CHARACTERIZATION OF BACTERIA WHICH PRODUCE COLONIES ATYPICAL OF THE COLIFORM GROUP ON VIOLET RED BILE AGAR[1]

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SUMMARY

Thirty-three strains of bacteria were isolated during routine analysis of dairy products at two Canadian centers from colonies on violet red bile agar VRB which measured less than 0.5 mm in diameter after incubation at 35 C for 24 hr. All isolates showed typical coli-form morphology and Gram reaction and from the results of biochemical test were identified as coliform biotypes. No strain survived laboratory pasteurization at 145 F for 30 min. Strains of presumptive intestinal orgin (*E. coli*) were typed serologically and one strain was found to be of serotype 026:B6, an important etiological agent of infantile diarrhoea. It is concluded that size of colony is not a valid criterion for discriminating between colonies of coliform and non-coliform bacteria on VRB.

Coliform organisms have probably received more attention than most other groups of bacteria occurring in dairy products, apart from the lactic acid bacteria, owing to their importance as "indicator" species in routine analysis. The presence of even a few coliforms per unit volume in a product immediately after adequate pasteurization suggests recontamination from improperly sanitized equipment or unsanitary handling, while a higher coliform density in a stored product may indicate a higher initial recontamination or inadequate refrigeration. To enable a reliable assessment of the sanitary quality of a product to be made therefore, accurate determination of its coliform density is essential. When such a determination is made by a plating method using a solid medium, clearly a major factor governing its accuracy is the precision of the colony count.

The American Public Health Association (APHA) has formulated and published *(1)* a detailed plating procedure for coliform tests of dairy products using the solid medium violet red bile agar (VRB). On this medium subsurface colonies of coliform bacteria, which by definition ferment lactose *(1)*, are purplish red in color and are usually surrounded by a reddish zone of precipitated bile *(3)*. According to the APHA recommendations *(1)*, only colonies which show these characteristics and which, in addition, measure 0.5 mm or more in diameter on uncrowded plates incubated at 32 or 35 C for 18-24 hr should be counted. Colonies with a diameter of less than 0.5 mm on such plates are not considered to be of coliform bacteria.

In the course of a survey of coliform biotypes in Canadian pasteurized dairy products (to be reported elsewhere) a number of strains isolated from colonies on VRB which were atypical of coliform colonies on the basis of their diameter (i.e. < 0.5 mm) were studied to determine whether failure to enumerate them in routine analysis was justified. The results obtained form the substance of this report.

MATERIALS AND METHODS

Isolates

Thirty-three strains of bacteria were isolated from colonies on VRB which measured less than 0.5 mm in diameter after incubation for 24 hr at 35 C. The isolations were made at two geographically separated Canadian centers during routine analysis of various pasteurized dairy products and were shipped to this laboratory, without prior incubation, in 5-ml screw-capped tubes containing VRB. Preliminary examination of the tubes on receipt showed that sufficient growth for subculturing the isolates had developed during transit. Each isolate was then streaked on a VRB plate and the inoculated medium was overlaid with 4-5 ml of sterile VRB. Plates were incubated at 37 C for 24 hr and a single discrete colony was picked into nutrient broth (NB). After incubation a smear was prepared from each broth culture and stained by Gram's method. The smears were examined for the Gram-negative short rod character typical of coliform bacteria. In this way an apparently pure culture of each isolate was obtained. To test the stability of its colony characteristics on VRB each purified isolate was serially transferred several times in pour plates of this medium; inoculated plates were incubated at 37 C for 24 hr between transfers.

Biochemical tests

A series of biochemical tests was carried out on each isolate using methods recommended in Manual of Microbiological Methods *(8)*. Each strain was treated in duplicate and control tests on uninoculated media were run in parallel with each set of tests performed. The tests used were those for *(a)* gas production in brillian green bile 2% (BGB) within 48 hr at 37 C; *(b)* growth in BGB at 44-45 C (modified Eijkman test); *(c)* indole production; *(d)* reduction of pH to 4.5 or lower in dextrose broth incubated for 48 hr at 37 C (methyl red test); *(e)* acetoin production (Voges-Proskauer test); *(f)* citrate utilization; *(g)* gelatin hydrolysis.

Classification

From results of these tests each isolate was classified according to a scheme which combined the systems proposed by Wilson *(10)* (Group I) and Mushin and Ashburner *(6)*

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TABLE 1. BIOCHEMICAL CLASSIFICATION OF 33 BACTERIAL STRAINS PRODUCING COLONIES ON VIOLET RED BILE AGAR ATYPICAL OF COLIFORM BACTERIA

Biochemical characters								
Gas from BGB at 37C	Growth at 44-45C	Indole produced	Dextrose broth pH 4.5	Acetoin produced	Citrate utilized	Gelatin hydrolyzed	Biotype	No. of strains found
							Group I:	
+	+	+	+	−	−	−	*E. coli* I	2
+	−	−	+	−	−	−	*E. coli* II	1
+	−	−	+	−	+	−	Intermediate I	4
+	−	−	−	+	+	−	*A. aerogenes* I	5
+	−	+	−	+	+	−	*A. aerogenes* II	4
+	−	+	+	−	−	−	Irregular I	1
+	−	−	−	+	−	−	Irregular V	4
+	+	−	−	+	+	−	Irregular VI	3
							Group II:	
+	−	+	+	+	+	−	Irregular B	2
+	−	−	+	+	+	−	Irregular E	1
+	−	+	−	+	−	−	Irregular F	1
+	−	+	−	−	−	−	Irregular G	1
							Group III:	
+	−	−	+	+	−	−	Irregular M	3
+	−	+	−	−	−	+	Irregular S	1
							Total	33

(Group II) with a system (Group III) set up during the present investigation to accommodate biotypes which could not be assigned to either of the former groups.

Laboratory pasteurization

The effect of laboratory pasteurization on the survival of the isolates was tested as follows: A 1-ml volume of sterilized skim milk, inoculated from a 24-hr NB culture to contain 50,000 - 700,000 cells per ml, as determined by a standard plate count (SPC) *(1)*, was sealed into a sterile 6 x 100 mm glass tube in such a way that heating of the milk was avoided. After cooling, the tubes were completely immersed in a water bath at 145 F. Since preliminary experiments using thermocouples sealed into similar tubes of skim milk had established an average warm-up time of 75 sec, the inoculated tubes were removed from the bath after 31 min and 15 sec, and immediately cooled in ice-water. In this way the cells were exposed to the temperature of the bath for exactly 30 min. Cooled tubes were incubated at 37 C for 24 hr; they were then opened aseptically without heating the milk and the contents were plated with VRB. Absence of colonies from the plates after incubation at 37 C for 48 hr indicated inability of the organism to survive laboratory pasteurization.

Serological typing

Strains classified as *Escherichia coli* I, *E. coli* II or Irregular I biotypes were tested against antisera to a number of well-established enteropathogenic *E. coli* serotypes *(9)*, using a modification of the procedure recommended in Difco Supplementary Literature *(4)*. Each test organism was grown at 37 C for 18 hr in brain heart infusion (BHI) and the culture was heated in a boiling water bath for 1 hr. The cell suspension was then standardized optically at 600 mμ against a cell suspension in BHI previously shown by a SPC to contain 5 x 10^8 cells per ml. The appropriate monovalent antiserum (Difco) was serially diluted in Kahn serological tubes with 0.85% sodium chloride solution. From an initial tenfold dilution of antiserum serial two-fold dilutions were prepared to a maximum of 1:2560 *(8)*. An additional tube containing only 0.5 ml of NaCl solution was included as a control. One-half milliliter of test cell suspension was then added to each tube and mixed with the diluted antiserum. The tubes were incubated in a water bath at 50 C for 18-20 hr and examined for agglutination every 2-4 hr. Agglutination at an antiserum dilution of 1:320 or greater showed the test organism to contain the homologous O antigen. This constituted identification of the serotype.

RESULTS

All 33 isolates studied showed typical coliform morphology, cell arrangement and Gram reaction when examined microscopically. Their colonies on VRB were typically red but in some cases lacked a halo of precipitated bile. On serial transfer in uncrowded VRB pour plates the small colony size characteristic of the isolates on initial isolation was generally maintained, although a few isolates showed occasional colonies with diameters in excess of 0.5 mm. These were sufficiently infrequent to suggest that colony diameters of less than 0.5 mm were typical of the strains.

Results of the biochemical tests are shown in Table 1. Responses of the various isolates to these tests were variable, yet the characters of 29 of the 33 strains studied fitted those of previously recognized coliform biotypes. Of these, 12% fell in the group of presumptive intestinal origin represented by *E. coli* I, *E. coli* II and Irregular I biotypes. The

4 biotypes classified in Group III have not previously been recorded. All isolates produced gas in BGB within 48 hr at 37 C.

Three of the 4 presumptive intestinal biotypes tested serologically failed to agglutinate with any of the antisera used; one, however, was identified as belonging to serotype 026:B6. This strain was an *E. coli* II biotype which had been isolated from homogenized milk showing 300 coliform colonies per ml on VRB. Under the test conditions it agglutinated at the highest dilution of homologous antiserum used, namely 1:2560.

None of the 33 isolates studied survived laboratory pasteurization at 145 F for 30 min.

Discussion

Violet red bile agar is not absolutely selective for the growth of coliform bacteria. According to Standard Methods *(1)* some strains of cocci may produce minute red colonies and Koburger *(5)* has reported that *Mimi polymorpha*, a Gram-negative cocco-bacillus, produces red spindle-shaped colonies less than 0.3 mm long on the medium. Barber and Fram *(2)*, in an investigation of false coliform counts on fruit ice cream, showed that sucrose carried over with the sample permitted development of colonies of non-coliform bacteria on several selective media, including VRB. If organisms other than coliforms produce colonies on VRB under conditions used for the coliform count, the question arises as to the basis on which such colonies may be omitted from consideration when colony counts are made, in order that errors in the coliform count may be minimized. In Standard Methods it is recommended that the distinction between colonies of coliform and non-coliform bacteria be made principally on the basis of colony size, the critical diameter being 0.5 mm. This recommendation is apparently made on the basis of the finding of Yale *(11)* that red colonies less than 0.5 mm in diameter rarely formed gas from lactose and therefore did not represent true coliforms.

In the present investigation 33 strains of bacteria present in dairy products were isolated from colonies on VRB with diameters less than 0.5 mm; these colonies would have been considered atypical of coliform colonies, and hence disregarded, in coliform counts in which Standard Methods recommendations were followed. That these isolates were indeed coliform bacteria is shown by the typical microscopic appearance and Gram reaction of their cells, by their ability to ferment lactose in BGB with production of gas and by their response to a number of biochemical tests. In no case did the biochemical characters of an isolate agree with those of *M. polymorpha (7)*. With the exception of 4 strains all were identified as recognized coliform biotypes, although the remaining 4 were also coliform strains. Support for the identification of at least one of the strains studied as a coliform is its recognition as an enteropathogenic *E. coli* serotype. Serotype 026:B6 is accepted as one of the more important etiological agents of infantile diarrhoea *(9)*.

The results of this study suggest that colony size is not a valid criterion for excluding certain colonies from enumeration in coliform counts using VRB. Failure to enumerate red colonies with diameters less than 0.5 mm, whether or not these colonies show a halo of precipitated bile, may lead to underestimation of coliform populations in dairy products and hence to erroneous assessments of their sanitary quality. Special significance in this respect attaches to the finding of an enteropathogenic *E. coli* serotype which produced an "atypical" colony on VRB.

It is suggested that further study of types of bacteria producing colonies on VRB should be undertaken, with a view to improving the selective properties of the medium or otherwise to facilitate discrimination between colonies of coliform and non-coliform bacteria.

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