czirok,E., Orskov,I and Orskov,F.(1982). O:K:H:F Serotypes of fimbriated Escherichia coli strains isolated from infants with diarrhoea. Infect. Immun. 37:519-525.

De,S.N. and Chatterjee,D.N.(1953). An experimental study of the mechanism of action of Vibrio cholerae on the intestinal mucous membrane. J.Pathol.Bacteriol. 66:559-562.

Dean,A.G., Ching,A.C., Williams,R.G. and Harden,B.(1972). Test for Escherichia coli enterotoxins using infant mice:application in a study of diarrhoea in children in Honolulu. J.infect.Dis. 125:407-411.

Deneke,C.F., Thorne,G.M. and Gorbach,S.L.(1979). Attachment pili from enterotoxigenic Escherichia coli pathogenic for humans. Infect. Immun. 26:362-368.

Deneke,C.F., Thorne,G.M. and Gorbach,S.L., (1981). Serotypes of attachment pili of enterotoxigenic Escherichia coli isolated from humans. Infect. Immun. 32:1254-1260.

Donta,S.T., Moon,H.W. and Whipp,S.C.(1974). Detection of heat labile Escherichia coli enterotoxin with the use of adrenal cells in tissue culture. Science: 183: 334-336.

Duguid,J.P. and Campbell,I. (1969). Cited by Wilson,G.S. and Miles,A.(1975).

Duguid,J.P. and Old,D.C. (1980). Adhesive properties of Enterobacteriaceae Cited by Gaastra and Graaf (1982)

Duguid,J.P., Smith,W., Dempster,G. and Edmunds,P.N.(1955). Nonflagellar filamentous appendages ("Fimbriae") and haemagglutinating activity in Bacterium coli. J.Pathol. Bacteriol. 70:335-348.

Duguid,J.P., Clegg,S. and Wilson,M.I. (1979). The fimbrial and non-fimbrial haemagglutinin of Escherichia coli. J.Med. Microbiol. 12:213-227.

Dupont,H.L., Formal,S.B., Hornick,R.B., Snyder,M.J., Libonati,J.P., Sheahan,D.G., LaBrec,E.H. and Kalas,J.P. (1971). Pathogenesis of Escherichia coli diarrhoea New.Eng.J. Med. 285:1-9.

Edwards,P.R. and Ewing,W.H.(1972). Identification of Enterobacteriaceae - 3rd Ed. Minnesota, Burgess Pub.Co.,

Echeverria,P. and Murphy,J.R. (1980). Enterotoxigenic Escherichia coli carrying plasmids for antibiotic resistance and enterotoxin production. J.Infect. Dis. 142:273-278.

Echeverria,P., Ulayangco,C.V., Ho,M.T., Verhaert,S., Komalarini,S., Orskov,F. and Orskov,I. (1978a). Antimicrobial resistance and enterotoxin production among isolates of Escherichia coli in the far east. Lancet 2:589-592.

Echeverria,P., Blacklow,N.R., Vollet,J.L., Ulyangco,C.V., Cuckor,G., Soriano,V.B., Dupont,H.L., Cross,J.H., Orskov,F. and Orskov,I.(1978b). Reovirus-like agent and Enterotoxigenic Escherichia coli infections in paediatric diarrhoea in the Phillipines. J.Infect. Dis 138:326-331.

Echeverria,P., Blacklow,N.R., Zipkin,C., Vollet,J.L., Olsson,J.A., Dupont,H.L. and Cross,J.H.(1979). Etiology of gastro enteritis among Americans living in the Phillipines. American J. Epidemiol. 109:493-501.

Echeverria,P., Mejia,P.A. and Duangmani,C.(1981) Effects of antibiotics on the prevalence of enterotoxigenic Escherichia coli in two populations in Phillipines. Antimicrob Agents Chemother. 19:290-293.

Evans,D.J.Jr. and Evans,D.G.(1977). Direct serological assay for the heat-labile enterotoxin of Escherichia coli, using passive immune haemolysis. Infect. Immun. 16:604-609.

Evans,D.G. and Evans,D.J.Jr. (1978). New surface associated heat-labile colonization factor antigen (CFA II) produced by enterotoxigenic Escherichia coli of serogroups O:6, O:8. Infect. Immun. 21:638-647.

Evans,D.G., Evans,D.J.Jr. and Pierce,N.F. (1973a). Differences in the response of rabbit small intestine heat-labile and heat-stable enterotoxins of Escherichia coli. Infect. Immun. 7:873-880.

Evans,D.J.Jr., Evans,D.G. and Gorbach,S.L. (1973b). Production of vascular permeability factor by enterotoxigenic Escherichia coli isolated from man Infect. Immun. 8:725-730.

Evans,D.G., Silver,R.P., Evans,D.J.Jr., Chase,D.G. and Gorbach,S.L.(1975). Plasmid controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect. Immun. 12:656-667.

Evans,D.R.Jr., Evans,D.G., Dupont,H.L., Orskov,F. and Orskov,I. (1977). Patterns of loss of enterotoxigenicity by Escherichia coli isolated from adults with diarrhoea: suggestive evidence for an irrelationship with serotype. Infect.Immun. 17:105-111.

Evans,D.G., Evans,D.J.Jr. and Tjoa,W.S.(1977). Haemagglutination of human group A erythrocytes by enterotoxigenic and enteropathogenic Escherichia coli isolated from adults with diarrhoea: correlation with colonization factor. Infect. Immun. 18:330-337.

Evans,D.G., Evans,D.J.Jr., Tjoa,W.S. and Dupont,H.L.(1978). Detection and characterization of colonization factor of enterotoxigenic Escherichia coli isolated from adults with diarrhoea. Infect. Immun. 19:727-736.

Evans,D.J.Jr., Evans,D.G. and Dupondt.H.L.(1979). Haemagglutination patterns of enterotoxigenic and enteropathogenic Escherichia coli determined with human, bovine, chicken and guinea pig erythrocytes in the presence and absence of mannose. Infect. Immun. 23:336-340.

Evans,D.G., Evans,D.J.Jr., Clegg,S., Pauley,J.A.(1979) Purification and characterization of the CFA/I antigen of enterotoxigenic Escherichia coli. Infect. Immun. 25: 738-748.

Evans,D.J.Jr., Evans,D.G., Young,L.S. and Pitt,J.(1980). Haemagglutination typing of Escherichia coli.Definition of seven haemagglutination types. J.Clin.Microbiol. 12: 235-242.

Fader,R.C. and Davis,C.P. (1980). Effect of piliation on Klebsiella pneumoniae infection in rat bladders. Infect. Immun. 30:554-561.

Fader,R.C., Avots - Avotins,A.E. and Davis,C.P.(1979) Evidence for pili mediated adherence to Klebsiella pneumoniae to rat bladder epithelial cells in vitro Infect.Immun. 25:729-737.

Fantasia,L.D., Mestrendria,L., Schrade,J.P. and Yatts,J. (1975). Detection and growth of enteropathogenic Escherichia coli in ripened cheese. Appl.Microbiol. 29:179-185.

Fisher,A. and Svitavsky,K.(1975). Occurrence of hemolytic strains of Escherichia coli and Staphylococcus in the air of large premises for pigs and dairy cows in the course of one year. Vet.Bull.Abst. 45:4799.

Fluckiger,E., Maes,G. and Winterer,H.(1980). Factors influencing the bacteriological quality of raw milk Document 120 Brussels : International Dairy Federation.

Foster,E.M. (1982). Food safety : Problems of the past and perspective of the future. J.Food Prot. 45:658 660.

Foster,E.M., Nelson,F.E., Speck.M.L., Doetsch,R.N. and Olson,J.C.(1958). Dairy Microbiology, London MacMillan and Co., Ltd.,

Frank,J.F. and Marth,E.H.(1978). Survey of soft and semisoft cheese for presence of faecal coliforms and serotypes of enteropathogenic Escherichia coli.J. Food.Prot. 41:198-200.

Frank,J.F., Marth,E.M. and Olson,N.F.(1978). Behaviour of enteropathogenic Escherichia coli during the manufacture and ripening of brick cheese. J.Food Prot. 41:111-115.

Fung,D.Y.C. and Cox,A.C.(1981). Rapid microbial identification system in food industry: Present and future. J.Food Prot. 44:877-880.

Gaastra,W. and Graaf de.F.K.(1982). Host specific fimbrial adhesins of noninvasive Enterotoxigenic Escherichia coli strains. Microbiol. Rev. 46:129-161.

Gahlot,D.P., Pal,R.W. and Kapoor,C.M.(1975). Microbiological quality of market milk in Hissar city. J.Food Sci. Technol. 12:68-70.

Gangarosa,E.J. (1978) Epidemiology of Escherichia coli in United States, J. Infect. Dis. 137:634-638.

Gehriger,G.(1980). Factors influencing the bacteriological quality of raw milk. Document 120 Brussels: International Dairy Federation.

Gianella,R.A. (1976) Suckling mouse model for detection of heat-stable Escherichia coli enterotoxin: characteristics of model. Infect. Immun. 14:95-99.

Gianella,R.A. (1978). Purification of the heat stable enterotoxin from Escherichia coli of human origin Gastroenterology, 74:1124-1127.

Gianella,R.A. and Drake,K.W.(1979) Effect of purified Escherichia coli heat-stable enterotoxin on intestinal cyclic nucleotide metabolism and fluid secretion. Infect.Immun. 24:19-23.

Gianella,R.A., Drake,K.W. and Luttrell,M. (1981). Development of radio immunoassay for Escherichia coli heat stable enterotoxin: Comparison with suckling mouse bioassay. Infect. Immun. 33:186-192.

Glatz,B.A. and Brudvig,S.A.(1980a). Enterotoxin production in milk by enterotoxigenic Escherichia coli. J.Food Prot. 43:298-299.

Glatz,B.A. and Brudvig,S.A.(1980b). Survey of commercially available cheese for enterotoxigenic Escherichia coli J.Food. Prot. 43:395-398.

Gnan,S. and Leudecke,L.O. (1982). Impedence measurements in raw milk as an alternate to the standard plate count J.Food. Prot. 45:4-7.

Goel,M.C., Kulshrestha,D.C., Marth,E.H., Francis,D.W., Bradshaw,J.G. and Read,R.B.Jr.(1971). Fate of coliforms in yogurt, butter milk, sour cream and cottage cheese during refrigerated storage. J.Milk Food Technol. 34:54-58.

Golton,D.M., Adrinson,R.W. and Thomas,C.V.(1982). Effects of pre-milking udder preparations on environmental bacterial contamination of milk. J. Dairy Sci. 65:1540-1543.

Gomes,J.A., Rodrigues,A.C., Simoes,H., Serafim,M.B. and Castro,A.F.P.de. (1979). Simplification of methods for the production and storage of specimens to be tested for heat-stable enterotoxin of Escherichia coli. J. Clin.Microbiol. 10:786-790.

Gorbach,S.L., Thorne,G.M. and Deneke,C.F.(1981). Personal communication. Cited by Mehlman and Romero (1982).

Greenberg,J.B., Sack,D.A., Rodriguez,W., Sack,R.B., Wyatt,R.G., Kalica,A.R., Horswood,R.L., Chanock,R.M. and Kapikian,A.Z.(1977). Microtiter solid-phase radioimmunoassay for detection of Escherichia coli heat labile enterotoxin. Infect. Immun. 17:541-545.

Griffith,M.W. and Phillips,J.D.(1982). Identification of bacteria of dairy origin using miniaturized test system. J.Appl.Bacteriol. 53:343-350.

Gross,R.J., Cravioto,A., Scotland,S.M.Cheasty,T. and Rowe,B.(1978). The occurrence of colonization factor (CF) in enterotoxigenic Escherichia coli. Cited by Kornäki and Marth (1982b).

Guerrant,R.L., Brunton,L.L., Schnaitman,T.C., Rebhun,L.I. and Gilman,A.G.(1974). Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology a rapid sensitive in vitro assay for the enterotoxins of Vibrio cholerae and Escherichia coli. Infect. Immun. 10:320-327.

Guerrant,R.L., Dickens,M.A., Wenzel,R.P. and Kapikion,A.Z. (1976). Toxigenic bacterial diarrhoea:nursury out break involving multiple bacterial strains. J.Paediat. 89:855-891.

Guinee,P.A.M., Jansen,W.H. and Agterberg,C.M.(1976) Detection of the K 99 antigen by means of agglutination and immunoelectrophoresis in Escherichia coli isolated from calves and its correlation with enterotoxigenicity. Infect. Immun. 13:1369-1377.

Gyles,C.L. (1979) Limitation of the infant mouse test for Escherichia coli heat-stable enterotoxin Can.J. Comp.Med. 43:371-379.

Gyles,C.L., Palchandhuri,S. and Maas,W.K.(1977). A naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. Science. 198:198-199.

Hadad,J.J. and Gyles,C.L. (1978) Detection of bovine enteropathogenic Escherichia coli by indirect fluorescent antibody technique. Am.J.Vet.Res. 39:1651 1655.

Hall,E.H., Brown,D.F. and Lewis,R.H.(1967). Estimation of market foods for coliform organisms. Appl.Microbiol. 15:1062-1069.

Hansen,S.R.(1973). Studies on the influence of udder cleaning methods on the bacterial flora of the teat skin, the mastitis incidence and the bacterial contamination of bulk milk. Nord.Vet.Med. 25:359-371.

Harrigan,W.F. and McCance,M.E.(1976). Laboratory methods in food and dairy microbiology, London, Academic Press.

Harrison,J. (1938) Numbers and types of bacteria in cheese Cited by Harrigan,W.F. and McCance, M.E.(1976).

Harvey,W.C. and Hills,H.H. (1951). Milk production and control P.577, 3rd Ed. London: H.K.Lewis and Co. Ltd.

Hartley,J.C., Reinbold,G.W. and Vedamuthu,E.R.(1968). Bacteriological methods for evaluation of raw milk quality. A review I: Use of bacterial tests to evaluate production conditions. J.Milk Food Technol. 31:315.

Hartley,J.C., Vedamuthu,E.R. and Reinbold,G.W.(1969). Bacteriological methods for evaluation of raw milk quality. A review II.Bacterial tests used to measure milk quality. J.Milk Food Technol. 32:4.

Hartman,I., Ziv,G. and Saran,Z.G.(1976). Application of Limulus amoebocyte lysate test to the detection of Gram-negative bacterial endotoxin in normal and mastitic milk. Res. Vet.Sci. 20:342-343.

Hartman,P.A. and Minnich,S.A.(1981). Automation for rapid identification of Salmonella in foods. J.Food Prot. 44:385

Hayek,L.J. and Willis,G.W.(1976). A comparison of two commercial methods for the identification of the Enterobacteriaceae:API 20E and the enterotube with conventional methods. J.Clin. pathol. 29:158-161.

Hemken,R.W., Fox,J.D. and Hicks,C.L.(1981). Milk iodine content as influenced by heat sources and sanitizer residues. J.Food Prot. 44:476-479.

Holmes,B., Willcox,W.R. and Lapage,S.P.(1978). Identification of Enterobacteriaceae by the API 20E system J.Clin.Pathol. 31:22-30.

Hosono,A., Yastuki,K and Tokita,F.(1977). Isolation and characterization of an inhibitory substance against Escherichia sp. cited by Cousins,C.M.(1982).

Hubble,I.P. and Mein,G.A.(1981). Non-iodophor methods for cleaning and sanitizing milking machines. Aust. J.Dairy Technol. 36:64-68.

ICAR (1972) Manual in dairy bacteriology. Reports Subcommittee on dairy education. Bangalore 8th Dairy Teachers Workshop.

ICMSF (1974) Microorganisms in Foods 2.Sampling for microbiological analysis "Principles and Specific Applications". Recommendations of the international commission on microbiological specifications for foods of the International Association of Microbiological Societies, Toronto, University of Toronto Press.

Indian Standards No.1479-Part I-1960. Dairy Industry methods of test for: rapid examination of milk New Delhi : Indian Standards Institution.

Indian Standards No.1479-Part III-1962. Methods of test for Dairy Industry.Bacteriological analysis of milk New Delhi : Indian Standards Institution.

Indian Standards No.1479-Part III-1977. Methods of test for dairy industry. Bacteriological analysis of milk (First revision) New Delhi. Indian Standards Institution.

Indian Standards No.5253 (1969). Guidelines for cleaning and sterilizing dairy equipment, New Delhi, Indian Standards Institution.

Indian Standards No.5402 - 1969 - Methods for standard plate count of bacteria in food stuffs. New Delhi. Indian Standards Institution.

Indian Standards No.5404 - 1969-Code for practice of handling of samples for microbiological analysis. New Delhi. Indian Standards Institution.

Indian Standards No.5887 - Part II- 1976 Food poisoning (Part I) isolation, identification and enumeration of *Escherichia coli*. First Revision, New Delhi, Indian Standards Institution.

Issacson,R.E.(1978) K 99 surface antigen of *Escherichia coli*: antigenic characterization. Infect. Immun. 22:555-559.

Issacson,R.E. and Moon,H.W.(1975). Induction of heat labile enterotoxin synthesis in enterotoxigenic *Escherichia coli* by Mitomycin C. Infect. Immun. 12: 1271-1275.

Jacks,T.M., Wu,B.J., Braemer,A.C. and Bidlock,D.E.(1973). Properties of the enterotoxic component in *Escherichia coli* enterotopathogenic for swine. Infect. Immun. 7: 178-1979.

Jacquet,J. and Chiki,A.(1977). Chemotypes and antibiotypes of coliform bacteria isolated in a Normandy farm with calf septicaemia. Vet.Bull.Abst. 27:48

Jay,J.M.(1977). The Limulus lysate endotoxin assay as a test of microbial quality of ground beef. J.Appl. Bacteriol. 43:99-109.

Jay,J.M.(1981). Rapid Estimation of microbial numbers in fresh ground beef by use of the Limulus test. J.Food Prot. 44:275-278.

Jay,J.M. and Margitic,S.(1979). Comparison of homogenising, shaking and blending on the recovery of microorganisms and endotoxins from fresh and frozen ground beef as assessed by plate counts and Limulus amoebocyte lysate test. Appl.Environ.Microbiol. 38:879-884.

Jay,J.M., Margitic,S., Shereda,A.L. and Covington,H.V.(1979) Determining endotoxin content of ground beef by the Limulus amoebocyte lysate test as a rapid indication of microbial quality. Appl.Environ. Microbiol. 38:885-890.

Jayasankar,S.R., Dundani,A.T. and Iya,K.K.(1966). Studies on psychrophilic bacteria in milk. (Cited by Cousins, C.M., 1982)

Joergensen,K.(1980). Factors influencing the microbiological quality of raw milk. Document 120 Brussels: International Dairy Federation.

Jones,G.W. and Rutter,J.M. (1974). The association of Haemagglutinating activity in porcine strains of Escherichia coli J.Gen.Microbiol. 84:135-144.

Juffs,H.S.(1973). Proteolysis detection in milk I. Interpretation of tyrosine value data for raw milk supplies in relation to natural variation, bacterial counts and other factors. J.Dairy Res. 40:371-381.

Juven,B.J., Gardian,S., Rosenthal,I. and Laufer,A.(1981). Changes in refrigerated milk caused by Enterobacteriaceae J.Dairy Sci. 64:1781-1784.

Kaloyanov,I. and Gogov,I. (1977). Coliform bacteria in raw and pasteurized milk. Food Sci. Technol. Abst. 3P 408 (3) 11 (1979).

Kapitany,R.A., Scoot,A., Forsyth,G.W., McKenzie,S.F. and Worthington,R.W.(1979). Evidence for two heat stable enterotoxins produced by enterotoxigenic Escherichia coli. Infect. Immun. 24:965-966.

Kaplan,H.M. and Timmons,E.H.(1979). The Rabbit: A model for the principles of mammalian physiology and surgery Academic Press, Sanfransisco.

Kaplan,M.M., Abdussalem,M. and Bijilenga,G.(1962). Disease transmitted through milk. In milk hygiene World Health Organization monograph series. 48. Geneva : World Health Organisation.

Kartashova,V.M., Danilenko,I.P. and Vatsik,P.I. (1974). Hygiene significance of determination of coli titre at 37°C Dairy Sci. Abst. 8182. 38

Kay,H.D.(1962) Pasteurization outlines a procedure and control. In milk Hygiene World Health organization monograph. 48. Geneva:World Health Organisation.

Klipstein,F.A. and Engert,F.G.(1975). Enterotoxigenic intestinal bacteria in tropical sprue. III.Preliminary characterization of Klebsiella pneumoniae enterotoxin J.Infect.Dis. 132:200-203.

Klipstein,F.A. and Schenk,E.A.(1975). Enterotoxigenic intestinal bacteria in tropical sprue II.Effect of the bacteria and their enterotoxins on intestinal structure. Gastroenterotology. 68:642-655.

Klipstein,F.A. and Engert,F.G.(1976a) Purification and properties of Klebsiella pneumoniae heat stable enterotoxin. Infect. Immun. 13:373-381.

Klipstein,F.A. and Engert,R.F.(1976b). Partial purification and properties of Enterobacter cloacae heat stable enterotoxin. Infect. Immun. 13:1307-1314.

Klipstein,F.A. and Engert,R.F.(1977). Immunological interrelationship between cholera toxin and the heat-labile and heat stable enterotoxins of coliform bacteria. Infect. Immun. 18:110-117.

Klipstein,F.A., Holdemen,L.V., Corcino,J.J. and Moore, W.E.C.(1973). Enterotoxigenic intestinal bacteria in tropical sprue. Ann.Intern.Med. 79:632-641.

Klipstein,F.A., Horowitz,I.R., Engert,R.F. and Schenk, E.A.(1975). Effect of Klebsiellapneumoniae enterotoxin on intestinal transport in the rat. J.Clin. Invest. 56:799-807.

Klipstein,F.A., Lee,C.S. and Engert,R.F.(1976). Assay of Escherichia coli enterotoxins by in vivo perfusion in rat jejunum. Infect. Immun. 14:1004-1010.

Klipstein,F.A., Engert,R.F. and Short H.B.(1977) Relative enterotoxigenicity of coliform bacteria. J.Infect. Dis. 136:205-215.

Klipstein,F.A., Guerrant,R.L., Wells,J.G., Short,H.B. and Engert,R.F.(1979). Comparison of assay of coliform enterotoxins by conventional techniques versus in vivo intestinal perfusion. Infect. Immun. 25:146-152.

Klipstein,F.A., Engert,R.F. and Houghten,R.A.(1983a). Properties of synthetically produced Escherichia coli heat stable enterotoxin. Infect. Immun. 39:117-121.

Klipstein,F.A., Engert,R.F. Clements,J.D. and Houghten, R.A.(1983b). Vaccine for enterotoxigenic Escherichia coli based on synthetic heat stable toxin cross linked to the B sub unit of heat labile toxin. J.Infect. Dis. 147:318-327.

Koallick,M. and Holke,R.(1980). Water consumption in Industrial Milk production. Dairy Sci. Abst. 104(1982)

Kornacki,J.L. and Marth,E.H.(1982a) Fate of non-pathogenic and enteropathogenic Escherichia coli during manufacture of Colby like cheese. J.Food Prot. 45:310-316.

Kornacki,J.L. and Marth,E.H.(1982b). Food borne illness caused by Escherichia coli. A review. J.Food Prot. 45:1051-1067.

Kraft,A.A.(1982). Trends in instruction of food microbialogists. J.Food Technol. 36:74-77.

Kraft,A.A. and Rey,C.R.(1979) Psychrophilic bacteria in foods. An up date. Food Technol. 23:66-71.

Kumar,A., Sharma,V.D. and Tnapliyal,D.C.(1982) Occurrence of Enteropathogenic Enterotoxigenic Escherichia coli in cow, calves and infants/children Ind.J.Comp.Imm. and Inf. Dis. 3:174-177.

Kutas,F. and Kovats,L.(1979) Effect of heat stable Escherichia coli Enterotoxin (ST) on infant rat model Acta Veterinaria Academiae Scientiaram Hungaricae tomus, 27:265-271.

Lagrange,W.S.(1979). Opportunity to improve milk quality J.Food Prot. 42:599-603.

Lallier,R., Lariviere,S. and St.Pierre,S.(1980). Escherichia coli heat-stable enterotoxin: Rapid method of purification and some characteristics of the toxin. Infect. Immun. 28:469-474.

Lariviere,S., Gyles,G.L. and Barnum,D.A.(1972). A Comparative study of the rabbit and pig gut loop system for the assay of Escherichia coli enterotoxin Can.J.Comp.Med. 36:319-328.

Levin,M.M. and Bang,F.B.(1964). The role of entero toxin in the extra cellular coagulation of blood. Cited by Jay, J.M. (1977).

Levine,M.M. and Rennels,M.B. (1978). Escherichia coli colonization factor antigen in diarrhoea. Lancet. 2:534.

Levine,M.M., Rennels,M.B., Daya,V. and Hughes,F.P.(1980) Haemagglutination and colonization factors in entero-toxigenic and enteropathogenic Escherichia coli that cause diarrhoea. J.Infect.Dis. 141:733-737.

Lovett,J., Bisha,J.M. and Spaulding,P.L.(1979). Escherichia coli enterotoxin production in beef broth at 15 to 50°C J.Food Prot. 42:838.

Luck,H.(1972) Bacteriological quality tests for bulk cooled milk. A review:Dairy Sci.Abst. 34:101-122.

Luzio,N.R.& Friedmann,J.J.(1973) Bacterial entero toxin in the environment. Nature U.K. 244:5410:49-51.

Mabbit,L.A.(1980a) Factors influencing the bacteriological quality of raw milk Document 120: Brussels: International Dairy Federation.

Mabbit,L.A.(1980b) Factors influencing bacteriological quality of raw milk (Summary) Document 120. Brussels: International Dairy Federation.

McCoy,J.H.(1966). Milk borne disease: Reviews of the progress of dairy Science. J.Dairy Res. 33:103-106.

MacFaddin,J.F.(1980). Biochemical test for identification of medical bacteria. 2nd Ed. Baltimore, Williams and Wilkins.

Marrier,R.J.G., Wells,R.C. Swanson,R.C. and Mehlman,I.J. (1973). An outbreak of enteropathogenic Escherichia coli food borne disease traced to imported French cheese. Lancet. 2:1378-1378.

McConnel,M.M., Willshaw,G.A., Smith,H.R., Scotland,S.M. and Rowe,B.(1979). Transposition of ampicillin resistance to an enterotoxin plasmid in an Escherichia coli strain of human origin. J.Bacteriol.139:346-355.

Mehlman,I.J. and Romero,A.(1982) Enteropathogenic Escherichia coli: Methods of recovery from food. Food Technol. 36(3):73-79.

Mehlman,A.J., Fishbein,M., Gorbach,S.L. and Olson,J.C.Jr. (1976). Pathogenecity of Escherichia coli recovered from food. J.Assoc.Off.Anal.Chem. 59:57-80.

Mercuri,A.J. and Cox,N.A.(1979). Coliform and Enterobacteriaceae isolates from selected foods. J. Food Prot. 42:712-714.

Merson,M.H., Orskov,F., Orskov,J., Sack,R.B., Huq,I. and Hoster,F.F.(1979). Relationship between enterotoxin production and serotypes in enterotoxigenic Escherichia coli. Infect. Immun. 23:325-329.

Merson,M.H., Sack,R.B., Islam,S., Sacklayen,G., Huda,N., Hua,I., Nulich,A.W., Yolken,R.H. & Kapikian,A.Z.(1980). Disease due to Enterotoxigenic Escherichia coli in Bangladesh adults: clinical aspects and controlled trial of tetracycline. J.Infect. Dis. 141:702-711.

Mikolajcik,E.M.(1979). Psychrotrophic bacteria and dairy product quality: Major organisms involved and defects produced. Cultural Dairy product J. 14:6-10.

Mikolajcik,E.M.(1980) Organisms of Public Health importance in fermented dairy products. Cultured Dairy Products. J. 15:15-17.

Misra,H.N. and Rao,C.C.P. (1975). Role of milk and milk products in the etiology of diarrhoea in infants. Izatnagar, U.P. Annual report of Division of Veterinary Public Health.

Misra,S.K. and Sinha,M.N. (1978). Incidence of coliforms in raw milk samples. Indian J.Dairy Sci. 31:378-380.

Moeller,R.W., Smith,I.M., Shoemaker, Alice,C. and Tjalma, R.A.(1963). Transfer of hospital staphylocci from man to farm animals. J.Amer.Vet.Med.Ass. 142:613.

Molenda,J., Kozyrczak,J., Skurski,A., Zwierzchowski,J. and Statoniewicz,Z.(1983). Influence of Immunization with enteropathogenic and non-enteropathogenic Escherichia coli on the occurrence of loop dilation in rabbits. Res. Vet.Sci. 34:1-4.

Moon,H.W., Whipps,S.C., Engstrom,G.W. and Baetz,A.L.(1970) Response of the rabbit ileal loop to cell-free products from Escherichia coli enteropathogenic for swine J.Infect.Dis. 121:182-187.

Moon,H.W., Whipp,S.C. and Baetz,A.L.(1971). Comparative effect of enterotoxin from Escherichia coli and Vibrio cholerae on rabbit and swine small intestine. Cited by Raskova,H. and Raska,K.(1980).

Moon,H.W., Fung,P.Y., Whipp,S.C. and Isaacson,R.E.(1978) Effects of age and ambient temperature on the resource of infant mice to heat-stable enterotoxin of Escherichia coli:assay modification. Infect. Immun., 25:127-132.

Moran,J.W. and Witter,L.D.(1976). An automated rapid test for Escherichia coli in milk, J.Food Sci. 41:165-167.

Morse,P.M., Jackson,H., McNaughton,C.H., Leggat,G., Landerkin,B. and Johns,C.K.(1968). Investigation of factors contributing to the bacterial count of bulk tank milk III. Increase in count from cow to bulk tank and effects of refrigerated storage and preliminary incubation. J.Dairy Sci. 51:1192-1206.

Muier,D.D., Kelly,M.E. and Phillips,J.D.(1978). The effect of storage temperature on bacterial growth and lipolysis in raw milk. J.Soc.Dairy Technol. 31:203-208.

Mundell,D.H., Anselmo,D.R. and Wishnow,R.M.(1975). Factors influencing heat-labile Escherichia coli enterotoxin activity. Infect. Immun. 14:383-388.

Nachum,R. and Shambrom,E.(1981). Rapid detection of Gram negative bacteriuria by Limulus amoebocyte lysate test assay, J.Clin. Microbiol. 13:158-162.

Nalin,D.R., Bhattacharjee,A.K. and Richardson,S.H.(1974) Cholero like toxic effect of culture filtrate of an Escherichia coli. J.Infect. Dis. 130:595-601.

Natarajan,A.M., Ramaraju,V.V., Nambudripad,V.K.N. and Ranganathan,B.(1983). A comparative study of different plating procedures and media for enumeration of coliform bacteria in milk. Paper submitted to the XIX Dairy Industry Conference, Madras.

Olsson,E.(1982). Cultural methods for the production of heat-stable enterotoxin by porcine strains of Escherichia coli and its detections by Infant mouse test. Vet.Microbiol. 7:253-260.

Orskov,I. and Orskov,F.(1977). Special O:K:H Serotypes among enterotoxigenic Escherichia coli strains from diarrhoea in adults and children. Occurrence of the CF (colonization factor) antigen and of haemagglutinating abilities. Med. Microbiol.Immunol. 163:99-110.

Orskov,I. and Orskov,F. (1980). Cholera and related diarrhoeas. 43rd Nobel symp. Stockholm, 1978. pp. 134-137.

Orskov,I., Orskov,F., Sojka,W.J. and Leach,J.M.(1961). Simultaneous occurrence of E.coli B and L antigens in strains from diseased swine.cited by Gaastra and Graaf (1982)

Orskov,I., Orskov,F., Smith,H.W. and Sojka,W.J.(1975). The establishment of K 99 a thermostable, transmissible, Escherichia coli K antigens previously called " KCO" possessed by calf and lamb enteropathogenic strains. Acta.Pathol.Microbiol. Scand. Sect. B:31-36.

Orskov,F., Orskov,I., Evans,D.J.Jr. Sack,R.B., Sack,D.A. and Wadstrom,T.(1976). Special Escherichia coli serotypes among enterotoxigenic strains from diarrhoea in adults and children. Med.Microbiol. Immunol. 162:73-80.

Palmer,J.(1980). Contamination of milk from the milk from the milking environment. Factors influencing the bacteriological quality of raw milk. Document 120, Brussels. International Dairy Federation.

Panhotra,B.R. and Agarwal,K.C.(1981). Haemagglutination and buccal epithelial cell adherence of enterotoxigenic Escherichia coli isolated from infants with acute diarrhoeal disease. Indian J.Med.Res. 74:187-191.

Panhotra,B.R., Agarwal,K.C. and Agnihotri,V.(1981) Enterotoxigenic and enteropathogenic Escherichia coli isolated from hospital food. Indian J.Med.Res. 74: 183-186.

Pitarangsi,C., Echeverria,P., Whitmire,R., Tirapat,C Formal,S., Dammin,C.J. & Tingtalapong,M.(1982). Enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides: Prevalence among individuals without diarrhoea in Thailand. Infect.Immun. 35:666-673.

Poelma,P.N., Romero,A., Andrews,W.H.(1977). Rapid identification of Salmonella and related food borne bacteria by the five biochemical multitest system. J.Food Sci. 42:677-680.

Poelma,P.N., Romero,A., Andrews,W.H. (1978). Comparative accuracy of the five biochemical system for identifying related food borne bacteria. Colaborative study. J. Assoc. Offi.Anal.Chem. 61:1043-1049.

Prasad,G., Khan,B.L. and Kulshrestha,D.L.(1980) Survival of Escherichia coli and Enterobacter aerogenes in dahi. Indian J.Dairy Sci. 33:497-500.

Prevention of food adulteration act (1954). Ministry of health, New Delhi, Government of India.

Prevention of food adulteration rules (Amended, 1979), Ministry of Health, New Delhi, Government of India.

Prier,R.B. and Spagna,V.A.(1983). Rapid evaluation of gonococcal and non gonococcal urethritis in man with Limulus amoebocyte lysate and a chromogenic substance J.Clin.Microbiol. 17:485-488.

Pruzzo,C., Debbia,E.A. and Satta,G.(1980). Identification of the major adherence ligand of Klebsiella pneumoniae in the receptor in coliphage 17 and alteration of Klebsiella, adherence properties by hydrogenic conversion. Infect. Immun. 30:562-577.

Ranganathan,B.(1973). Studies on Enterotoxigenic Escherichia coli in milk and milk products. National Dairy Research Institute, Karnal, Hissar; Summer Institute on Enterobacteriaceae.

Rangasamy,P.W. (1980). The influence of production methods of milk quality, Dairy Sci. Abst. 44:4064.

Rao,S.K.R. and Nambudripad,V.K.N.(1978). Mastitis survey in the region. Annual Report : National Dairy Research Institute, Karnal P.163.

Raskova,H. and Raska,K.(1980). Enterotoxins from Gram negative bacteria relevnt for Veterinary Medicine. Vet.Res.Commun. 4:195-224.

Rendos,J.J., Eberhart,R.J. and Kesler,E.M.(1975). Microbial populations of teat ends of dairy cows and bedding material. J.Dairy Sci. 58:1492-1500.

Richard,L., Guerrant,R.L. and Sauer,K.T.(1981). Selective use of microbiological procedure for identification of etiological agents of diarrhoeal illness. J:Food safety 3:(1981) 145-164.

Richardson,G.H., Marth,E.H., Marshall,R.T., Mesur,J.W., Ginn,R.E., Wehr,H.M. Gase,R. and Bruhn,J:C.(1980). Update of the fourteenth Ed. of Standard methods for examination of dairy products. J.Food Prot. 43:324-328.

Richter,R.(1981). Microbiological problems in dairy foods in the 1980's.J.Food Prot. 44:471-475.

Reinhart,D.J., Naborns,W., Kennedy,C. and Griggs,B.M.(1981) Limulus amoebolysate and direct sampling methods for surveillance of operating Nebulizers. Appl.Environ. Microbiol. 42:850-855.

Robertson,E.A: and Maclowry,J.E.(1974). Mathematical analysis of the API Enteric profile register using a computer diagnostic model. Appl.Microbiol. 28:691-695.

Robichaud,N., Lallier,R. and Lariviere,S.(1978). Comparison of the various test systems for the detection of Escherichia coli enterotoxin. Proceedings of the second international symposium on neonatal diarrhoea. Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada.

Robins,Browne,R.M., Levine,M.M., Rowe,B. and Gabriel,E.M. (1982). Failure to detect conventional enterotoxins in classical enteropathogenic (Serotyped) Escherichia coli of proven pathogenecity. Infect. Immun. 38:798-801.

Rondinini,G.(1982). Control of environmental hygiene in the dairy sector from production to processing, Dairy Sci. Abst. 44:2331.

Sack,R.B.(1978). The epidemiology of diarrhoea due to enterotoxigenic Escherichia coli. J.Infect. Dis. 137: 639-640.

Sack,R.B.(1980) Enterotoxigenic Escherichia coli: Identification and characterization. J.Infect.Dis. 142: 279-286.

Sack,R.B.(1981) Escherichia coli and acute diarrhoeal diseases. Ann.Intern.Med. 94:129-137.

Sack,R.B., Gorbach,S.L., Banwell,J.G., Jacob,B., Chatterjee,B.D. and Mitra,R.C.(1971). Enterotoxigenic Escherichia coli isolated from patients with severe cholera-like disease. J.Infect. Dis. 123:378-385.

Sack,D.A., Mersen,M.H., Wells,J.G.(1975). Diarrhoea Associated with heat-stable enterotoxin producing strains of Escherichia coli. Lancet, 2:239-241.

Sack,R.B., Sack,D.A., Mehlman,I.J., Orskov,F. and Orskov,I. (1977). Enterotoxigenic Escherichia coli isolated from food J.Infect. Dis. 135:313-317.

Sadruddin,F.A., Krovacek,K. and Wadstrom,T.(1981). Enterotoxigenic bacteria in food and water from an Ethiopian Community. Appl.Environ.Microbiol. 41:1010-1019.

Sanderson,E.(1976). Genetic relatedness in the family Enterobacteriaceae. Annu.Rev. Microbiol. 30:327-349.

Schonberg,F.(1951) Factors influencing the bacteriological quality of raw milk. Cited by Joergensen,K.(1980).

Schoreder,S.A., Caldwell,J.R., Vernon,T.M., White,P.C., Granger,S.I. and Bennet,J.V.(1968). A water borne outbreak of gastroenteritis in adults associated with Escherichia coli. Lancet, 1:737-740.

Scotland,S.M., Day,N.P., Cravioto,A., Thomas,L.V. and Rowe,B.(1981). Production of heat-labile or heat stable enterotoxin by strains by Escherichia coli belonging to serogroups 044, 0114 and 0128. Infect. Immun. 31:500-503.

Serafim,M.B., Pestana de Castro,A.F., Lemos des Reis,M.H. and Trabulsi,L.R.(1979). Passive immune haemolysis for detection of heat-labile enterotoxin produced by Escherichia coli isolated from different sources. Infect. Immun. 24:606-610.

Sheldrake,R.F. and Hoare,R.J.T.(1982) Post-milking disinfection of bovine teats. Effects of dipping versus spraying on sub-clinical mastitis. Atmosphere iodine concentration and volume of disinfectant used. J.Dairy Technol. 37:95-98.

Shore,E.G., Dean,A.G. and Holik,K.J.(1974). Enterotoxin producing Escherichia coli and diarrhoeal disease in adult travellers: a prospective study. J.Infect.Dis. 129:577-582.

Singh,R.S. and Ranganathan,B.(1974). Occurrence of Enterotopathogenic Escherichia coli serotypes in milk and milk products, Milchwisseneschaft. 29: 529-532.

Singh,R.S. and Ranganathan,B.(1978). Incidence and distribution of Escherichia coli in dairy products. Indian J.Dairy Sci. 31(1):92.

Smith,H.W. and Halls,S.(1967a). Observation by the ligated segment and oral inoculation methods of Escherichia coli infection in pigs, calves, lambs and rabbits. J.Pathol.Bacteriol. 93:499-529.

Smith,H.W. and Halls,S.(1967b). Studies on Escherichia coli enterotoxin. J.Pathol.Bacteriol. 93:531-543.

Smith,H.W. and Linggood,M.A.(1972). Further observations on Escherichia coli enterotoxins with particular regard to those produced by atypical strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of K.antigens possessed by calf and lamb strains. J.Med.Microbiol. 5:243-250.

Smith,P.B., Tomfohrde,K.M., Rhoden,D.L. and Balows,A.(1972) API 20E system a multitube method for identification of Enterobacteriaceae. Appl.Microbiol. 24:449-452.

Smythe,V.R.(1960) Factors influencing the bacteriological quality of raw milk. Cited by Tolle,A.(1980).

Starvic,J.I., Speirs,J.L., Konowalchuk,J. and Jeffrey,D. (1978). Stimulation of cyclic AMP secretion in Vero cells by enterotoxins of Escherichia coli and Vibrio cholerae. Infect. Immun. 21:514-517.

Staples,S.J., Asher,S.E. and Gianella,R.A.(1980). Purification and characterization of heat-stable enterotoxin produced by a strain of Escherichia coli pathogenic for man. J.Biol.Chem. 255:4716-4721.

Stirm,S., Orskov,F., Orskov,I and Mansa,B., (1967). Episome carried surface antigen K 88 of Escherichia coli. II.Isolation and chemical analysis. J.Bacteriol. 93:731-739.

Swanson,E.C. and Collins,M.T.(1980). Use of API 20E system to identify Veterinary Enterobacteriaceae. J.Clin.Microbiol. 12:10-14.

Swartling,P. (1967) Neth.Milk and Dairy.J. 21:87-102. Cited by Gehriger,(1980).

Tenney,J.H., Smith,T.F. and Washington,J.A.II (1979) Sensitivity, precision and accuracy of the YI adrenal cell enterotoxin assays. J.Clin.Microbiol. 9:197-199.

Thomas,S.B.(1958). Psychrophilic microorganisms in milk and dairy products, Part I. Cited by Cousins,C.M., (1982).

Thomas,S.E.(1964). Investigation of the bacterial content and microflora of farm dairy equipment. J.Soc.Dairy Technol. 17:210.

Thomas,L.V., Cravioto,A., Scotland,S.M. and Bowe,B.(1982) New Fimbrial antigenic type (E.8775) that may represent a colonization factor in Enterotoxigenic Escherichia coli in humans. Infect. Immun. 35:1119-1124.

Thorne,G.M., Deneke,C.F. and Gorbach,S.L.(1979) Haemagglutination and adhesiveness of toxigenic Escherichia co isolated from humans. Infect. Immun. 23:690-699.

Tiripathi,R.C. and Soni.J.L. (1982) Antibiotic sensitivi test with Escherichia coli isolates from cases of ne natal calf diarrhoea. Indian Vet.J. 59:413-416.

Todd,E.C.D.(1978) Food borne diseases in Canada, 1975. Annual summary,J.Food Prot. 41:910-918.

Tolle,A.(1980). Factors influencing the bacteriologica quality of raw milk. Document 120 Brussels. Inter national Dairy Federation.

Tullock,E.F.Jr., Ryan,K.J., Formal,S.B. and Franklin,A. (1973). Invasive enteropathogenic Escherichia col dysentry: An outbreak in 28 adults. Ann.Intern.Md. 79:13-17.

Twedt,R.M. and Boutin,B.K.(1979). Potential Public Health Significance of non Escherichia coliforms in foo J.food prot. 42:161-163.

Tzipori,S., Smith,M., Halpin,C., Makin,T. and Krakowka,F. (1983). Intestinal changes associated with rota virus and Enterotoxigenic Escherichia coli infection in calves. Vet.Microbiol. 8:35-53.

Verstrete,W. and Votes,J.P. (1974). The microbial flora of air ecology analysis and removal. Microbiol Abst.4

Wachsmuth,K., Wells,J., Shipley,P. and Falkow,S.(1979). Heat-labile enterotoxin production in isolates from a shipboard outbreak of human diarrhoeal illness. Infect.Immun. 24:791-797.

Wadstrom,T., Smyth,C.J., Faris,A., Jonsson,P., Freer,J.H. (1978). Hydrophobic absorptive and haemagglutinating properties of Enterotoxigenic Escherichia coli with different colonizing factors. K.88, K.99 and colonization factor antigens and adherence factor. Proceedings 2nd International symposium on Neonatal diarrhoea VIDO University of Saskatschewan, Canada.

Waes,G.M. and Bossuyt, (1982). Usefulness of Benzalkon Crystal violet - ATP method for predicting the keepin quality of pasteurized milk. J.Food Prot. 45:928-931.

Walsh,S.M. and Bissonnette,G.K.(1983). Chlorine induced damage to surface adhesins during sublethal injury of Enterotoxigenic Escherichia coli. Appl.Environ. Microbiol. 45:1060-1065.

Whipp,S.C., Moon,H.W. and Lyon,N.C.(1975). Heat-stable Escherichia coli enterotoxin production in vivo. Infect. Immun. 12:240-244.

Wilson,G.S. and Miles,A.(1975). Topley and Wilson's principles of Bacteriology, Virology and Immunity, 6th ed. Vol.1 London, Edward and Arnold.

Winslow,R.T.(1982). The food microbiologists' role in th professional execution of industry's calls for a safe wholesome food supply. Food Technol. 36:60-62.

Yoger,J.F. and Kershaw,E.(1974). Immunofluorescent procedure for the detection of enteropathogenic Escherichia coli in soft ripened cheese. Dairy Sci. Abst. 38:6577.

Yolken,R.H., Greenberg,H.B., Merson,M.H., Sack,R.B. and Kapikian,A.Z.(1977). Enzyme-linked immunosorbent assay for detection of Escherichia coli heat-labile enterotoxin. J.Clin.Microbiol. 6:439-444.