STUDIES ON COLIFORM BACTERIA ISOLATED FROM MILK

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STUDIES ON COLIFORM BACTERIA ISOLATED FROM MILK

By

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OUTLINE OF CONTENTS

INTRODUCTION

THE COLIFORM GROUP OF BACTERIA

The coliform group is antigenicly heterogeneous and has widely diversified metabolic activities. For these reasons, classifications proposed for the group have been many and complicated. The 5th Edition of Bergey's Manual of Determinative Bacteriology (9), classifies the group in two genera under the family Enterobacteriaceae. The two genera, Escherichia (Type species: Escherichia coli) and Aerobacter (Type species: Aerobacter aerogenes) are differentiated from other members of the family by their ability to ferment lactose with gas production[1]. A third genus, Citrobacter, was proposed by Werkman and Gillen to include the intermediates classified by Bergey as Escherichia freundii (31). But this genus has not been recognized. The lines of demarcation between the genera are intergrading, and many bacteriologists today consider the group more naturally divided into species within a single genus rather than into two or three genera. British bacteriologists include the coliform bacteria in the genus Bacterium. (9)(31)(41)

All members of the coliform group are widely distributed in nature. However, Esch. coli normally is found in the intestinal tract of animals, while Aer. aerogenes is found

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[1]Recently [illegible] found in the [illegible]

on grains and plants, in the soil and to a varying degree in the intestinal tract. Because of their association with pathogenic bacteria in the intestinal tracts of man and animals, the bacteria in this group have assumed public health significance in the detection of fecal contamination or sewage pollution of water supplies. In dairy products, the presence of large numbers of coliform bacteria is regarded as indicating contamination from barns or dirty dairy equipment, or of storage at too high temperatures. (9)(28)(41)

The group has been referred to under a wide variety of names: "Escherichia-Aerobacter," [illegible] and [illegible] (11). In 1937, [illegible] (1[illegible]) introduced the term "[illegible]," [illegible] by the American Public Health Association in the *Standard Methods for the [illegible] and* [illegible] (3) and the *Standard Methods for the [illegible] of Dairy Products* ([illegible]).

During 19[illegible] and 19[illegible] [illegible] bottled milk and cream from [illegible] the [illegible] [illegible] eight months, from October until [illegible] [illegible] milks, [illegible] cream [illegible].

[illegible] in four [illegible] brilliant green bile (2%) [illegible] broth[a] and lauryl sulfate

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[a] Formulae [illegible] [illegible] 19[illegible] ([illegible]).

tryptose lactose broth[3] were used for presumptive tests on all samples. Lauryl sulfate tryptose dextrose broth[4] was used on the first two series of samples indexed, and formate ricinoleate broth[5] on the remaining eight series. Eosin-methylene blue agar[5] plates were streaked from the positive-presumptive tubes (those tubes showing gas in 48 hours at 37°C.) Wherever possible, typical Esch. coli or Aer. aerogenes colonies were fished from the plates after 24 or 48 hours incubation at 37°C. and planted into lactose broth for confirmed refermentation tests. Occasionally an atypical colony was fished from a plate into lactose broth.

Agar[6] slant cultures were made from those lactose tubes which showed gas production within 48 hours at 37°C. About 80 cultures were diluted and plated. New slant cultures were made from isolated single colonies on the dilution plates. Stock slant cultures were stored at room temperature and transfers were made from them as needed.

Five hundred cultures that fermented lactose with gas formation within 48 hours at 37°C. were isolated. It is with these cultures that the following three studies on methods of detection and identification of coliform organisms from water and milk were made during the years 1942-44.

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[3]The formula for tryptose lactose broth is given by Darby, C.W. and Hallmann, W.L., J.Am.Water Works Assoc., 31:689, 1939. The addition of lauryl sulfate to the tryptose lactose broth is discussed by these authors in Am.J.Pub.Health, 31:127, 1941.

[4]Dextrose was substituted for lactose in the lauryl sulfate tryptose broth.

[5]Formulae of these media are given in Standard Methods for the Examination of Water and Sewage, 8th Ed., 1936 (1).

[6]See appendix for formula and preparation of nutrient agar.

STUDY I

EFFECTS OF 30°C. and 37°C. INCUBATION ON THREE BIOCHEMICAL DIFFERENTIAL TESTS

Introduction

The coliform group of bacteria is defined as including "all aerobic and facultative anaerobic, gram-negative, non-spore-forming bacilli which ferment lactose with gas formation" by both the Standard Methods for the Examination of Water and Sewage, 8th Edition, 1936 (1) and the Standard Methods for the Examination of Dairy Products, 8th Edition, 1941 (3). However, as both these Standard Methods limit their presumptive analysis tests to one temperature of incubation, 37°C., only those bacilli which ferment lactose with gas formation at 37°C. are detected in the routine examination of water supplies and dairy products for coliform bacteria.

In recent years, data have been reported indicating that not all coliform bacteria produce gas from lactose at 37°C., and therefore are not detected in examination of water and dairy products conducted at 37°C. only. Hiscox (20) isolated from milk a member of the coliform group that fermented lactose with vigorous production of gas at temperatures up to and including 30°C., but which produced no gas at all or, at most, a scarcely perceptible amount at 37°C. He also cited a similar organism isolated from butter by Grimes and Hennerty. Levine, Carpenter and Coblentz (26) described 68 of 196 coliform strains isolated

from chlorinated waters, as producing gas slowly, if at all, at 37°C. but very luxuriantly, as a rule, at 30°C. Their detection, they stated, would be facilitated by lowering the temperature of incubation for presumptive tests to 30°C.

These reports indicating that some coliform bacteria produce gas from lactose more frequently and abundantly at temperatures below 37°C. raise the question whether more coliform bacteria might not produce positive reactions to biochemical differential and identification tests at lower incubation temperatures.

Reports on the effect of incubation temperature on biochemical differential tests made on "aberrant coliforms"[1] differ. Hiscox (20) found his bacillus to give the same citrate, indole and Voges-Proskauer reactions at either 37°C. or 20°C. Stuart, Mickle and Borman (38), on the other hand, state that their biochemical reactions with "aberrant coliforms"[1] varied with the temperature of incubation.

Observations of 221 normal coliform strains led Levine (25) to conclude that a 30°C. incubation temperature would yield more positive reactions than 37°C. in Voges-Proskauer tests of such strains.

The following study was made on a large number of

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[1] "Aberrant coliforms" as used by Stuart, Mickle and Borman (38) includes those coliform bacteria which produce less than 20 per cent gas in lactose in 48 hours at 37°C.

typical 37°C.-lactose-fermenting coliform cultures to determine the effect of 30°C. and 37°C. incubation temperatures on three biochemical tests, citrate utilization, indole production and the Voges-Proskauer reaction.

The Citrate Utilization Test

The citrate utilization test was proposed by Koser (21)(22), who found, in 1923, that Aer. aerogenes could utilize the citrate radical as a source of carbon and produce visible growth in a chemically defined medium in which citrate supplied the only source of carbon. Esch. coli, although multiplying in such a medium, shows no visible growth until after seven to fourteen days (33).

The Test for Indole Production

The production of indole from tryptophane is characteristic of Esch. coli (36). Ehrlich's original test for indole production was modified by Kovács (1928), and further simplified by Ruchhoft, Kallas, Chinn and Coulter (33). Ruchhoft et al. found that this test for differentiating Esch. coli was an extremely sensitive one, and that the indole once formed in a culture medium was very stable.

The Voges-Proskauer Reaction[2]

Aer. aerogenes ferments glucose with the formation of acetylmethylcarbinol. In the presence of atmospheric oxygen caustic alkali oxidizes the acetylmethylcarbinol to diacetyl,

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[2]Named for Voges and Proskauer who first observed the reaction in 1898.

which condenses with certain constituents of peptone to give the red coloration characteristic of the Voges-Proskauer reaction (24)(36). Unfortunately, the red coloration develops very slowly, and it was customary to wait 12 to 24 hours, or longer, after addition of the hydroxide, before recording results (24)(33). However, in 1936, Barritt (6) suggested for a more delicate test, the addition of α-naphthol and a change in the concentration of the potassium hydroxide. This modification permits reading of the test in two to four hours after the addition of the alkali.

Procedure

Three hundred and thirty coliform cultures isolated from milk and cream in the coliform index survey were used in this study. Each of the cultures fermented lactose with gas formation within 48 hours at 37°C.

The temperature of the 37°C. incubator was held during this study at 37.0°C. or 37.5°C. ±0.5°, the 30°C. incubator at 30°C. ±1.° Air temperature at the top of each incubator was recorded as the incubator's temperature. Neither incubator had a fan.

Koser's citrate medium[3] was used for the citrate utilization test, tryptone broth[3] for the indole production test, and dextrose dipotassium phosphate medium[3] for the Voges-Proskauer reaction.

All three test media were seeded at room temperature from agar[3] slant transfers of the stock cultures. After

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[3]See appendix for formulae and preparation of media.

seeding, the tubes were shaken and placed in the 30°C. or the 37°C. incubator.

Tubes of Koser's citrate medium showing visible growth (turbidity) in 24, 48 or 96 hours incubation were recorded as positive, but only those tubes which did not show visible growth in 96 hours were recorded as negative.

Two or three drops of Kovács amyl alcohol indole reagent[4] were added to each tryptone broth tube after 48 hours incubation at either temperature. A cherry-red ring forming after a few minutes at the surface of the medium indicated the presence of indole and a positive test.

Forth-eight hours incubation at both 30°C. and 37°C. was also used for the Voges-Proskauer reaction. After incubation, 0.6 ml. of α-naphthol reagent[4] and 0.2 ml. of 40 per cent potassium hydroxide[4] were added per ml. of medium to each culture tube. The development of a crimson to ruby-red color within two to four hours indicates in this test the presence of acetylmethylcarbinol. Vigorous shaking speeded the formation of the red color, and tubes could be read as positive (red color), negative (no color) or partial (inbetween shades of orange) within one-half hour.

Results of all tests were recorded as follows: + (positive), − (negative) or ± (partial).

Results and Discussion

Of the 330 cultures tested, 238 grew visibly in Koser's citrate medium at both 30°C. and 37°C. Seventy-four cul-

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[4] See appendix for the composition of reagents.

tures showed no visible growth in four days at either temperature. Thus, 312 or 94 per cent of the cultures gave the same citrate utilization test at either temperature. The remaining 18 cultures grew better in the citrate medium at the lower temperature, all 18 showing decided growth at 30°C. and only one a slight visible growth at 37°C. See Table 1.

A much smaller percentage of the cultures produced indole. Of the 90 cultures which produced indole at either temperature, 89 did so at both temperatures and one at 30°C. only. No partial reactions were recorded for this test, and the number of reactions agreeing after incubation at both temperatures was 329, or practically a 100 per cent agreement. Results are tabulated in Table 1.

For the Voges-Proskauer test, 191 cultures produced enough acetylmethylcarbinol to give positive reactions when incubated at either temperature. One hundred and thirty cultures were negative reactors after 48 hours incubation at both 30°C. and 37°C. From Table 1 it can be noted that six positive reactors were positive only at 30°C. and one only at 37°C. However, 321 or 97 per cent of the cultures tested gave the same reactions after both 30°C. and 37°C. incubations.

In the first 110 cultures studied, 37°C. incubation tests were made soon after the isolation of the cultures; 30°C. tests were made four to six months later. On the remaining 220 cultures, the 30°C. and 37°C. tests were made at the same time and were seeded from the same agar slant

transfer of the stock cultures. Tests were repeated on cultures not giving the same results after 30°C. and 37°C. incubation. The final results on all repeated tests were used in tabulating the results of the citrate, indole and Voges-Proskauer reactions (Table 1) and in typing the cultures (Table 3). Results of the repeated tests made on the 220 cultures, whose original 30°C. and 37°C. tests were made simultaneously, are tabulated in Table 2.

Stuart et al., as quoted by the 9th Edition (unpublished) of the Standard Methods for the Examination of Water and Sewage (2) would classify coliform bacteria into three groups[6], Aer. aerogenes, Esch. coli, and Intermediate, on the basis of the three biochemical tests: citrate utilization, indole production and the Voges-Proskauer reaction. Stated in the order just mentioned, those cultures giving +++ , +-+ and --+[7] test reactions are classified as Aer. aerogenes by Stuart. Those reacting as -+- are classified as Esch. coli by him, as are those cultures giving negative reactions in all three tests but which give positive Eijkman tests[8]. Most of the --- type cultures in this study gave negative Eijkman tests in Study II. Many of them after 30°C. incubation gave the reactions of typical Intermediates. Accordingly, the --- type culture has been considered here

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[6] Stuart et al.(39) in the original publication point out that this grouping is one of convenience and carries no taxonomic recommendations.

[7] + stands for a positive test, - a negative test.

[8] See Study II for a discussion of the Eijkman Test.

as belonging to the Intermediate group. Stuart considers the Intermediate group to be composed of two types, +-- and ++- . In addition to those two types, the --- type already discussed and the type -++ not mentioned in the Standard Methods, 9th Edition, have been included in the Intermediate group in this study. With the two exceptions stated, the 330 cultures were classified by Stuart's classification as given in the 9th Edition (unpublished) of the Standard Methods for the Examination of Water and Sewage (2)[9]

According to their reactions after incubation at the usual 37°C. temperature, the cultures were grouped into the eight possible types arising from the three tests. The types were then arranged into the three groups of Stuart's classification. One hundred and ninety-three were classified as Aer. aerogenes, 58 as Esch. coli and 79 as Intermediate by the 37°C. incubation reactions.

After 30°C. incubation, 21 cultures gave different reactions to one or more tests, and the classification of seven was affected. Three Esch. coli were transferred at this temperature to the Intermediate group. One Intermediate was classified as Esch. coli, and three Intermediates as Aer. aerogenes. Classification based on 30°C. incubation typing would have been changed to the following: Aer. aerogenes, 196; Esch. coli, 54, and Intermediate, 78. See Table 3 for typing and classification of the cultures used in Study I.

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[9] This classification was also followed in classifying the cultures used in Study II and in Study III.

Summary

Three hundred and thirty typical 37°C.-lactose-fermenting coliform cultures were studied; 312 gave the same citrate utilization test, 329 the same indole test, and 321 the same Voges-Proskauer reaction, whether incubated at 30°C. or 37°C.

Twenty-one cultures differed in one or more biochemical test reactions following 30°C. and 37°C. incubations. Seven of these would have had their classification affected by the differences.

A tendency was exhibited in all three biochemical tests for more cultures to produce positive reactions when incubated at 30°C. than when incubated at 37°C.

Table 1

Biochemical Tests

Citrate Utilization

Cultures Tested	30° 37° + +*	30° 37° ± ±	30° 37° + −	30° 37° + ±	30° 37° − +	30° 37° ± +	30° 37° − −	30° 37° ± −	30° 37° − ±	Total
Number	238		17	1			74			330
Per cent	72.1		5.2	0.3			22.4			100

Indole Production

Cultures Tested	30° 37° + +*	30° 37° ± ±	30° 37° + −	30° 37° + ±	30° 37° − +	30° 37° ± +	30° 37° − −	30° 37° ± −	30° 37° − ±	Total
Number	89		1.				240			330
Per cent	27.0		0.3				72.7			100

Voges-Proskauer Reaction

Cultures Tested	30° 37° + +*	30° 37° ± ±	30° 37° + −	30° 37° + ±	30° 37° − +	30° 37° ± +	30° 37° − −	30° 37° ± −	30° 37° − ±	Total
Number	191		3.	3	1		130	2		330
Per cent	57.9		0.9	0.9	0.3		39.4	0.6		100

* + (Positive Test); − (Negative Test); ± (Partial Test).

Table 2

Repeated Tests

Test	Citrate Utilization							Indole Production					Voges-Proskauer Reaction				Total
Type	30° 37° + −				30° 37° − +	30° 37° + ±	30° 37° ± −	30° 37° + −			30° 37° − +		30° 37° + −			30° 37° − +	
Type Upon Repeating	+ −	− −	+ +	+±	+ +	+ +	− −	+ −	− −	+ +	− −	+ +	+ −	− −	+ +	− +	
Number Changing Type	5	4	4	1	1	3	6	1	5	1	1	4	1	7	2	1	
Total Repeats	24							12					11				47

Table 3

Classification of Coliform Bacteria

at 37°C. and at 30°C. **Study I**

Group	Aer. aerogenes			Esch. coli	Intermediate				Total
Type *	+ + +	+ - +	- - +	- + -	+ - -	+ + -	- + +	- - -	
Cultures at 37°C.	26	157	10	58	52	3	2	22	330
Changes at 30°C.			6	3			1	11	21
— — to — — — type		← 6		3 →		←		1	
		←						1	
			←					2	
					←			7	
				←			1		
Cultures at 30°C.	26	164	6	56	59	7	1	11	330

* Types refer to the biochemical test results and are arranged in the following order: Citrate utilization, indole production and Voges-Proskauer reaction.

STUDY II

THE EIJKMAN TEST

Introduction

In 1904, Eijkman reported that *Esch. coli* from warm-blooded animals fermented glucose-peptone broth with gas formation at 46°C. and that that temperature either inhibited or destroyed other bacteria commonly found in water (23).

In the succeeding three years, Eijkman's report was confirmed by four European workers: Christian, [illegible]mann, Thomas and Bulir ([illegible]).

Leiter ([illegible]), in 19[illegible], made one of the first careful investigations of the tenets of Eijkman's original thesis. He reported the Eijkman reaction to be a fairly constant characteristic of [illegible] strains of *Esch. coli* isolated from warm-blooded animal feces. Leiter further demonstrated the close correlation of the 46°C. Eijkman test with indole production and non-utilization of citrate.

[illegible]

[illegible]

coli. They reduced the glucose concentration from 1.4 per cent to 0.3 per cent and added a phosphate buffer. In the original medium, the pH after fermentation was 4.5, whereas in the modified medium it remained at a pH of 5.6. In their modified medium, Perry and Hajna found that all strains of Esch. coli tested produced gas after 24 hours at 46°C., and that they remained viable for 96 hours or longer.

As a result of Hajna's experiments in 1937 (17) on the fermentation of a large number of mono- and disaccharides by Esch. coli, Hajna and Perry (18) substituted lactose for the glucose in their first modification of Eijkman's medium. Later, they changed the protein from peptone to tryptose ([illegible]).

In 1934, Levine, Epstein and Vaughn ([illegible]) questioned 46°C. as the optimum temperature for the Eijkman test. Performing parallel incubations, with temperatures in the medium of [illegible]°C. to 44°C. and [illegible]°C. to [illegible]°C., they obtained good growth of Escherichia strains in both Eijkman medium and standard lactose broth at the lower temperature. At the higher temperature, the gas production of the Escherichia strains was inhibited. [illegible] were [illegible] inhibited at [illegible]°C. to [illegible]°C.

Further investigation of the incubation temperature was made the following year by Wilson et al. Using the modified Eijkman medium of Williams, Weaver and Scherago ([illegible]), and MacConkey's lactose bile salt broth, they substantiated the work of Levine et al. ([illegible]). The close correlation of the [illegible]°C. Eijkman test with indole production and non-utilization of citrate was demonstrated. These workers

stressed the importance of a carefully controlled, uniform temperature in the medium and the need for using a water bath rather than an air incubator. (7)

Minkevich, Alexandrov and Soboleva (30), using Bulir's mannitol medium, in 1936 recommended 43°C. to 43.5°C. incubation for the Eijkman test. They determined that 46°C. did not hinder gas production of Escherichia cultures when in massive inoculations but that it repressed their development when in small numbers.

In 1939, Hajna and Perry (19) experimented with lower incubation temperatures for the Eijkman test. In their medium they found that many Aerobacter and Intermediate strains produced gas at 44°C., but very few did so at 46°C. On the other hand, of 1,374 Esch. coli strains tested, only five did not produce gas from lactose at 46°C. These workers favor the use of an incubator with a temperature in the medium of 45.5°C. to 46°C.

Using a water bath and Perry and Hajna's medium, Stuart et al. (39) found that among a large number of coliform strains, Aer. aerogenes and Intermediates seldom produced gas at 45.5°C. and that Esch. coli seldom failed to do so.

To date the Eijkman test has had a checkered career in the hands of many investigators[1]. Some condemn it entirely;

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[1] In this introduction, only a few of the many investigations reported in the literature of the Eijkman test have been mentioned. For a detailed review of the literature on this test, see Leiter (23) for work previous to 1929 and Datty-Smith's comprehensive survey (7) of the work from 1929 to 1942.

others claim it to be the most valuable single test for the detection of typical Esch. coli. Apparently the results obtained are influenced to a great extent by the technique with which the test is performed (7)(35). One most important factor in technique is temperature of incubation, and the optimum temperature seems to vary with the medium employed. Moreover, it is influenced by the length of the lag period between the medium's planting and its rise to incubation temperature (30)(35).

The 9th Edition (unpublished) of the Standard Methods for the Examination of Water and Sewage (2) recommends an incubation temperature of 45.5°C. and Perry and Hajna's modified tryptose-lactose medium for the performance of the Eijkman test.

It was the purpose of this study to determine how the Eijkman test performed as recommended by the Standard Methods, 9th Edition, would correlate with the type and classification of a large number of coliform strains. Type and classification were based on citrate utilization, indole production and the Voges-Proskauer reaction.

Procedure

The procedure followed was that recommended by the 9th Edition (unpublished) of the Standard Methods for the Examination of Water and Sewage (2).

The cultures used in the study were isolated from milk and cream in the coliform index survey and had been growing on artificial media for two weeks to several months. All produced gas from lactose within 48 hours at 37 °C. Previous

to testing, fresh agar[2] slant transfers were made of the cultures. The transfers were incubated 24 hours at 37°C. before plantings were made from them into the Eijkman medium.

Perry and Hajna's tryptose-lactose modification of Eijkman's original medium was used.[2] It was tubed in 10 ml. quantities in Durham fermentation tubes.

The tubes of Eijkman medium were seeded at room temperature, shaken, and immediately placed in a 45°C. water bath. The temperature of the bath was held between 45.0° and 45.5°C. Throughout the test the water level was maintained above the level of the medium in the tubes.

At first, an attempt was made to incubate the cultures in a 45°C. air incubator, but with very irregular results. The reactions reported in this study are those of water-bath incubated cultures only.

Gas production, the criterion of a positive test, was determined by displacement of the medium in the inserts of the fermentation tubes. Any amount of displacement (from a bubble to 100 per cent) was considered as positive. Readings were made after 24, 48 and 96 hours. Those cultures not producing gas in 96 hours at this temperature were recorded as negative.

Results and Discussion

Over a period of several months, Eijkman tests were made on 320 coliform cultures. Sixty-one of the cultures fermented lactose with gas formation at 45°C. The remaining

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[2]See appendix for formulae and preparation of media.

259 either failed to grow in the modified Eijkman medium at this temperature or, if growing, did not produce gas. Only 19 per cent of these coliform cultures, isolated from raw and pasteurized milk and cream, produced positive Eijkman tests.

Typed according to their reactions to the citrate utilization, indole production and Voges-Proskauer tests, 65 cultures (20 per cent) were classified as Esch. coli; 186 (58 per cent) were classified as Aer. aerogenes and 69 (22 per cent) as Intermediate[3].

Only 51, or 78 per cent, of the 65 Esch. coli cultures produced positive tests. Fourteen cultures in this group were negative to the Eijkman test. On seven of these fourteen, the test was repeated, but with the same negative results.

Among the 186 Aer. aerogenes cultures, five produced gas at the Eijkman temperature. All five were citrate positive, indole negative, Voges-Proskauer positive type cultures and were isolated at one time from two milk samples of the same dairy. They therefore present the possibility of originating from a single source.

In the Intermediate group, it is interesting to note that three of the five positive Eijkman cultures were the only three indole producing cultures in the group. One culture negative to all three biochemical tests[4] and one

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[3] See Study I for a discussion of the three tests and the modified Stuart et al. method of classification.

[4] This culture would have been classified by Stuart et al. (2)(39) as an Esch. coli.

positive to the citrate test only, produced the other two positive Eijkman tests.

The Eijkman reactions of the 320 cultures are tabulated in Table 4.

With the 255 Aer. aerogenes and Intermediate cultures included in the study, the Eijkman test presented excellent correlation. However, among the Esch. coli only 78 per cent gave positive Eijkman tests.

Williams, Weaver and Scherago (42), working with the Eijkman test in 1933, experienced certain changes in the fermentation characteristics of pure strains of Esch. coli after several transfers on artificial media. To some extent, the property of gas production was lost. At that time, these workers suggested over-cultivation on artificial media as a possible explanation of the poor results obtained by many workers with the Eijkman test. Although Leiter (23) had only four years previously stated that the Eijkman reaction was a fairly constant characteristic of pure strains of Esch. coli, the remarks of Williams, Weaver and Scherago merit consideration in a study in which 14 of 65 Esch. coli cultures did not produce gas from lactose at 45°C.

Conclusions

The Eijkman test performed as recommended by the 9th Edition (unpublished) of the Standard Methods for the Examination of Water and Sewage (2) seldom produces positive reactions from cultures in the Aer. aerogenes or Intermediate groups. On the other hand, it does not produce positive reactions from all Esch. coli cultures.

Table 4

Eijkman Reactions of 320 Coliform Cultures

Classification	Aerobacter aerogenes			Esch. coli	Intermediate				Total	Per cent
Type	+ + +*	+ - +	- - +	- + -	+ - -	+ + -	- + +	- - -		
Cultures Tested	32	145	9	65	47	2	1	19	320	100
Cultures Eijkman Positive		5		51	1	2	1	1	61	19
Cultures Eijkman Negative	32	140	9	14	46			18	259	81

*Reactions to the citrate, indole and Voges-Proskauer tests, in that order.

STUDY III

PASTEURIZATION STUDIES

Introduction

Early work on the resistance of Esch. coli to heat indicated that this bacterium would be killed in much less than 30 minutes at 62.5°C., a time and temperature commonly employed in pasteurization of milk and cream. Van Geuns reported that it was killed in one minute at 62.5°C. (4), Kitasato and Weisser, at 60°C. in fifteen and ten minutes respectively (34), and Chantemesse and Widal, in five minutes at 60°C. to 61°C. or in fifteen minutes at 59°C. (34).

However, in 1906, DeJong and DeGraff described heat-resistant strains of Esch. coli which were not killed in 30 minutes by temperatures below 70°C. to 72°C. (4).

Shippen (34), in 1915, isolated coliform bacteria from raw and pasteurized milk samples. After determining the thermal death points on some of the cultures, he concluded that the thermal death points of these bacteria varied in different strains.

Ayers and Johnson (4) at about the same time heated Esch. coli cultures, massively inoculated into milk, for 30 minutes at increasing temperatures. Twelve cultures (7 per cent) survived 62.8°C., but only one survived 65.6°C. In 1924 (5), these workers reported further pasteurization studies in which they determined that the majority of Esch. coli were killed at temperatures lower than 57.2°C. when held for 30 minutes but that a few resistant cells were

destroyed in 30 minutes only by temperatures above 65.6°C.

Finkelstein (16), in 1919, stated that pasteurization by the holding process destroyed practically all coliform bacteria in milk. Yet he reported an average survival of 42 per ml.

As early as 1903, Ringeling had attempted to use the presence of coliform bacteria in pasteurized milk as an indication of improper pasteurization or subsequent contamination (4). With the report of Ayers and Johnson (4) in 1914-15, showing that a few coliform strains had the ability to survive pasteurization time and temperature, the group came into disrepute as an index of proper pasteurization and subsequent handling of dairy products. However, in 1927, Swenarton (40) suggested that a test for coliform bacteria in pasteurized milk could be used to good advantage in checking up on dairy plant performance.

Beavens (8), in 1930, examined 100 samples of pasteurized milk, taken from the vat after holding at approximately 62.8°C. for 30 minutes. In 32 per cent of the samples, coliform organisms had survived the pasteurization.

Yet, in 1932, McCrady and Langevin (29) found that the coliform group was practically absent from one ml. quantities in the pasteurizing vat after holding but that they often reappeared in the milk because of contamination in the cooling or bottling process.

Fabian and Coulter (15), performing laboratory pasteurization in 1930, found no coliform cultures surviving in skim milk for 30 minutes at 62.8°C.

Stark and Patterson (37), in 1936, inoculated 505 pure Escherichia and Aerobacter cultures into milk in densities of about 100,000,000 bacteria per ml. All 505 were destroyed in the milk by 62.8°C. for 30 minutes. These workers stated that their results with the 505 cultures tended to show that the presence of coliform bacteria in properly pasteurized milk is mainly due to recontamination.

That same year, Chilson, Yale and Eglinton (13), after working with 271 samples of raw and pasteurized milk, felt that the test for the coliform group should supplement the agar plate count for the detection of contamination of pasteurized milk. They claimed that properly pasteurized milk that had not been recontaminated should have no coliform organisms in one ml. quantities.

The 8th Edition of the Standard Methods for the Examination of Dairy Products (3) reflected, in 1941, the reports of these later investigators. Coliform organisms, it stated, were practically eliminated from milk and cream by proper commercial pasteurization. The presence of these organisms in one ml. samples of pasteurized milk was considered cause for suspecting improper pasteurization or subsequent contamination.

In the pasteurization investigations previous to and including 1930, isolations of coliform bacteria from the pasteurizing vat immediately after holding were reported, and strains of coliform bacteria with the ability to survive pasteurization time and temperature were described.

If coliform bacteria do survive pasteurization in quantities that could be detected in laboratory tests, the value of their presence would be limited as an index of proper pasteurization, and they could not be taken as evidence of contamination of the milk after pasteurization.

In the coliform index survey of the bottled milk and cream of fourteen dairies in the Lansing milkshed, a large number of coliform cultures had been isolated, in most instances from pasteurized samples. This study was undertaken to find out if such coliform cultures could survive pasteurization at 62.5°C. for 30 minutes and, if so, in what degree they might possess that ability.

Procedure

The cultures used in this study had all been isolated in the coliform index survey. Three hundred and ninety-one cultures were included in the study. One hundred and ninety-nine had been isolated from milk, and 192 from cream. Three hundred and seventy-four came from pasteurized samples, and only 17 from raw milk and cream. All the cultures fermented lactose with gas formation at 37°C. within 48 hours. Typed on the basis of their reactions to the citrate utilization, indole production and Voges-Proskauer tests, the cultures were classified as follows: Aer. aerogenes, 237 (61 per cent); Intermediate, 91 (23 per cent); Esch. coli, 63 (16 per cent).[1]

After isolation, the cultures were stored at room

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[1] See Study I for a discussion of the three tests and the method of classification followed.

temperature on nutrient agar[2] for periods ranging from two weeks to several months. Previous to laboratory pasteurization, each culture was carried through a series of three 24-hour nutrient broth[2] transfers at 37°C. From the last of these broth transfers, the cultures were planted into autoclaved skim milk for pasteurization.

Pasteurized skim milk was obtained from the college dairy the day it was separated. It was autoclaved that same day at 12 pounds pressure for 15 minutes. Occasionally the milk was autoclaved at 15 pounds for 10 minutes. In no case did the milk show extensive caramelization or the presence of coliform bacteria. The milk was tubed aseptically in five ml. quantities in large (20 mm. diameter) sterile cotton-plugged culture tubes and brought to temperature in a constant-temperature water bath.

A deep water bath in continual agitation was used in the pasteurization work. The thermostatically controlled temperature varied ±0.5°C. At all times the water level was above the level of the milk in the tubes.

One ml. or one-half ml. of a 24-hour broth culture was pipetted into the heated milk tube. The tube was immediately replaced in the 62.5°C. ±0.5° water bath and held at that temperature for 30 minutes plus or minus one minute.

At the end of the holding time, two ml. of the pasteurized milk were removed. One ml. was placed in a lactose broth[2] fermentation tube, and an agar[2] pour plate was made of the other. The lactose tubes were read after 24, 48 and

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[2] See Appendix for formulae and preparation of media.

96 hours incubation at 37°C. The agar plates were counted after 48 hours at 37°C. Survival was indicated by colonial growth on the agar plates and gas formation in the lactose tubes.

Eight cultures were pasteurized in decreasing densities at 62.5°C., three at 69°C. to 70°C., and four for increasing time intervals. In these experiments, a series of dilutions were made from the 24-hour broth culture. One ml. of each dilution, including the original broth culture, was planted in five ml. of milk for pasteurization.

Results

To eliminate the less resistant cultures from the group of 391, all cultures were planted in excessive quantities (1 ml. or 0.5 ml. of a 24-hour broth culture per 5 ml. milk) for the initial laboratory pasteurization. One hundred and seven cultures were eliminated by this process; 284 (73 per cent) survived the pasteurization under these abnormal conditions.

Correlating the survivors as to type, it was found that 76 per cent of the Aer. aerogenes had survived, 55 per cent of the Intermediates, and 86 per cent of the Esch. coli. These percentages would indicate that Esch coli is slightly more resistant to pasteurization than the other two groups. Eijkman tests had been made on 306 of the 391 cultures. It is interesting to note that among the survivors were 52 of the 55 positive Eijkman cultures in the group. (Table 5)

No correlation was found among the survivors with their original source. Cultures isolated from milk showed about

the same percentage surviving the initial pasteurization as did those isolated from cream. This was true whether they came from raw or pasteurized samples. Neither did the density of coliform organisms in the sample seem to affect the resistance of cultures isolated from it.

The cultures which were used for further pasteurization studies had all survived the initial laboratory pasteurization. Originally they were isolated from the milk and cream of seven different dairies. All but one came from pasteurized samples.

Because the densities of the bacteria in the initial laboratory pasteurization were greatly in excess of those occurring normally in raw milk, eight cultures were pasteurized in decreasing densities. The densities varied from 140,000,000 to 275 bacteria per ml. of milk before pasteurization. For each culture, the number of bacteria surviving pasteurization increased with the number present. When the logarithms of the numbers of bacteria per ml. surviving were plotted against the logarithms of the initial counts, they fell along straight line curves.

Bigelow (10) finding that thermal death time curves for spore-forming bacteria were logarithmic and parallel, suggested that for non-spore-forming bacteria they might also be logarithmic and approach parallelism. The pasteurization survival curves of the two cultures which are plotted on Graph 1 corroborate his suggestion.

In Table 6 are tabulated, for each of the eight cultures, the bacterial count per ml. of the greatest dilution showing survival and the count of the next higher dilution in which

all bacteria were killed. The range of the greatest dilutions showing survival was 2,800 to 15,500 bacteria per ml. The range in which all were killed was from 275 to 1,200 bacteria per ml. All plantings above 2,800 per ml. showed survival, and all below 1,200 per ml. were killed by the pasteurization.

By raising the temperature to 69°C. to 70°C., but otherwise using the same pasteurization procedure, it was found that three coliform cultures could survive 30 minutes at this higher temperature, but only if present in greater densities than were required for survival at 62.5°C. From Table 7 it can be seen that plantings from 45,000 to 550,000 bacteria per ml. showed survival at the 69°C. to 70°C. pasteurization, but that in one planting of 53,000 all were killed. At 62.5°C. plantings from 2,800 to 15,500 survived 30 minutes.

The resistance of four cultures to increased holding time was also ascertained. Cultures were held at first for 30, 45 and 60 minutes at 62.5°C. It was soon apparent that 15-minute increments were too small to noticeably affect the survival of the coliforms. Whereupon, two cultures were held for 3 hours at 62.5°C., withdrawing samples at half-hour intervals after the first hour. In both cultures, resistant coliforms were found that would survive 62.5°C. for three hours or longer. With one culture, a planting of 5,200,000 bacteria per ml. survived for three hours, one of 520,000 for two hours, and one of 52,000 for one hour. (Table 8)

To test the reliability of this pasteurization procedure, six raw milk producers' samples were pasteurized in six ml. quantities. At the same time, the coliform indices of the samples were determined. Coliform bacteria from the two samples with coliform indices of 100,000+ survived the pasteurization, as did one of the two samples with an index of 10,000. Neither of the two samples with indices of ten or less showed survival. (Table 9)

The results with the raw milk samples agree with the density range for survival arrived at with the cultures grown on artificial media, although indicating that the artificially grown cultures might be slightly more resistant than those in raw milk.

Summary

Pasteurization studies were made on 391 coliform cultures isolated from raw and pasteurized milk and cream. Even when present in quantities greatly in excess of those found normally in raw milk, 107 of the cultures failed to survive 62.5°C. for 30 minutes; 284 survived.

For the eight cultures studied, the number of bacteria surviving pasteurization increased with the number present. Coliform organisms survived in plantings ranging from 2,800 to 15,500 bacteria per ml. but not in plantings ranging from 275 to 1,200 per ml.

Three cultures survived 69°C. to 70°C. for 30 minutes, but only in densities greater than were required to survive the 62.5°C. pasteurization.

Two cultures contained organisms capable of surviving 62.5°C. for three hours.

Coliform bacteria in three raw milk producers' samples survived 30 minutes at 62.5°C.

Conclusions

Many coliform cultures isolated from milk and cream can survive pasteurization at 62.5°C. for 30 minutes if present in quantities greatly in excess of those occurring in milk and cream under ordinary conditions. Some cultures are capable of resisting increased pasteurization temperatures and times when present in such excessive amounts. A few coliform strains do exist which can survive pasteurization when in densities that are found in high-count raw milk samples.

Acknowledgment

I wish to thank Dr. W. L. Mallmann for his suggestions and guidance in this work.

Table 5

Pasteurization Survival when Present in Excessive Quantities

Correlation with Type and Classification

Group	Aer. aerogenes				Esch. coli	Intermediate					Totals	Per cent.
Type	+ + +*	+ - +	- - +	Total	- + -	+ - -	+ + -	- + +	- - -	Total		
Cultures Pasteurized	34	191	12	237	63	61	3	2	25	91	391	100
Cultures Viable	26	143	11	180	54	33	3	2	12	50	284	73
Cultures Killed	8	48	1	57	9	28			13	41	107	27

*Reactions to the citrate, indole and Voges-Proskauer tests in that order.

Correlation with Eijkman Test

Eijkman Test	+	-	Total
Cultures Pasteurized	55	251	306†
Cultures Viable	52	171	223
Cultures Killed	3	80	83

†Eijkman tests were not made on 85 of the cultures pasteurized.

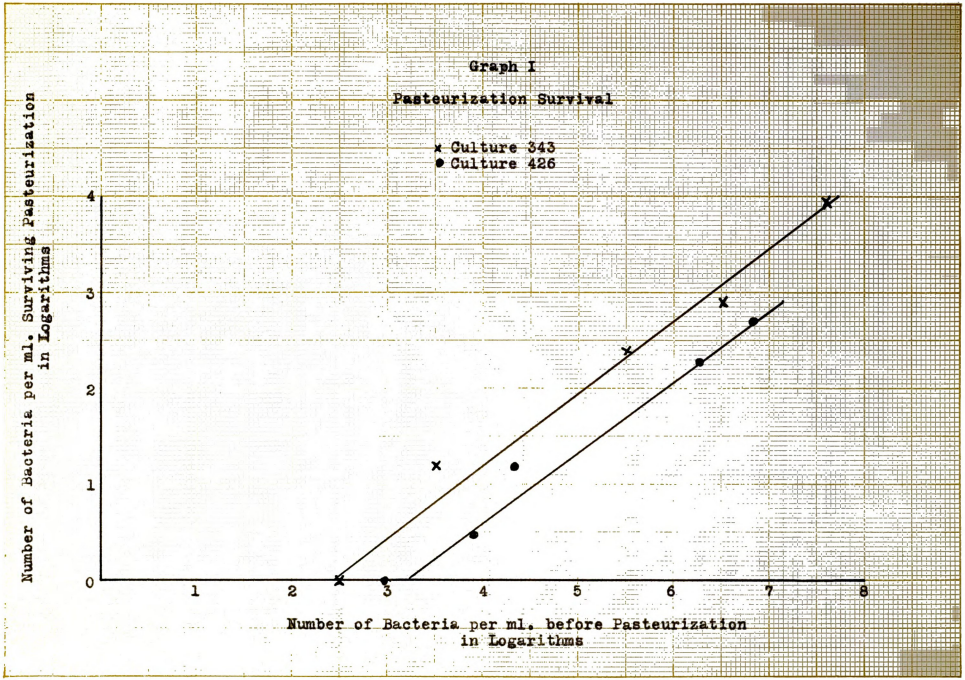
Graph I
Pasteurization Survival
x Culture 343
● Culture 426
Number of Bacteria per ml. Surviving Pasteurization in Logarithms
0
1
2
3
4
1
2
3
4
5
6
7
8
Number of Bacteria per ml. before Pasteurization in Logarithms

Table 6

Pasteurization Survival
62.5°C. - 30 Minutes

Culture†	Type*	Eijkman Test	Largest No. Bacteria per ml. Showing No Survival	Smallest No. Bacteria per ml. Showing Survival
343	+ + +	-	325	3,330
380	- + -	+	—	10,500
426	+ - +	-	1,000	8,200
435	+ + +	-	275	2,800
1002	- + -	+	650	10,000
1006	- + -	+	750	5,200
1013	+ - +	-	325	15,500
1014	+ - -	-	1,200	12,500
Range			275 - 1,200	2,800 - 15,500

Table 7

Survival of Coliform Bacteria in Milk
69° to 70°C. - 30 Minutes

Culture†	Type*	Eijkman Test	Largest No. Bacteria per ml. Showing No Survival	Smallest No. Bacteria per ml. Showing Survival
435	+ + +	-	53,000	550,000
647×	+ - +	-	40,000	400,000
723	- + -	-	4,500	45,000

*Reactions to citrate, indole and Voges-Proskauer tests, in that order.

†All cultures were dilution plated except 647 and 723.

×Culture 647 was isolated from raw milk.

Table 8

Survival of Coliform Bacteria in Milk at 62.5°C.

for Varying Lengths of Time.

Culture†	Type	Eijkman Test	No. Bacteria per ml.	Survival for 1	1½	2	2½	3 hrs.
435	+++*	–	170,000,000	+	+	+	+	+
			17,000,000	+	+	–	+	+
			1,700,000	–	+	–	–	+
			170,000	–	(+)	–	not run	
1013	+-+	–	52,000,000	+	+	+	+	+
			5,200,000	+	+	+	+	+
			520,000	+	+	+	not run	
			52,000	+	not run			

†* Explained under Table 7

(+) Survival detected in lactose broth, but not on agar plate.

Table 9

Pasteurization Survival of Coliform Bacteria in Raw Milk - Producers' Samples.

Samples	Coliform Bacteria per ml.	Coliform Bacteria Surviving Pasteurization in 1 ml.	5 ml.
1	10,000	-	-
2	10,000	(+)	+
3	10	-	-
4	100,000+	-	+
5	less than 10	-	-
6	100,000+	+	+

(+) Was not able to confirm the presence of coliform bacteria.

APPENDIX

MEDIA AND REAGENTS

Media and reagents were prepared according to the Standard Methods for the Examination of Water and Sewage, 8th Edition, 1936 (1), and 9th Edition (unpublished) (2) with the exception that sodium chloride (0.5%) was added to the nutrient agar, tryptone broth, nutrient broth and lactose broth.

Formulae of Media

Nutrient Agar

Agar	15 gm.
Sodium chloride	5 gm.
Beef extract (Bacto)	3 gm.
Peptone (Bacto)	5 gm.
Distilled water	1000 ml.

Koser's Citrate Medium

The dehydrated medium, Bacto citrate medium, was used for most citrate utilization tests. Occasionally the medium was prepared according to the following formula:

Sodium ammonium phosphate (microcosmic salt)	1.5 gm.
Potassium dihydrogen phosphate	1.0 gm.
Magnesium sulfate	0.2 gm.
Sodium citrate (crystal)	3.0 gm.
Distilled water	1000 ml.

Tryptone Broth

Tryptone (Bacto)	10 gm.
Sodium chloride	5 gm.
Distilled water	1000 ml.

Dextrose Dipotassium Phosphate Medium

Proteose-peptone (Bacto)	5 gm.
Dextrose, C.P.	5 gm.
Dipotassium phosphate	5 gm.
Distilled water	1000 ml.

Eijkman Test Medium

Tryptose (Bacto)	15	gm.
Dipotassium phosphate	4	gm.
Potassium dihydrogen phosphate	1.5	gm.
Sodium chloride	5	gm.
Lactose, C.P.	3	gm.
Distilled water	1000	ml.

Nutrient Broth

Sodium chloride	5	gm.
Beef extract (Bacto)	3	gm.
Peptone (Bacto)	5	gm.
Distilled water	1000	ml.

Lactose Broth

Sodium chloride	5	gm.
Beef extract (Bacto)	3	gm.
Peptone (Bacto)	5	gm.
Lactose, C. P.	5	gm.
Distilled water	1000	ml.

Reagents

Kovács Amyl Alcohol Indole Reagent

p-dimethyl-amino benzaldehyde	5	gm.
Amyl alcohol	75	ml.
Hydrochloric acid (conc.)	25	ml.

α-Naphthol Reagent

5% solution of α-naphthol in absolute ethyl alcohol. (95% ethyl alcohol was used rather than absolute).

40 Per cent Potassium Hydroxide

40% aqueous solution of potassium hydroxide.

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