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Detection and Growth of Enteropathogenic *Escherichia coli* in Soft Ripened Cheese[1]

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The organism most frequently encountered during the 1971 outbreak of enteropathogenic *Escherichia coli* (EPEC) in soft ripened cheese was a strain that failed to ferment lactose broth within 48 h. Since existing methods for *E. coli* are dependent upon fermentation of this sugar, such strains can remain undetected, particularly when present in low numbers. Therefore, a cultural testing procedure was developed to insure isolation of both lactose-positive and -negative strains. This method used GN broth, modified by substituting lactose and arabinose for glucose and D-mannitol, as an enrichment medium. MacConkey agar, used as a plating medium, was modified by substituting arabinose for half the lactose. The cultural procedure was used in conjunction with a fluorescent antibody method to screen cheese for the presence of presumptive enteropathogenic *E. coli*. Suspected isolates were subjected to further biochemical and serological testing and identified as members of specific serogroups. These methods were used for the analysis of over 2,000 wheels of cheese; over 10% of the samples tested were found to contain strains belonging to six different serogroups associated with diarrheal diseases. No attempt was made to confirm pathogenicity by in vivo tests. Enumeration of *E. coli* in cheese showed that numbers increased during storage. Cheese with less than 10 organisms/g initially increased to over 10^5 at room temperature and over 10^3 at 4 C within 10 days. With higher initial counts, levels up to 10^9 were found at 4 C. These studies showed that the high levels of *E. coli* encountered in these products cannot be used as a direct indicator of post-processing contamination.

In the United States, enteropathogenic types of *Escherichia coli* (EPEC) are usually associated with cases of infantile diarrhea. Studies from India and Vietnam by Gorbach et al. (6) and Japan by Sakazaki et al. (9) showed that EPEC cause disease not only in children but in adults as well. Dupont et al. (2) performed studies with several types of EPEC to determine the effects of these organisms on animals and human volunteers. Their work demonstrated that EPEC can cause disease in man by at least two mechanisms: by production of a cholera-like enterotoxin and by an invasion of intestinal epithelial lining. Before November 1971, there were reports of water-borne outbreaks of EPEC in the United States, but no confirmed outbreaks were associated with foods (1). However, from 30 October to 10 December 1971, over 200 persons in more than 90 separate outbreaks suffered acute food poisoning symptoms after eating imported Camembert or Brie cheese.

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Our laboratory investigated several complaints of food poisoning in which Camembert cheese imported from France was implicated. None of the common food poisoning organisms were detected; however, high levels of *E. coli* were encountered and, with the methods used, were frequently the only organism found. This led to the serotyping of these organisms and their subsequent identification as EPEC. However, no in vivo tests were performed to confirm pathogenicity. Therefore, the term EPEC as used in this paper refers only to those strains belonging to serogroups having known association with diarrheal disease as outlined by Ewing (4). The most frequently detected type was a strain of O124:B17 *E. coli* that did not ferment lactose within 48 h. This strain was found usually by direct plating of the cheese on agar media and was also recovered from individuals who had contracted the disease (7).

These isolates all failed to ferment lactose broth within 48 h; however, after that time fermentation did occur with some strains. Since not all isolates were tested after 48 h, the term

"lactose negative" will be used throughout the remainder of this text. Methods for analysis of *E. coli* normally used fermentation of lactose as a selective factor. Therefore, slow or nonfermenting strains could remain undetected particularly when present in low numbers. With this in mind, existing methods for analysis of *E. coli* were evaluated and a tentative procedure for the detection of EPEC was developed that would not exclude lactose-negative strains. In addition, studies were initiated to determine whether the high levels of *E. coli* in the cheese were due to improper processing or simply to the ability of these organisms to grow in the product under normal conditions of handling.

MATERIALS AND METHODS

Cultures. Cultures of *E. coli* were obtained from the collection of the Food and Drug Administration (FDA), New York District. One strain from each of the following 20 serogroups was used in the media evaluation studies: O86:B7, O124:B17, O128:B12, O112:B11, O125:B15, O126:B16, O119:B14, O127:B8, O100, O70, O66, O62, O46, O44, O40, O32, O21, O18, O17, and O11. The lactose-negative strain of *E. coli* O124:B17 was isolated from imported cheese samples. All cultures were routinely grown in heart infusion broth (Difco) at 37 C. Working cultures were transferred daily, whereas stock cultures were maintained on heart infusion (Difco) slants stored under refrigeration.

Media. Lauryl tryptose broth, brilliant green lactose bile broth, EC broth, GN broth (Hajna) and MacConkey agar were obtained from Difco and prepared according to the manufacturer's instructions. A modification of MacConkey agar was also used. In this, 5 g of lactose and 5 g of arabinose were substituted for the 10 g of lactose per liter normally present. A modification of GN broth was also used in which 1 g of lactose and 1 g of arabinose per liter were substituted for the glucose and D-mannitol.

Antisera. Isolates were screened as presumptive EPEC by typing with *E. coli* OB poly A and poly B and with *E. coli* OK poly C antisera, all from Difco. Monovalent *E. coli* O, OB, and OK antisera (Difco) were used in the identification of the specific serogroups present. Difco polyvalent fluorescein-labeled anti-*E. coli* globulin (poly A, B, and C) was used for the screening of cheese samples by the fluorescent antibody (FA) method. A 1:4 dilution of these conjugates was determined to be an appropriate working dilution by the method of Goldman (5).

Identification. *E. coli* isolates were identified as possible enteropathogenic types on the basis of biochemical characteristics and serological titrations as outlined by Ewing (4).

Enumeration of E. coli. Samples of imported soft ripened cheese collected at the time of entry at the Port of New York were analyzed for the numbers of *E. coli* present immediately upon receipt in the laboratory. Serial dilutions out to 10^{-7} were prepared in potassium phosphate buffer at pH 7.2. The same enumeration procedure was used in studies on the growth of *E. coli* in stored cheese samples. Each dilution was inoculated into three tubes of lauryl tryptose broth containing inverted durham tubes. Cultures producing gas in 24 and 48 h at 37 C were then subcultured into EC broth and incubated in a constant-temperature water bath at 45.5 C. The presence of gas was recorded after 24 and 48 h. The most probable number (MPN) of *E. coli* was then calculated per gram of original cheese.

FA procedure. Three smears were prepared from GN broth cultures by using a 2-mm loop onto multiwell Teflon-coated slides (5). Smears were air dried; the slides immersed in xylene for 3 min, drained and placed in Kirkpatrick fixative solution (ethyl alcohol-chloroform-formalin [60:30:10]) for 3 min, and air dried. Treatment of the slides with xylene removed lipids that interfered with fixation. The slides were then rinsed in 95% ethyl alcohol and air dried. The three smears were then treated separately, each with one drop of poly A, B, or C *E. coli* conjugate. The slides were placed into moist chambers and incubated for 30 min. Excess conjugate was drained and the slides were rinsed in phosphate-buffered saline (pH 7.5). The slides were washed twice in a phosphate-buffered saline bath for 5 min and rinsed in distilled water. After air drying, a drop of buffered glycerol saline at pH 7.5 was placed on the slide and covered with a no. 1 cover slip. Slides were examined with an American Optical Series 20 Microstar microscope equipped with a 50-W mercury vapor lamp. A cardioid dark-field condenser, BG 12 exciter filter, and an OG 1 barrier filter completed the system. Fluorescent cells were rated on a scale of 1+ to 4+. Slides with cells rated 3+ or 4+ (bright cell outline with clear lumen) were considered positive.

RESULTS

In preliminary studies it was found that strains of EPEC grew well in GN broth. It was thought that selectivity for EPEC might be improved by substituting lactose and arabinose for the glucose and D-mannitol normally present in this medium as carbon sources. According to Edwards and Ewing (3), *Edwardsiella*, *Providencia*, *Serratia*, and *Proteus* for the most part will not ferment either lactose or arabinose. However, 91% of *E. coli* ferment lactose and 99% use arabinose. Thus, chances of encountering strains of *E. coli* incapable of growth on one or the other of these sugars should be minimal.

GN broth, the modified GN broth, and several other media normally used for selection of *E. coli* were compared for ability to support the growth of EPEC. The test organisms were a lactose-negative and a lactose-positive strain of *E. coli* type O124:B17 and a nonpathogenic strain of *E. coli*. The two O124:B17 strains came from samples of cheese known to have caused diarrhea, and it is highly likely that they were in fact pathogenic. The three strains were grown for 24 h in heart infusion broth, and 0.01 ml of each culture was inoculated into tubes contain-

ing 10 ml of the test media. The tubes were incubated for 18 h at 37 C and viable cells were estimated by plate counts on standard methods agar.

The results in Table 1 show no significant difference in the levels of growth achieved by the nonpathogenic strain of *E. coli* in any of the media tested. Some inhibition of the lactose-positive pathogenic strain was noted in EC broth at 45.5 C, possibly because of inhibition by bile salts and the elevated temperature. All other media yielded comparable amounts of growth with this strain. The differences in growth levels for the pathogenic lactose-negative strain were much more pronounced. Growth was substantially less in the broths having a lactose base; however, cell numbers in GN broth and the modified GN broth were higher and of about the same value. Evidently, with the pathogenic lactose-negative strain the amount of growth in the modified GN broth was not affected by the fact that only the arabinose and not the lactose present in that medium could be utilized.

In another experiment, a modified MacConkey agar was tested as a plating medium. Although lactose-negative EPEC will grow on MacConkey agar with the normal formulation, colonies do not appear as typical *E. coli* since acid is not produced from the lactose present. Normally these colonies would not be picked for routine screening. The medium was therefore modified by deleting half the lactose and substituting an equivalent amount of arabinose. This modification eliminated the need to pick lactose-negative isolates on MacConkey agar. This modified medium was tested by streaking cultures representing 20 different serogroups of *E. coli* on the plates and noting colonial morphology and color reaction. Strains were tested both singly and in combinations of a lactose-negative with a lactose-positive type. All strains gave typical colonial morphology and color reaction. No differences in selectivity were noted on plates streaked with the mixed cultures. When typical *E. coli* colonies were randomly picked from such plates and transferred to phenol red lactose broth, approximately equal numbers of lactose-positive and lactose-negative cultures were recovered.

TABLE 1. *Growth of pathogenic and nonpathogenic strains of E. coli in various media*

Medium	Strains		
	Pathogenic lactose +	Pathogenic lactose −	Non-pathogenic lactose +
Lauryl sulfate tryptose broth	7.1[a]	5.7	7.1
EC broth	6.4	5.5	7.2
Brilliant green lactose broth	7.1	5.5	7.2
GN broth	7.2	7.2	7.3
GN broth (modified)	7.3	7.5	7.5

[a] Growth is expressed as $\log_{10}$ number of cells per milliliter after 18 h at 37 C.

On the basis of these observations, the cultural procedure as outlined in Fig. 1 was devised for the screening of EPEC in soft cheese. On day 1, a slurry consisting of 50 g of cheese in 50 ml of modified GN broth is prepared. After 1 h of incubation, a loopful of the slurry is streaked onto modified MacConkey agar plates. One hundred and fifty milliliters of modified GN broth is then added and the slurry is incubated for 18 to 24 h at 37 C. On day 2, a loopful of the slurry is streaked onto a second set of modified MacConkey agar plates. The first plates are examined, and if typical colonies are present a minimum of 10 are picked. Each colony is used to inoculate a slant of Simmons citrate agar, a slant of heart infusion agar, and a tube of KCN broth. On day 3, if the citrate and KCN tubes are negative, the heart infusion slant is used as a source of cells for serological screening. The second set of modified MacConkey agar plates is used as above in cases where the initial direct plating does not yield presumptive *E. coli* based upon negative reactions in citrate and KCN. Isolates that are both KCN and citrate negative and give a positive reaction with *E. coli* OB antisera before and after boiling are considered presumptive positives. These cultures are then identified biochemically and serologically as outlined in the *CDC Laboratory Manual* (4).

The above procedure has been used successfully during the last 2 years for routine screening of EPEC at the New York District FDA laboratories. Over 2,000 cheese samples were examined, and approximately 10% of the samples were found to contain *E. coli* belonging to serogroups associated with diarrheal diseases. The majority of these isolates were detected shortly after the initial food outbreak and belonged to the following serogroups: O124:B17 (lactose negative); O124:B17 (lactose positive); O112:B11; O124:B15; O128:B12; and O127:B8. Since not all strains in a given serogroup are virulent, actual pathogenicity of an isolated strain should be confirmed by a test such as the ileal loop procedure.

After the initial samples generated by the outbreak had been analyzed, attempts were also

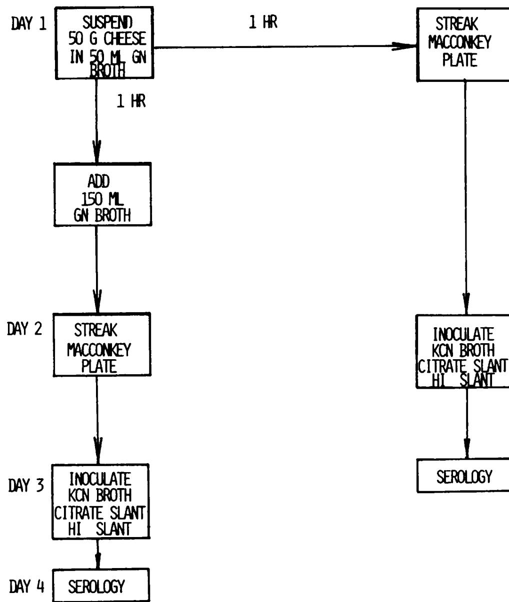

FIG. 1. *Diagrams of screening procedure used for detection of enteropathogenic E. coli in soft ripened cheese. GN broth was modified by substitution of lactose and arabinose for glucose and D-mannitol. MacConkey agar was modified by substitutions of arabinose for half the lactose. Cells from slants of heart infusion agar were used for serological screening.*

made to screen cheese samples for EPEC by using immunofluorescent techniques. We felt this would be feasible since the conjugates available did cover serogroup O124:B17, the organism implicated in the food poisonings. A slurry of cheese was prepared in modified GN broth and incubated for 18 to 24 h at 37 C as previously described (Fig. 1). After incubation, 0.5 ml was inoculated into 5 ml of modified GN broth and incubated for 4 h at 37 C. This second enrichment was found necessary to reduce the carry-over of product that fluoresced brightly under ultraviolet light. Three smears were prepared and stained with poly A, B, or C *E. coli* FA conjugate as described in Materials and Methods.

Several hundred cheese samples were screened by this procedure. A number of positive enrichment cultures were detected, and these were identified as EPEC types by the cultural, serological, and biochemical procedure outlined in Fig. 1. No false FA negatives were found, but a false-positive rate of approximately 20% was encountered.

These results indicate that the FA method might be developed as a routine screening procedure. If the 4-h enrichment culture is FA negative, then the analysis could be terminated; those cultures giving a positive reading would be streaked on modified MacConkey agar for subsequent confirmation.

In some of the first cheese samples analyzed at the time of the food poisoning outbreak, counts in excess of 3×10^7 *E. coli* were detected. Studies were undertaken to determine the significance of these high levels. Fifty-five imported cheese samples (approximately 1 lb [454 g] each) were collected at the port of entry and brought to the laboratory under ice. Although the cheese was refrigerated during shipment, the actual temperature and any previous storage history was not known. On arrival in the laboratory, the samples were placed in a refrigerator at 4 C equipped with a continuous recording thermometer. Samples were individually removed from the refrigerator and cut into two sections. One section was returned immediately to the refrigerator. A portion of the other section was used to determine the initial level of *E. coli* using the MPN procedure. The remainder of this section was stored at ambient room temperature. Although the actual temperature of the refrigerated cheese sections was not known, it was assumed that they reached 4 C very rapidly since they were relatively small in size and were stacked to allow maximum air circulation. After 2, 4, 8, and 10 days of storage, the levels of *E. coli* in both sections were determined again by the MPN procedure. Precautions were taken to insure that there was no significant increase in temperature of the cheeses when they were removed from the refrigerator for enumeration of *E. coli*.

The initial MPN values varied from less than 10 to 10^5 *E. coli*/g. The numbers of *E. coli* in all 55 samples increased upon storage. Data representing three typical cheese samples with high, medium, and low initial levels are shown in Fig. 2.

The rate of growth for *E. coli* in the portions maintained at room temperature was significantly greater than for those kept refrigerated. However, given a significant period of time, sections of cheese maintained under refrigeration often achieved the same levels as their counterparts kept at room temperature.

Apparently, the initial level of *E. coli* did not influence the ability of the organisms to eventually propagate. Samples that had 10 or less *E. coli* per g and even some with levels initially undetectable demonstrated an increase in *E. coli* upon holding. After 10 days of storage, samples held at room temperature had more than 10^6 *E. coli*/g. The refrigerated counterpart had more than 10^3 *E. coli*/g.

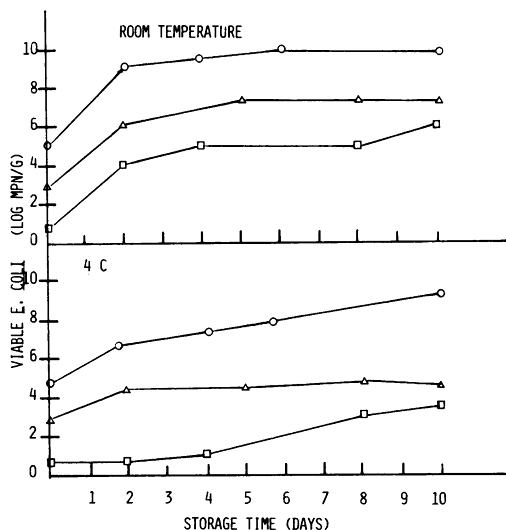

FIG. 2. *Most probable number of E. coli per gram at various times during storage of soft ripened cheese. Each type of symbol refers to a separate cheese sample that was divided in half and stored at two different temperatures. The data shown represents typical results for cheese samples with high* (○), *medium* (△), *and low* (□) *initial levels of E. coli.*

DISCUSSION

The cheese samples collected as a result of the outbreak in 1971 and analyzed by our laboratory were found to contain as many as 3×10^7 EPEC/g. In the F.R.I. Annual Report of The University of Wisconsin, Trenk and Deibel also found *E. coli* in soft ripened cheese at levels greater than 100,000/g. Due to the high numbers present in the cheese, more than 90% of the EPEC detected in the original outbreak could be isolated by direct plating of the product. The strains isolated by direct plating were frequently not of the same type as encountered after incubation of the product in an enrichment broth. Selective agars streaked from enrichment broths, such as lauryl tryptose, brilliant green lactose bile, and EC, were predominantly populated with lactose-positive organisms in serogroups not associated with disease, and in many instances the negative or late lactose-fermenting *E. coli*, O124:B17, generally found by direct plating, was not present. Studies conducted by Sakazaki et al. (9) further substantiate the need to look for lactose-negative *E. coli*. Over an 8-year period, of the 764 types of *E. coli* that these workers found associated with disease in man, 393 were negative lactose-fermenting strains.

The need to pick at least 10 colonies per plate must be emphasized. The methods used for isolation do not select for only pathogenic types but for all *E. coli* that might be present. Furthermore, many isolates may autoagglutinate even in saline solution, making serological identification impossible. From samples consisting of 10 packages of cheese in which 10 colonies were picked per cheese, as few as three isolates were identified as EPEC.

Therefore, initially we chose a method using a direct plating of the cheese slurry on MacConkey agar and enrichment in GN broth with subsequent streaking on MacConkey agar. In both instances, colonies appearing lactose negative or positive were picked for biochemical and serological screening. Later this procedure was changed to use of the modified MacConkey agar and modified GN broth. On the modified MacConkey agar it was assumed that all or almost all *E. coli* strains would produce an acid reaction regardless of whether they were lactose positive or negative. Organisms belonging to the genera *Edwardsiella*, *Providencia*, *Serratia*, and *Proteus* would not give acid reactions, and if still present after enrichment in modified GN broth they would be readily distinguishable from *E. coli*. One disadvantage to the use of modified MacConkey, however, is that if lactose-negative *E. coli* are present in low numbers, they may be missed by random picking of 10 typical colonies. At this point it is not known which is the most advantageous, exclusion of the above four genera or the ability to distinguish lactose-positive and -negative strains of *E. coli* early in the analysis. If one were attempting to specifically isolate a lactose-negative strain, the nonmodified agar would be the obvious choice. For routine screening we prefer the modified agar medium; however, perhaps both should be used.

Analysis of cheese manufactured by the firm implicated in the original food poisoning outbreak revealed that the problem was not confined to only one type of product. EPEC was found in multiple lots of Camembert, Brie, and Coulommiers cheese. At that time all products manufactured by the firm were removed from the market and a survey of all soft cheeses entering the Port of New York was instituted. During a 1-month period more than 10% of the soft ripened cheeses tested were found to contain EPEC types and the problem was not limited to one manufacturer. Once again these organisms were often encountered in very high numbers.

The curing process required for soft cheese production and the physical properties of the final product are significant in explaining how high levels of *E. coli* can develop. Soft ripened cheese has a moisture content generally greater

than 50%. After the formation of the curd, the curing or ripening is caused by decomposition of protein due to the activity of molds. This activity reduces the acidity of the curd and the pH rises from about 4.9 to 7.5. Thus, the acidic products produced originally by the growth of bacteria are neutralized and an ideal environment is created for the growth of *E. coli*. Those cheeses found to contain over 6×10^7 *E. coli*/g had pH values in the range of 7.2 to 7.4. A neutral pH after this much growth is normally not encountered in food products, and acids produced by bacteria usually cause inhibition and eventually reduction in cell numbers.

It is well known that *E. coli* can propagate in dairy products. For example, Olson et al. (8) and Watrous et al. (11) demonstrated that coliform levels can increase in milk upon holding at 45 F after pasteurization. These studies were conducted on milk that had no detectable coliforms immediately after pasteurization, and precautions were taken to prevent post-processing contamination. Presumably, very low levels of *E. coli* were actually present after pasteurization but were not detected by the cultural methods, and these were capable of subsequent growth. The MPN of *E. coli* in cheeses stored at 4 C and at room temperature was determined in the studies reported here by ability to produce gas in EC broth at 45.5 C. Although this procedure is not a confirmatory one, it is frequently used as an indication of the presence of *E. coli*. Evidently, *E. coli* is capable of growing in soft ripened cheeses when low levels are present initially, even under controlled refrigeration. Another possibility is that the increase in numbers does not indicate growth but represents injured *E. coli* cells that were able to repair themselves at 4 C and ultimately grow in EC broth at 45.5 C. In any event, our studies emphasize the need for methods sensitive enough to detect extremely low levels of EPEC. At this point it is impossible to determine the relative significance of pasteurization and subsequent sanitation as regards the initial numbers of *E. coli* detected in the finished product.

The FA procedure tested here has been successful in detection of *E. coli* O124:B17 in enrichment cultures inoculated with cheese samples. The average cheese sample as received by FDA consists of 10 individual packages. Assuming 10 colonies are picked for each package tested, a sample would generate 100 cultures for identification. If the FA procedure could be developed as a routine screening method, the need for costly and time-consuming biochemical and serological testing of hundreds of negative isolates could be eliminated. This is not possible at the present time because the conjugates now available commercially are limited in their coverage of the EPEC types and would need to be expanded for use in a broad screening application.

In the United States, food-borne strains of *E. coli* capable of causing serious disease in adults have so far only been associated with soft ripened cheese. Nevertheless, other foods might harbor and transmit these organisms, and testing laboratories must consider routinely that *E. coli* is a possible food-borne pathogen. In considering testing methods to be used, it must be emphasized that serotyping simply identifies an organism as a member of a group previously associated with the disease and does not establish that it is actually pathogenic. All organisms belonging to that group may not be pathogenic. Furthermore, it is known that factors associated with virulence can be transmitted from donor strains to nonpathogenic strains. For example, Smith and Halls (10) have shown that a genetic factor, presumably a plasmid, responsible for enterotoxin production in certain strains of *E. coli* can be transmitted to other non-enterotoxigenic strains of *E. coli*. The recipient strains were capable of causing diarrhea in pigs. Therefore, it is possible that new pathogenic strains can develop that have serotypes not previously associated with disease. At present, pathogenicity can only be established by testing in animals by a procedure such as the ileal loop technique. Unfortunately, such a procedure is too time-consuming and expensive to use for routine analysis of all suspected isolates. Thus, existing procedures for detection of *E. coli* must be reevaluated and perhaps new and simpler methods devised for identification of the pathogenic strains.

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