Fisk. Dir. Skr., Ser. Ernæring. Vol. 1 : No. 3, 67-74.

CLASSIFICATION OF COLIFORM BACTERIA ISOLATED FROM MARINE ENVIRONMENT AND MARINE FISH PRODUCTS

By

MATHANA SANGJINDAVONG[1] and JAN GJERDE[2]
Institute of Vitamin Research
Directorate of Fisheries, Bergen

ABSTRACT

A classification of strains of coliform bacteria isolated from marine environments was performed as an aid in the hygienic control of products of marine origin. From a marine fish farm and from frozen marine fish fillets 170 strains of coliform bacteria and 70 strains of faecal coliform bacteria were isolated and classified into genera. In the coliform group *Klebsiella* was found most frequently (50%), followed by *Escherichia* (20%), *Enterobacter* (20)% and *Citrobacter* (10%). In the faecal coliform group 50 out of 70 strains (70%) were identified as *Escherichia coli*, wereas the genus *Klebsiella* accounted for the rest (30%). No other genera were isolated in this group. In 7% of the primary isolation for coliform bacteria false positive results were observed. No positive false results were found in the faecal coliform count.

INTRODUCTION

The sanitary quality of foods and water is normally evaluated by the determination of coliform bacteria. Contamination of coliform can be of enteric origin, but may also come from soil and water. Coliform bacteria are defined as facultative anaerobe, gram negative rods which ferment lactose with production of gas, and include genera belonging to the family *Enterobacteriaceae*. The accuracy of methods commonly used for testing the number of coliform in food and water is limited by false reactions which can occur (ANON., 1953; RAJ and LISTON, 1961). By testing presumptive coliform bacteria isolated from fish surfaces, bacteria belonging to the genus *Vibrio* were found to show similar reactions to the

[1] Present address: Kasetsart University, Bangkok 9, Thailand.

[2] Present address: Central Laboratory, Directorate of Fisheries, P.B. 185, N-5001 Bergen.

coliform bacteria in Violet red bile agar (ROSEN and LEVIN, 1972). False reaction in the primary isolation can occur also by inoculating seawater in MacConkey broth and glutamate medium for isolation of the coliform bacteria (ALIVISATOS and PAPADAKIS, 1975).

The socalled elevated temperature test for the faecal coliform bacteria was introduced to improve simple methods for the detection of organisms related to the hygienic condition (THATCHER, 1973). The faecal coliform bacteria include *Escherichia coli* and other strains of the coliform group which can ferment lactose at temperatures between 44°C and 45.5°C. Detection of faecal coliform is considered to represent recent contamination of bacteria of faecal origin. The object of this investigation was firstly to determine whether single strains of bacteria not belonging to the family *Enterobacteriaceae* could give positive reactions in primary isolations, and secondly to isolate strains of coliform bacteria and faecal coliform bacteria from marine environment and marine fish products and classify them in genera by biochemical and physiological tests.

In all, 38 samples of seawater, mussels and fish fillets were examined, and a total of 170 strains of coliform bacteria and 70 strains of faecal coliform bacteria were isolated and classified in genera.

MATERIALS AND METHODS

SAMPLING

Bacteria were isolated from samples of seawater and blue mussels (*Mytilus edulis*) collected from fish farms raising salmon and rainbow trout on the west coast of Norway, and further from raw frozen fish fillets. The water samples were collected at depths of two meters using sterile equipment, and the mussels were taken at the nets surrounding the ponds. The total elapsed time between sampling and inoculation was less than three hours for these samples. The samples of fish fillets were commercial products obtained at the local market and kept in a freezer until the bacteriological examination.

ENUMERATIONS AND ISOLATION OF COLIFORM BACTERIA

Three rows of three tubes, tenfold dilution in MacConkey broth (Oxoid CM 5a) were used for primary isolation. Physiological saline was used as diluent. After incubation at 37°C for 24 to 48 hours the positive tubes were plated on Eosin methylene blue agar (EMB) (Oxoid CM 69) and incubated at 37° C for 24 hours. The colonies appearing on EMB agar were restreaked on EMB plates to isolate pure cultures. Gram staining and

microscopical examination gave further control for purity. Gram negative rods were reinoculated in MacConkey broth and incubated at 37°C for 48 hours. Pure strains giving positive results in the tubes were tested for the oxidase reaction (KOVACS, 1956) and their ability to ferment glucose in Hugh and Leifsons medium (Merck no. 10282). Gram negative rods which fermented lactose and produced gas in MacConkey broth, fermented glucose in Hugh and Leifsons medium, and were oxidase negative were considered as coliform bacteria. All tubes which were found positive for acid and gas were controlled for the presence of coliform bacteria.

ENUMERATION AND ISOLATION OF FAECAL COLIFORM BACTERIA

Positive tubes from the primary isolation in MacConkey broth were transferred to Eijkmans Lactose broth (Difco 0017—01) and incubated in thermostated waterbaths at 44.5°C for 48 hours. The tubes giving positive reaction in this test were further plated on EMB agar and incubated at 37°C for 24 hours. The colonies appearing on EMB agar were controlled for purity and reinoculated in Eijkmans Lactose broth and incubated at at 44.5°C for 48 hours. Pure strains which gave positive results in the tubes were tested for the oxidase reaction and their ability to ferment glucose in Hugh and Leifsons medium. Gram negative rods which fermented lactose and produced gas in Eijkmans lactose broth at 44.5°C, fermented glucose in Hugh and Leifsons medium, and were oxidase negative were considered as faecal coliform bacteria. All positive tubes were tested for the existence of pure strains of faecal coliform bacteria.

IDENTIFICATION OF ISOLATES

The strains were identified by their morphological features and reactions in biochemical tests. Media for morphological and cultural examination were based on Nutrient agar (Oxoid CM3). Cultures of ages not more than 18 hours were examined for motility by phase contrast microscopy. The size, shape and cellular arrangement of the preparations were noted. Details of the biochemical tests employed are shown in Table 1. All tubes were inoculated with cultures of ages between 18 and 24 hours and the incubation temperature for all tests was 37°C.

Table 1. Description of biochemical tests employed

Test	Growth medium	Incubation period (days)
Indole production	Sim medium (Merck 5470)* Test reagent: Kovacs indolereagenz (Merck (9293)*	5
Methyl red and Voges Proskauer	M.R.V.P. medium (Oxoid CM 43)**	5
Citrat Utilization	Koser Citrate medium (Oxoid CM 65)**	5
Nitrate reduction	Nitrate broth (Merck 5462)* Test reagent: Griess-Ilosvays reagenz (Merck 9023)*	4
Casein hydrolysis	Calcium-caseinat Agar (Merck 5409)*	4
Urease production (Christensen, 1946)	Christensens Urea Agar	4
Lysine decarboxylase (Möller, 1955)	Lysine decarboxylase broth (Merck 5266)*	4
Ornithine decarboxylase (Möller, 1955)	Ornithine decarboxylase broth (Merck 6934)*	4
Tween 80 hydrolysis (Sierra, 1957)	Tween 80 agar	4
KCN growth on (Möller, 1954)	KCN broth (Merck 5412)* with 0.5% aqueous solution of potassium cyanide	1
Hydrogen Sulfide production	Sim medium (Merck 5470)*	4
Arabinose (acid)	Nutrient broth (Oxoid CM67)** with 1% wt/vol. concentration of the compounds	2
Rhamnose (»)	» » »	2
Raffinose (»)	» » »	2
Sorbitol (»)	» » »	2
Glucose (acid+gas)	» » »	2
Mannose (» »)	» » »	2
Inositol (» »)	» » »	2
Glycerol (» »)	» » »	2
Starch hydrolysis (» »)	» » »	7

* Merck katalog
** Oxoid katalog

Table 2. Test responses of coliform bacteria.

Number of strains tested	Klebsi-ella 88	Entero-bacter 36	Esche-richia 34	Citro-bacter 12
Motility	0	28	30	12
Indole production	14	0	34	0
Methyl red	20	8	34	12
Voges Proskauer	49	8	0	0
Citrate utilization	49	20	0	12
Nitrate reduction	88	36	34	12
Casein hydrolysis	0	11	0	0
Urease production (Christensen, 1946)	48	8	0	0
Lysine decarboxylase (Möller, 1955)	70	14	32	0
Ornithine decarboxylase (Möller, 1955)	0	36	30	0
Tween 80 hydrolysis (Sierra, 1957)	0	0	0	0
KCN growth on (Möller, 1954)	78	36	0	12
Hydrogen Sulfide production	0	0	0	12
Arbinose (acid)	78	36	34	12
Rhamnose "	78	22	34	12
Raffinose "	76	35	27	3
Sorbitol "	75	23	34	12
Glucose (acid+gas)	88	36	34	12
Mannose "	88	36	34	12
Inositol "	73	12	0	0
Glycerol "	77	20	34	12
Starch hydrolysis "	64	11	33	0

RESULTS AND DISCUSSION

A total of 1026 tubes were inoculated for the coliform count and 150 tubes were found positive for acid and gas. In 11 of these tubes (7%) no coliform bacteria were isolated. From these tubes faint growth on EMB agar were observed. When the pure cultures from EMB agar were reinoculated in MacConkey broth none produced acid and gas after incubation for 48 hours at 37°C.

CLASSIFICATION OF THE ISOLATED STRAINS

The classification of the *Enterobacteriaceae* is normally accomplished by investigation of biochemical reactions. Some of the genera in the family are, however, near related and similar reactions within the genera are found.

The coliform bacteria in this investigation were classified according to EDWARDS and EWING (1972). The Edwards and Ewing's classification system arose from comparative studies of biochemical reactions given by relatively large numbers of cultures from each of the genera in the family

Table 3. Test responses of faecal coliform bacterica*.

Number of strains tested	Escherichia 50	Klebsiella 20
Motility	38	−
Indole production	+	−
Methyl red	+	−
Voges Proskauer	−	15
Citrate Utilization	−	19
Nitrate reduction	+	+
Casein hydrolysis	−	−
Urease production (Christensen, 1946)	−	+
Lysine decarboxylase (Möller, 1955)	46	+
Ornithine decarboxylase (Möller, 1955)	45	−
Tween 80 hydrolysis (Sierra, 1957)	−	−
KCN growth on (Möller, 1954)	−	+
Hydrogen Sulfide production	−	−
Arabinose (acid)	+	+
Rhamnose "	+	+
Raffinose "	40	+
Sorbitol "	+	18
Glucose (acid+gas)	+	+
Mannose " "	+	+
Inositol " "	−	18
Glycerol " "	+	+
Starch hydrolysis " "	48	+

*Symbols: +, all strains in a group positive, −, all strains in a group negative Figures, number of strains in a group positive.

Enterobacteriaceae. In our study the classification of the strains were done on the basis of overall similarity with the genera listed in Edwards and Ewing's manual.

In Table 2 are listed the number of isolated coliform bacteria in each genus giving positive response to the tests employed. Thirtyfour strains were found to be nearly identical with the genus *Escherichia*, and 12 strains were found to be identical with the genus *Citrobacter*. The remaining 124 strains were found to have overall similarity with genera *Klebsiella/Enterobacter* though only 70 of them fitted the classical IMVC reactions (− − + +). Since the major differences between these genera are motility and presence or absence of ornithine decarboxylase, none motile strains with positive ornithine decarboxylase test were classified as *Klebsiella*, and motile negative ornithine decarboxylase strains were classified as *Enterobacter*. Of the faecal coliform group 50 strains out of 70 were identified as *Escherichia*, and 20 were identified as *Klebsiella* (Table 3). No other genera were detected.

Classification of coliform bacteria from the environment by morphological features and biochemical tests does not give fully satisfying results. Some near related genera complicate the identification of unknown strains. The strains isolated in this study gave, however, nearly similar reactions to the genera listed in Edwards and Ewing's manual in most cases. The majority of the coliform bacteria isolated belonged to the *Klebsiella* (50%). The genera *Escherichia*, *Enterobacter* and *Citrobacter* represented 20%, 20% and 10% respectively. Among the faecal coliform group species of *Escherichia* were isolated most frequently (70%) while *Klebsiella* represented 30% of the strains.

Bacteria of the coliform group are not normally detected from unpolluted marine environment. The coliform bacteria isolated in this investigation may originate from the feed prepared from trash fish, and from contamination of fish fillets during processing.

Positive false reactions were observed in 7% of the tubes where no pure strain of coliform bacteria were isolated. As no bacteria outside the family *Enterobacteriaceae* were found which gave similar reaction as the coliform bacteria in MacConkey broth, the positive reactions in the primary isolation are probably caused by mixed bacterial populations (Schiff et al., 1970). However, false reactions were found in a relatively low percentage.

Using biochemical reactions and morphological features, isolated strains of the coliform group were found belonging to genera within the family *Enterobacteriaceae* which ordinarily are isolated from food products. Most of the strains in the faecal coliform group were identified as *Escherichia coli* as expected, but also the genus *Klebsiella* was represented in this group.

ACKNOWLEDGEMENT

One of the authors (M.S.) wishes to thank the Norwegian Agency for International Development (NORAD) and professor O.R. Brækkan of the Vitamin Research Institute for making this study possible.

REFERENCES

Anon, 1953. The effect of anaerobic spore-bearing organisms on the validity of the presumptive coliform test as used in the bacteriological examination of water. J.Hyg., *51*, 2–68.

Alivisatos, G.P. and Papadakis, J.A., 1975. MacConkey and Glutamate Media in the Bacteriological Examination of Seawater. J. Appl. Bact. *39*, 287–293.

Christensen, W.E., 1946. Urea decomposition as a mean of differentiating *Proteus* and paracolon bacteria from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. *52*, 461–466.

EDWARDS, P.R. and EWING, W.H., 1972. Identification of *Enterobacteriaceae*. Burgess Publishing Company, Minneapolis, Minnesota, 362 pp.

KOVACS, N., 1956. Identification of *Pseudomonas pyocyanea* by oxidase reaction. Nature *178*, 703.

MÖLLER, VAGN, 1955. Simplified tests for some amino acid decarboxylases and for the Arginine Dihydrolase system. Statens Serumsinstitutt, Copenhagen, Denmark. 158–172.

— 1954. Diagnostic use of the braun KCN test within the *Enterobacteriaceae*. Statens Serumsinstitutt, Copenhagen.

RAJ, H. and LISTON, J. 1961. Detection and Enumeration of Faecal Indicator Organisms in Frozen Sea Foods. I. *Escherichia coli*. Appl. Microbiol. *9*, 171–174.

ROSEN, A. and LEVIN, R.E., 1970. Detection and Incidence of *Escherichia coli* om Storage Pen Surfaces of Fishing Trawlers. Appl. Microbiol. *20*, 103–106.

SCHIFF, L.J., MORRISSON, S.M. and MAYEUX, J.V., 1970. Synergistic False-Positive Coliform Reaction on M-Endo MF Medium. Appl. Microbiol. *2*, 778–781.

SIERRA, G., 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations of the influence of contact between cells and fatty acid substances. Antonie van Leeuwenhock. J. Microbiol. Serol *23*, 15–22.

THATCHER, F.S. and CLARK, D.S., 1973. Microorganisms in foods; their Significance and Methods of Enumeration. University of Toronto Press., Canada, 25–28.