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STUDIES OF THE FECAL STREPTOCOCCI

PART I--THE ISOLATION OF ENTEROCOCCI FROM NATURAL SOURCES

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For a number of years the fishing industry has been dissatisfied with the bacteriological methods for detecting domestic pollution. The present methods frequently indicate that an area is unfit for shellfish production or that a fishery product is not suitable for human consumption even though there is no other evidence to indicate that this may be so. The College Park laboratory of the Division of Commercial Fisheries has been engaged in a search for a more reliable criterion of pollution than the coliform bacteria. This report is the first of a series dealing with research on indices of fecal pollution.

The fecal streptococci may prove to be the solution to the problem of a more certain index of fecal pollution. This group of organisms, commonly known as the enterococci, was first reported by Lawes and Andrews (1) in 1894. Six years later, Houston (2) began to lay stress upon the fact that those organisms were readily found in polluted waters and seemingly absent in non-polluted samples. The work of these and other British workers led Suckling (3) in 1943 to state:

> "Streptococci are used as an indicator of pollution on the same grounds as Bacterium coli, namely:
>
> 1. They are present in faeces and sewage and are found in known polluted waters.
>
> 2. They are not found in pure waters, virgin soil and sites out of contact with human and animal life.
>
> 3. They do not multiply outside the animal body (except in such media as milk.)"

In the United States, Winslow and Hunnewell (4) made the first report on these organisms in 1902. Since then several methods of isolation have

been suggested and in 1939, Darby and Mallmann (5) suggested the use of a selective medium which would permit these organisms to grow without competition from cettain other bacteria.

Four years later, Hajna and Perry (6) proposed their "SF medium" which they reported to be highly selective for the enterococci if the incubation temperature was 45° C.

In 1945, White and Sherman (7) indicated that a medium containing pencilli was highly selective for this group of organisms. The possibilities of this medium for routine use in sanitation bacteriology led us to investigate it in detail. This report is the first of a series of studies we have made on the enterococci as indices of fecal pollution.

MATERIALS AND METHODS

White and Sherman employed direct plating methods in using the sodium azide-penicillin medium for the isolation of enterococci from milk. This procedure was tested and proved to be very selective for isolating this group of organisms from sewage and polluted waters. One difficulty was that the streptococci grow as extremely small pin-point colonies and frequently it was difficult to differentiate between the colonies and small specks of debris in the test sample. As a result, various dyes and indicators were incorporated into the medium, among them, methylene blue to which the enterococci are very tolerant.

It was found that on the addition of 0.001% methylene blue the debris is stained a deep blue color whereas the colonies are white with a blue spot in th center. This permitted a distinction of the colonies from other material. It was also found that the amount of penicillin could be doubled without inhibiting the enterococci and at the same time would increase the barrier against the staphylococci and non-fecal streptococci.

The modified sodium azide-penicillin medium of Sherman and White, as used in these experiments, had the following composition:

0.5% yeast extract
0.5% tryptone
0.5% glucose
0.03% sodium azide
0.001% methylene blue
1.5% agar
650 Oxford Units penicillin per lite

The ingredients, with the exception of the penicillin, are put into solution and the pH adjusted to 7.4-7.6. After being autoclaved at 15 pounds pressure for 15 minutes, the medium is cooled to 45° C., and the penicillin added just before the plates are poured. Incubation should be at 37° for 48-72 hours.

Plates should be poured from a dilution that will result in not less than 10 nor more than 100 colonies per plate. When more than 100 colonies appear on a plate, the colonies are extremely small and atypical. The colonies formed are of four types, fusiform deep, triangular fusiform deep, circular surface, and a filmy diffuse colony lying between the agar and the glass of the dish. All of these show a blue center and white edge.

The verification of these colonies is based on the characteristics describe by Sherman (8). Since the colonies formed on the plates are extremely small

and usually deep in the agar, it is suggested that the colony be removed with one stroke of a needle and transferred to a slant-broth preparation of 0.5% glucose, 0.5% tryptone and 0.5% yeast extract. The slants are prepared as usual by the addition of 1.5% agar to part of the broth. After gelation of the slant, enough sterile broth is aseptically added to cover one-half of the gel surface when the tube is vertical. At the time of inoculation the needle is first dipped into the broth and then streaked on the dry slant. After 24 hours incubation at 37° C., a sediment should appear in the broth and pin-point colonies on the slant. A Gram stain is made from the broth culture. If a typical sediment is present in the broth, if colonies are formed on the agar slant, and if large, Gram-positive, ovoid cocci in chains of two to seven cells are found microscopically, the presence of characteristic fecal enterococci is confirmed.

From the broth, inoculations are made into (a) a sterile tube of broth to be incubated at 45° C., (b) a similar tube of broth to be incubated at 10° C., and (c) another tube of the same broth containing 6.5% NaCl. After these inoculations have been made, 5-10 ml. of 3% H_2O_2 is added to the slant-broth to determine the production of catalase. For a positive completed test, the organism should grow at 45° C., at 10° C., and in the presence of 6.5% NaCl; and it should also be catalase negative. Various materials have been tested quantitatively for enterococci by this procedure. At the same time, the Most Probable Number of coliform organisms was determined using lactose fermentation tubes and Hoskins' Tables (9).

RESULTS

The average enterococci count of 140,000 per gram found for nine samples of human feces was approximately one hundredth of the average coliform count (See Table I). As has been reported by many workers, the coliform scores and the types encountered are highly variable. It should be noted that, while all the samples showed the presence of enterococci, one sample gave coliforms of the Aerobacter type only.

The raw sewage tested was collected every two weeks over a seven-month period from the inlet of a local sewage-disposal system. There was a marked tendency for both enterococci and coliform counts to be higher during the summer months, the percentage increases being of about the same magnitude in each case. The average enterococci count was 8200 per gr., and that of the coliforms was 631,000 per gm., these counts demonstrating again the approximate one to one hundred ratio. This sewage was collected from a domestic area having no commercial or street waste-water.

The polluted water samples tested were collected from certain areas of the Chesapeake Bay where a salinity of approximately 1.5% exists. Station A was right at the source of the pollution, and here the enterococci count was twenty times that of the coliform, being 603 per ml. against 30. This may be significant in showing that freshly, heavily polluted waters contain more enterococci than coliforms. The stations making up the group B were all some distance from the pollution source and gave an average count per ml. of 17 enterococci to 165 coliforms. Here a 1:10 ratio exists.

The feces samples from domestic animals were collected at nearby farms. All of them showed the presence of enterococci, with cows showing the lowest average and sheep, horse, and pig, following in increasing order. The samples from wild animals were collected from the intestinal tract of animals trapped on the Patuxent Wildlife Refuge. Raccoon excreta showed the highest enterococci count with 3,230,000 per gm., with skunk and opossum following in decreasing order. An approximate 1:10 ratio between coliform and enterococci exists with these samples as well.

The soil samples were collected from the Patuxent Wildlife Refuge. Five samples of virgin soil were taken from wooded areas where there has been no cultivation or human habitation for at least 50 years. These samples showed no evidence of enterococci or coliform bacteria. Five samples of pasture soils each showed approximately equal numbers of enterococci and coliforms.

DISCUSSION

The sodium azide-pencillin plate method provides a reliable quantitative method for the isolation of enterococci from various natural sources. Occasionally an atypical colony will appear on the medium, such as small, mold colonies which sometimes appeared on the plated cow feces. These colonies are easily distinguished from those of the streptococci. The only false positive test that occurred was caused by a long, slender, non-spore-forming, Gram-positive rod grown from the waters of the Chesapeake Bay.

In fecal samples, the enterococci are outnumbered approximately a hundred times by the coliform organisms. However, it is of interest that samples from station A (polluted waters) taken right at the source of pollution show the average enterococci count to be twenty times that of the coliforms. Then, as the distance from the point of pollution increases, the numbers of enterococci decrease more rapidly than the numbers of coliforms so that beyond a hundred yards, or more, the coliforms are greater in number.

The enterococci and coliform scores are higher for the wild animals than for the domestic animals. The only explanation known is that the water content is generally less in the feces of most wild animals.

The five virgin-soil samples showed no evidence of fecal pollution whereas pasture soils did. Two soil samples not listed in the table were collected from an uncultivated field where a polluted river had overflowed several months previously. There were no enterococci present, but Aerobacter-type coliforms were found.

SUMMARY

1. A plate method using a modified sodium azide-pencillin medium is presented as a presumptive test for the isolation of enterococci from natural sources.

2. A slant-broth preparation giving pin-point colonies on the slant, growth sediment in the broth, and a Gram-positive, ovoid, streptoccoccus smear constitutes a positive confirmation.

3. Growth at 45° C. and 10° C., growth in 6.5% NaCl, and a negative catalase test are considered as a positive completed test.

4. Enterococci were present in all the samples of human, domestic-animal and wild-animal feces that were tested.

5. Soils of virgin, wooded areas do not show the presence of enterococci, whereas the soil of pasture land does.

6. The preliminary data reported in this paper indicate that the enterococci may serve as a more specific and reliable index of fecal pollution than the coliform bacteria even though the former may be present in fewer numbers than the latter in polluted areas.

Table I

COMPARISON OF COUNTS OF ENTEROCOCCI AND COLIFORM

BACTERIA FROM VARIOUS SOURCES

	No. of Samples	Enterococci per ml. or gm.	Coliforms per ml. or gm.
Human feces	9	140,000	11,000,000*
Raw sewage	17	8,200	631,000
Polluted Waters A*	3	603	30
Polluted Waters B**	15	17	165
Cow feces	5	5,600	110,000*
Horse feces	6	201,000	110,000*
Sheep feces	4	101,000	110,000*
Pig feces	3	1,880,000	11,000,000*
Raccoon feces	3	3,230,000	110,000,000*
Skunk feces	2	2,100,000	11,000,000*
Opossum feces	2	1,100,000	11,000,000
Virgin soil	5	0	0
Pasture soil	5	6	4

* Samples of sea water collected at point where sewage entered water.
** Samples of sea water collected at least 50 yards from shore.

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PART II--RECOMMENDED PROCEDURE FOR DETECTING THE PRESENCE OF ENTEROCOCCI

INTRODUCTION AND DEFINITION

The enterococci comprise a group of streptococci that have as their normal habitat the intestinal tract of man and other warm blooded animals. They are large, ovoid, Gram positive streptococci appearing usually in chains of two to seven cells. The group consists of four species, Streptococcus faecalis, Streptococcous liquifaciens, Streptococcus zymogenes and Streptococcus durans, all of which belong to Lancefield's group D. They are characterized by the fermentation of dextrose which the production of acid, ability to grow at both 10° C and 45° C as well as in the presence of 6.5% NaCl and by failing to produce catalase. They will grow in the presence of 0.04% sodium azide and 650 Oxford units of penicillin per liter.

The test here described consists of two parts, a presumptive test in which the production of acid and growth turbidity in a sodium azide enrichment medium after incubation at 45° C is interpreted as evidence of the presence of enterococci. The positive presumptive tests are then confirmed by

inoculating a slant-broth preparation of a penicillin-sodium azide medium. Pin point colonies on the slant, growth sediment in the broth, the presence of Gram-positive ovoid streptococci in the broth and a negative catalase test is interpreted as confirmed positive evidence of the presence of enterococci.

MATERIALS

Materials needed for the preparation of the media and completion of the test include:

1. Distilled Water
2. Difco or B.B.L. Dehydrated Yeast Extract
3. Difco or B.B.L. Dehydrated Tryptone
4. Dextrose
5. C. P. Sodium Chloride
6. C. P. Sodium Azide
7. Methylene blue
8. Brom thymol blue
9. Penicillin
10. Agar
11. Hydrogen peroxide

PREPARATION OF MEDIA

1. Presumptive Enrichment Medium (normal strength).

0.5% Dextrose
0.5% Tryptone
0.5% Yeast Extract
0.04% Sodium azide
0.0032% Brom thymol blue

To save weighing of small amounts, the sodium azide is prepared in a 1% aqueous solution and 40 ml. added to each liter of medium. For the same reason a 1.6% alcohol solution of brom thymol blue is prepared and 2 ml. of this used in each liter of medium.

The medium is adjusted with NaOH to a pH of 8.0, tubed in 8 ml. amounts and autoclaved at 15 pounds pressure for 15 minutes.

2. Presumptive Enrichment Medium (concentrated).

A concentrated medium, using the same ingredients as the normal strength medium, is prepared increasing the percentage of each ingredient five fold. The medium is adjusted with NaOH to pH 8.5, tubed in large tubes in 2 ml. amounts and autoclaved at 15 pounds pressure for 15 minutes.

3. Slant-broth Confirmation Medium

A. Slants

0.5% Dextrose
0.5% Tryptone
0.5% Yeast Extract
0.04% Sodium Azide
0.001% Methylene blue
1.5% Agar

The methylene blue is added by using 1 ml. of a 1% aqueous solution. The medium is adjusted to pH 8.0, tubed in amounts for long surfaced slants, autoclaved at 15 pounds pressure for 15 minutes and slanted.

B. Broth

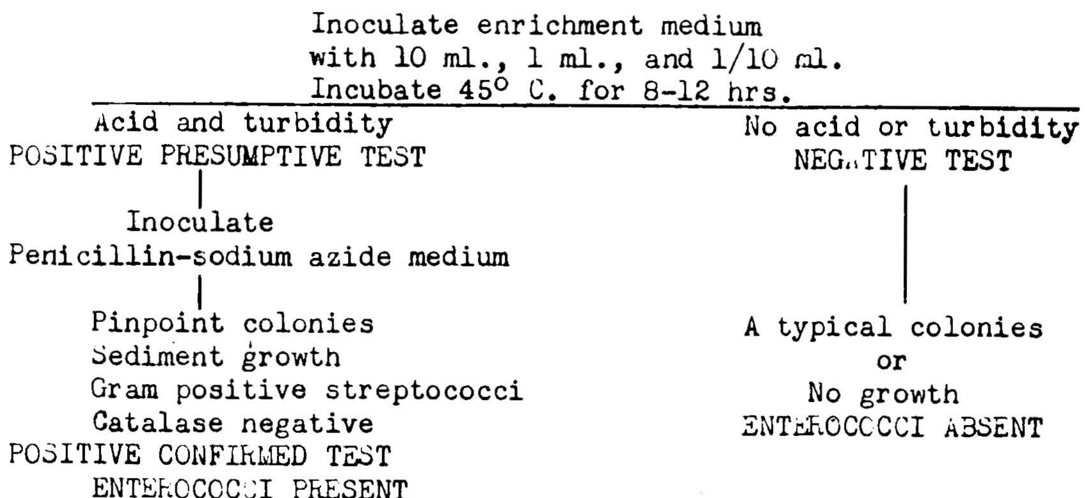
The broth medium differs from that of the slants in having no agar and by having 6.5% NaCl added after the adjustment to pH 8.0. It is autoclaved in flasks at 15 pounds pressure for 15 minutes. After cooling to room temperature, 650 Oxford units of pencillin per liter is added. A sterile distilled water dilution of pencillin is added by means of a sterile 1 ml. syringe. Enough of this broth is then added aseptically to each slant to cover approximately one half of the surface of the slant.

PROCEDURE FOR WATER ANALYSIS

It is recommended that one tube of the concentrated medium be inoculated with 10 ml. of the sample and that tubes of the normal strength medium be inoculated with 1 ml. and 1/10 ml. amounts. These are then incubated in a water bath at 45° C and observed periodically after 8 hours for the production of acid, as shown by the indicator, and for growth as indicated by turbidity. The production of acid and turbidity is interpreted as a positive presumptive test.

As soon as a positive presumptive reaction appears, a loopful of the material is transferred to the broth of the slant broth preparation and the loop zig-zagged on the surface of the slant as the loop is withdrawn. These tubes are then incubated at 37° C and observed after 12 hours for pinpoint colonies on the slant surface and for a growth sediment in the broth. After observations a Gram stain is made of the broth and then 5 ml. of H_2O_2 added to the slant-broth preparation for the catalase tests. Pinpoint colonies on the slant, a sediment growth in the broth, large Gram-positive ovoid streptococcus and a negative catalase test is interpreted as confirmatory evidence of the presence of enterococci.

SAMPLE



DISCUSSION

This recommended procedure has proved very satisfactory with both sea and fresh water. It must be mentioned that at first difficulty may be encountered in interpreting the production of acid in the 10 ml. inoculated concentrate presumptive test but if it is observed by reflected light rather than by transmitted light, little experience is needed to clearly differentiate between positive and negative tubes.

The test has also been used on crabmeat, oyster and frozen food samples where the foods have been ground with phosphate buffer solution in sterile Waring Blendors. Here the food material sometimes interfers with the interpretation of the presumptive test. Our procedure has been to inoculate the presumptive tubes as stated before and after 12 hours of incubation at 45° C. to transfer a loopful of each presumptive tube to a slant-broth preparation to determine the presence or absence of the enterococci.

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