Studies on the pathogenicity and Heat-Resistant Characteristics of *Escherichia coli* in milk and milk products.
SUMMARY AND CONCLUSIONS

The present study pertains to an investigation on the incidence of *Escherichia coli* with particular reference to the pathogenic serotypes occurring in milk and milk products produced and handled under different conditions. Attention has also been directed towards a study of the incidence of heat-resistant types among the pathogenic and non-pathogenic strains of *E. coli*.

The heat-resistant characteristics of three selected *E. coli* cultures comprising two pathogenic and one non-pathogenic strains have been studied in detail. The effect of several factors such as the type of substrate, growth temperature, initial cell concentration and pH of the medium on the heat-resistant characteristics of the above cultures has also been reported.

2. A total of 285 samples of milk and milk products were examined for the incidence and distribution of coliforms, as well as for the presence of *E. coli*. The samples included 128 samples of raw and 57 of pasteurized milk (both of cow and buffalo), 30 of cream and butter, 35 of ice-cream and *kulfi* and 35 samples of cheese and *burfi*.

3. The incidence of coliforms in samples of milk and milk products examined in this study showed considerable variation. In cow milk samples coliform counts varied from 500 to 50,000 per ml, while buffalo milk samples exhibited higher counts ranging from 50,000 to 1,960,000 organisms per ml. In the case of pasteurized milk, 5 cow and 3 buffalo milk samples were free from coliforms. High coliform counts of more than 5,000 per ml were observed in two samples of pasteurized cow and 4 samples of pasteurized buffalo milk.
Ten samples of cream and 7 samples of ice-cream and kulfi showed a coliform count of more than 100,000 per gram. One of the kulfi samples exhibited maximum coliform count of 3,450,000 per gram. All the 15 samples of processed cheese obtained from the Institute's Dairy did not contain any coliform bacteria. Ten out of 15 samples of cheddar cheese showed coliform counts ranging from 100 to 1000 per gram, while in the rest, the counts varied from 1000 to 20,000 per gram. In the butter samples which included both salted and unsalted types, the coliform counts varied from 250 to 8,200 per gram. Only 5 burfi samples were examined and in these the coliforms did not exceed 500 per gram.

4. A total of over 1200 colonies were isolated from 285 samples of milk and milk products and were examined for the presence of *E. coli*. In all, 167 isolates representing one from each sample of milk and milk products were taxonomically identified as strains of *E. coli*.

5. All the 167 *E. coli* strains were further categorised into 4 groups of biotypes on the basis of biochemical tests. One hundred and thirteen strains which have been grouped as *E. coli* I biotype were found to be positive for Indole and Eijkman test, besides being motile. Thirtyfour cultures differed from the above strains only in respect of being non-motile and hence they were categorised as group II of *E. coli* I biotype. Nine cultures were grouped as *E. coli* II biotype as they showed negative reaction for Indole production and Eijkman test, while 11 isolates which produced indole and exhibited a negative Eijkman test were grouped as *E. coli* III biotype. Among the several biochemical tests included in the classification of *E. coli* biotypes, Eijkman test
appeared to be the most reliable and rapid single test for identification of \textit{E. coli} type I.

6. Considerable variation was observed among the 4 groups of \textit{E. coli} biotypes with respect to fermentation of carbohydrates such as lactose, glucose, mannitol, maltose, sucrose, salicin, adonitol, dulcitol and inositol. On the basis of differences in fermentation reactions, a further division into 7 groups was made. However, it was observed that on the basis of biochemical and sugar fermentation tests it was not possible to clearly differentiate between pathogenic and non-pathogenic \textit{E. coli} strains.

7. The different \textit{E. coli} biotypes were serologically grouped by using 15 standard enteropathogenic \textit{E. coli} antisera, namely 026:B6, 055:B5, 086:B7, 0111:B4, 0119:B14, 0126:B16, 0127:B3, 0128:B12 and 020, 044, 080, 0112, 0114, 0124 and 0125. These antisera were procured from different laboratories in India and abroad. It was found that cultures were typable against only 10 out of 15 antisera. The remaining 5 antisera for which the cultures did not show any response, were 044, 080, 086:B7, 0114 and 0125.

8. As regards the distribution of enteropathogenic \textit{E. coli} biotypes, it was found that out of 147 \textit{E. coli} cultures categorised under group I and II of \textit{E. coli} I biotype, 49 were found to be serologically positive. It was interesting to note that from among the 20 isolates belonging to \textit{E. coli} II and III biotypes, only two cultures each of the above two groups were serologically positive against 026:B6, 055:B5 and 0126:B16 serogroups. Thus, it was evident that out of 167 cultures, 53 constituting 31.1\% of the \textit{E. coli} strains were serologically positive and hence
considered pathogenic.

9. Out of the 128 samples of raw milk examined, 30 showed the presence of 10 enteropathogenic serotypes, namely 020, 026:B6, 055:B5, 0111:B4, 0112, 0119:B14, 0124, 0126:B16, 0127:B8 and 0128:B12. Among the 57 pasteurized milk samples, 9 contained 5 enteropathogenic \textit{E. coli} serotypes, namely 026:B6, 055:B5, 0111:B4, 0126:B16 and 0128:B12. In regard to the other milk products it was observed that 9 samples of ice-cream and kulfi indicated the presence of 6 enteropathogenic serotypes, namely 026:B6, 055:B5, 0111:B4, 0119:B14, 0126:B16 and 0127:B8. Milk products such as cream, butter and cheese contained very few enteropathogenic \textit{E. coli} serotypes. The public health significance of enteropathogenic \textit{E. coli} serotypes has been discussed with particular reference to the high incidence of two serotypes 055:B5 and 0126:B16 in milk and milk products.

10. All the 53 enteropathogenic \textit{E. coli} serotypes were tested for agglutination titres ranging from 1:40 to 1:2560 and only those showing the titre of 1:320 and above were considered as belonging to a particular serogroup. On this basis, 35 cultures gave a titre of 1:320, 15 gave 1:640 and only 3 gave the highest titre of 1:1280.

11. The pathogenicity of \textit{E. coli} strains was also tested by mice inoculation experiments. The inoculated organisms were recovered from the internal organs of lethal cases. A culture was designated "virulent" when all the three or at least two out of three mice were killed within a period of 48 hours. A culture was regarded as "partially virulent" (doubtful pathogenicity) when one out of three inoculated mice was killed after 24 hours.
"Avirulent" cultures were those which did not kill the mice even one week after their inoculation. Based on the above criteria, 50 out of 53 enteropathogenic \textit{E. coli} strains were found to be virulent types, while only 2 cultures belonging to serotypes 0112 and 0119:B14 were proved to be partially virulent thereby showing their doubtful pathogenic reactions in mice. One culture of serotype 0126:B16 was found to be of avirulent type.

12. One hundred and fourteen \textit{E. coli} strains were serologically untypable. Among these, 24 each were found to be virulent and partially virulent, while 66 isolates were avirulent.

13. From distribution of different samples of milk and milk products showing the presence of \textit{E. coli} strains which were pathogenic to mice, it was observed that 45 out of 128 samples of raw milk, 12 of pasteurized milk, 5 of cream and butter, 9 of ice-cream and 4 of cheese contained \textit{E. coli} cultures which were virulent to mice.

14. When all the 167 isolates of \textit{E. coli} strains were tested for haemolysis on bovine blood agar, only 17 were found to be of Beta-haemolytic type. It was also observed that 15 haemolytic strains were lethal to mice. Three of the haemolytic strains belonged to important enteropathogenic serotypes, namely 055:B5, 0119:B14 and 0126:B16. Although these three serotypes have been generally considered to be of non-haemolytic type, the present investigation has indicated their occurrence in both haemolytic and non-haemolytic forms. The importance of the occurrence of haemolytic strains of \textit{E. coli} in milk and milk products has been stressed in regard to their public health importance.
15. All the 167 isolates of *E. coli* were exposed to laboratory pasteurization (63°C/30 minutes) in cow skim milk both by the tube and ampoule methods. The number of strains surviving pasteurization were determined by the growth of heat-treated cells in the above substrate.

16. Fifty-four strains from among the 167 *E. coli* cultures survived pasteurization by the tube method, while only 6 cultures resisted pasteurization treatment by the ampoule method. Out of 54 heat-resistant strains, 17 were pathogenic to mice. Four cultures of this group belonged to enteropathogenic serotypes, namely 055:B5, 0126:B16 and 0127:B8. One culture out of 6 survived pasteurization treatment by the ampoule method and it belonged to the enteropathogenic serotype, 0127:B8.

17. As regards the distribution of samples of milk and milk products indicating the presence of heat-resistant strains of *E. coli*, it was found that 22 samples of raw milk, 16 of pasteurized milk, 5 of cream and butter, 9 of ice-cream and kulfi, and 2 of cheese showed the presence of *E. coli* cultures which were resistant to laboratory pasteurization.

18. A point of particular epidemiological interest in the present study is the heat-resistant characteristics of pathogenic strains of *E. coli* isolated from a variety of milk and milk products. It is likely that the enteropathogenic *E. coli* serotypes which had initially gained entry into milk during its production and handling might not have been destroyed due to improper pasteurization or the organisms might have subsequently gained entry into the products due to post-pasteurization contamination. In view of this, the presence of even a few such organisms in dairy products
may constitute a potential public health danger.

19. Three cultures of \( E. coli \) comprising both pathogenic and non-pathogenic types were selected for detailed studies of their heat-resistant characteristics. Among these, two cultures, namely 0127:B8 (pathogenic) and NP (non-pathogenic) were isolated from pasteurized dairy products in this laboratory. The third culture, namely 0111:B4 (pathogenic) was procured from International \( E. coli \) Reference Laboratory, Copenhagen, Denmark.

20. The above three cultures were subjected to heat-treatment at various time-temperature combinations. In addition, the effect of several factors such as type of substrate, growth temperature, pH etc. on the heat-resistant characteristics of the organisms were also studied.

21. The temperatures of heat-treatment included in this study were, 50°C(122°F), 55°C(131°F), 60°C(140°F) and 63°C (145°F). The time-interval during heat-treatment was 10 minutes at 50 and 55°C and 5 minutes at 60 and 63°C.

22. The heat-resistance of \( E. coli \) cultures was expressed in terms of thermal death rate (D values) and thermal death time (z values). D values were calculated by the graphic (experimental) as well as by the regression equation method based on statistical procedures. When the survivor curves were showing linearity, a straight line regression equation was fitted. In case of survivor curves exhibiting deviations from linearity, second degree curves were fitted. For purposes of comparison standard pasteurization curves with reference to milk and milk products were also fitted.

23. Marked differences in the heat-resistant characteristics were observed in the three \( E. coli \) cultures when subjected to
heat-treatment in cow skim, cow whole and buffalo milk. Culture 0111:B4 was found to be the most heat-sensitive as compared to the other two cultures, 0127:B8 and NP.

In cow milk (skim and whole), culture 0111:B4 survived for 30 minutes at 55°C and for 5 minutes at 60°C, while in buffalo milk, the same organisms showed survival times of 40 minutes at 55°C and 10 minutes at 60°C. Thus, an increase in thermal death time of 10 minutes at 55°C and 5 minutes at 60°C was noticed in case of buffalo milk. The other two cultures, 0127:B8 and NP exhibited a higher heat-resistant capacity in buffalo milk (63°C/20 minutes) as compared to that in cow milk (63°C/15 minutes). Among the three cultures examined, the non-pathogenic strain, NP exhibited the highest heat-resistance in both cow and buffalo milk.

The results on the thermal destruction of E.coli in milk have been considered to be of special interest to the dairy industry since the variation in the heat-resistant characteristics of E.coli strains in respect of cow and buffalo milk may significantly affect the subsequent keeping quality of these products. It has also been suggested in this connection that it is necessary to examine the adequacy of the present time-temperature combination of heat-treatment of cow milk, for the efficient pasteurization of buffalo milk in India.

A comparison of the D values obtained in respect of three E.coli cultures in milk indicated that the values were significantly much higher in cultures 0127:B8 and NP than for culture 0111:B4. However, it was observed that such differences in D values were more marked at lower than at higher heat-treatments. It was also noted that between cow and buffalo milk,
D values obtained in latter were found to be higher than in the former.

26. The thermal death time curves (z-values) obtained in case of culture Oll114 were, 8.3, 9.0 and 10.2°F in regard to cow skim milk, cow whole milk and buffalo whole milk respectively. On the other hand, z-values for other two cultures 01271B8 and NP were respectively 17.5, 18.0 and 19.2°F in the former case and 18.8, 19.0 and 20.3°F respectively in the latter case in all the three types of milk.

27. A critical examination of the thermal death time curves of the three E. coli cultures in relation to the standard pasteurization curve in buffalo whole milk showed that in cultures 01271B8 and NP, the curves were running parallel to each other and intersected the pasteurization curve at HTST level showing thereby the possibility of survival of these two organisms during pasteurization of buffalo milk.

28. From the results on the thermal destruction of E. coli cultures suspended in cream containing varying percentages of butterfat (20, 25 and 50%), no significant differences were observed in the number of survivors in case of two types of cream containing 20 and 25% butterfat. But the protection offered against heat-destruction by these two substrates was similar to that of buffalo milk. Maximum protection against heat-destruction was noticed in case of cream containing 50% butterfat. It was also noted that the degree of protection offered by cream was more evident at lower temperatures of heat-treatment (50 and 55°C).

29. The z-values obtained for three E. coli cultures in cream were higher than that noted in case of milk. In case of
culture 0111:B4, the z-values obtained were 10.5, 10.5 and 11.0 for cream containing 20, 25 and 50 percent butterfat respectively. The corresponding z-values for 0127:B8 and NP cultures in the three types of cream were, respectively, 20.0, 20.0 and 20.5°F for the former strain and 21.75, 22.0 and 22.5°F respectively for the latter organism. The protective effect of fat in relation to thermal destruction of microorganisms has been discussed.

30. Among the 10 different substrates examined in the present study, ice cream mix offered the highest protection against heat-destruction of the three E. coli strains. The rate of destruction of the cells at 50 and 55°C was found to be very much less in ice cream mix as compared to that observed in other milk and milk products. Even the most heat-sensitive culture 0111:B4 which did not withstand heat-treatment at 63°C even for 5 minutes was able to survive at 55°C for 60 minutes and at 60°C for 20 minutes in the same substrate.

31. An examination of the thermal resistance curves in ice cream mix showed that none of the three cultures may survive pasteurization normally given to the ice cream mix (155°F/30 minutes or 175°F/25 seconds). The z-values obtained in the case of E. coli 0111:B4 suspended in ice cream mix was found to be 13.5°F, while in case of the other two cultures the z-values in the same heating menstruum were 22.0°F and 23.5°F respectively. The increased heat-resistance of E. coli cultures observed in the current study has been attributed to its high solids content. It has been pointed out that one or more of the ingredients in ice cream mix comprising milk solids sugar, fat and stabilizer
may offer sufficient degree of protection to the bacterial cells during heat-treatment.

32. In order to appreciate the extent of protective effect offered by milk and milk products against heat-inactivation of E. coli, other substrates such as nutrient broth, distilled water and saline were also tried as heating menstrua in the present study. The rate of cell death in the three E. coli strains was much faster in nutrient broth as compared to that obtained in milk and milk products. The z-values for the three organisms 0111:B4, 0127:B8 and NP in nutrient broth were 7.25, 14.25 and 15.5°F, respectively.

33. The number of survivors resulting from the heat-treatment of E. coli cultures were significantly small in distilled water and saline as compared to those observed in nutrient broth and in dairy products. The z-values for the three E. coli cultures 0111:B4, 0127:B8 and NP found in distilled water and saline were respectively 4.5, 12.75 and 13.75°F in the former case and 4.0, 9.5 and 10.75°F respectively in the latter case. The possible mechanism of cell death in heat-treated saline suspension of E. coli has been discussed in relation to the similar reports by other workers.

34. Since growth temperature is known to influence the heat-resistant characteristics of microorganisms, four preincubation temperatures such as 22, 30, 37 and 44°C were included in the heat-resistance trials. The three E. coli cultures grown at 22°C and 44°C were found to be more susceptible to heat-destruction than those grown at 30 and 37°C. Maximum D and z-values were observed in case of cultures grown at 30°C followed by growth temperature of 37°C.
35. Lowest D and z-values were obtained in all the three cultures that were exposed to a preincubation temperature of 44°C. The z-values for the three \textit{E. coli} cultures 0111:B4, 0127:B8 and NP grown at 30°C were 9.0, 19.0 and 22.0°F respectively, while in case of the cultures grown at 44°C the z-values were 4.5, 14.0 and 15.75°F respectively. It has been suggested that the lowest survival value of \textit{E. coli} cells grown at preincubation temperature of 44°C may have to be explained in terms of the possible damage to the cell wall followed by leakage of cellular material.

36. The results on the effect of growth temperatures on the heat-resistant characteristics of \textit{E. coli} strains seem to indicate that the growth temperature may have a great influence on the ability of the organism to survive pasteurization. The significance of low temperature storage of milk in relation to the destruction of microorganisms during pasteurization has been discussed.

37. The effect of initial cell concentration at three levels, namely 100,000, 1,000,000 and 10,000,000 per ml on the survival of \textit{E. coli} cells exposed to heat-treatment was also studied. It was observed that irrespective of the types of the \textit{E. coli} strains used, the increase in the level of initial cell concentration significantly affected the heat-resistant characteristics of the organisms. Culture 0111:B4 was inactivated after 40 minutes at 55°C and after 10 minutes at 60°C when the initial cell concentration was increased from 100,000 to 1,000,000 per ml, prior to heat-treatment of the cells. A further increase of initial cell concentration to 10,000,000 per ml in the case of the above
organism resulted in an increase in the survival time to the extent of 60 minutes at 55°C and 20 minutes at 60°C. No appreciable change in the heat-resistant characteristics was observed in case of the other two cultures 0127:B8 and NP, when the initial cell concentration was increased from 100,000 to 1,000,000 per ml. However, a further increase of cell concentration to 10,000,000 per ml in the case of the above two organisms resulted in an extended survival time of 5 minutes at 63°C.

38. The z-values in case of culture 0111;B4 in respect of three levels of cell concentration were 8.3, 9.5 and 10.0°C respectively. In case of the other two relatively heat-resistant strains 0127:B8 and NP, the z-values for the three cell concentrations were respectively 17.5, 17.5 and 19.5°C in the former strain, while for the latter the z-values for the above three cell concentrations, were 18.8, 19.0 and 20.5°C respectively.

39. The significance of increased heat-resistance observed in dense populations of microorganisms has been discussed. It may be possible that leakage of materials from heat-injured cells may have the stimulating effect on the subsequent growth of such cells.

40. Three levels of pH namely 6.0, 7.0 and 8.0 were included in the thermal resistance study of E. coli strains suspended in cow skim milk. It was observed that maximum heat-inactivation occurred at pH 6.0 followed by pH 8.0, while minimum effect was noted at pH 7.0. The z-values obtained for the culture 0111;B4 grown at pH 6.0, 7.0 and 8.0 were 5.5, 8.25 and 5.25°C respectively. In regard to the other two cultures 0127:B8 and NP, the z-values at the above three pH levels were, 15.5, 17.5
and $15.75^\circ F$ respectively for the former strain and $16.5$, $18.25$
and $17.0^\circ F$ respectively in case of the latter strain. The above
results seem to indicate that maximum heat-resistance was
observed in the bacterial cells when exposed to a neutral pH
environment.

The reactivation of heat-treated *E. coli* cells was
studied by their subsequent growth and activity in the heating
menstruum after incubation for 10 days at $37^\circ C$. The behaviour
of cells exposed to $60$ and $63^\circ C$ has been studied in this connection.
The results of the present study using a solid medium (yeast
extract agar) and also cow skim milk for the reactivation of
heat-injured cells of *E. coli* have shown that greater reactivation
of cells occurred in cow skim milk than in the solid medium.
Similar results were also obtained in regard to the reactivation
of heat-injured cells in other types of milk and milk products.
The data pertaining to the effect of growth temperature, initial
cell concentrations and pH in respect of reactivation of heat-
treated cells followed the same trend.

It was a matter of particular interest to note that
the recovery of heat-inactivated cells of *E. coli* was possible
only when the thermal injury was evident unto a certain extent,
since in case of drastic heat-treatment the recovery mechanism
of the cell is, perhaps, irreparably lost irrespective of the
type of nutritionally rich medium used for reactivation.