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Isolation and Characterization of Fecal Streptococci from Surface Waters in South Dakota

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ISOLATION AND CHARACTERIZATION OF FECAL STREPTOCOCCI FROM SURFACE WATERS OF SOUTH DAKOTA

BY

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FROM SURFACE WATERS OF SOUTH DAKOTA

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

_____ Thesis adviser Date

_____ Head, Bacteriology Department Date

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INTRODUCTION

Organisms referred to as "fecal streptococci" or "enterococci" have been studied as possible indicators of pathogenic bacteria of fecal origin since their discovery. In Bergey's Manual of Determinative Bacteriology (1957), the "enterococci" are classified as Streptococcus faecalis, Streptococcus faecalis var. liquefaciens, Streptococcus faecalis var. zymogenes and Streptococcus durans. Streptococcus bovis and Streptococcus equinus do not meet all the criteria of the enterococcus group. However, they are found in great numbers in certain fecal material. These organisms, and the "enterococci" are collectively referred to as "fecal streptococci." The unavailability of satisfactory methods and of selective media has prevented the acceptance of these organisms for indicative purposes. Recently, media which give satisfactory results and methods which rapidly produce these results have been developed. Now, many have become interested in this area, and are suggesting reevaluation of this group as indicators.

Standard Methods (APHA, 1965) has long utilized the Most Probable Number (MPN) multiple tube method to statistically determine the number of coliform organisms in a sample. These coliform organisms have found common usage as indicators of pathogens in the routine analysis of water for potability. The fecal coliform test, also described in Standard Methods, demonstrates the presence of an indicator known to be from the intestinal tract of warm-blooded

animals. The enumeration of this organism is also an MPN procedure. The fecal streptococci could well become established as indicators concerned with detecting the source of contamination. These organisms might best be utilized as a supplementary test rather than a substitute for the other tests currently being used. In Great Britain, fecal streptococci are used routinely in the analysis of water for drinking purposes.

The development of the membrane filter has provided this phase of bacteriology with an invaluable tool which allows the rapid, numerical quantification of organisms from variable amounts of water. It provides surface colonies as well as a differential test on a medium containing a reducible indicator.

In this study, the distribution of the fecal streptococci in three types of surface water was studied using media currently recommended for biochemical characterization of isolates and the membrane filter technique. The differentiation of certain members of the fecal streptococci on the basis of colonial appearance on membrane filters was also studied, with the hope that the results of these studies would help investigators to estimate the sources of pollution by distinguishing between human and farm animal wastes.

LITERATURE REVIEW

The enterococci were apparently first recognized by Lawes and Andrews in 1894 and were named in 1899 by Thiercelin (Mundt, 1964). Houston demonstrated in 1900 that streptococci are present in polluted water, however, they appeared to be absent in nonpolluted samples (Tanner, 1944; Slanetz, Bent and Bartley, 1955). He also advanced four premises which are considered to be valid today: 1) they are abundant in the feces of man, 2) they are absent even in fairly large quantities of pure water, 3) they fail to multiply in pure water, and 4) they are unable to survive for long in pure water (Tanner, 1944). The inability of this group to multiply after leaving the intestinal tract was also reported by Lattanzi and Mood (1951). The number of fecal streptococci present in polluted water was reported to be much lower than the number of Escherichia coli found (Lattanzi and Mood, 1951). Litsky, Rosenbaum and France (1953), however, pointed out that advancements in media for detection of fecal streptococci have shown their numbers to be much greater than was previously reported, therefore the use of fecal streptococci as indicators of pollution should be reevaluated.

Selectivity of media for isolation of fecal streptococci has been successfully obtained by employing potassium tellurite, thallium acetate or sodium azide (Kenner, Clark and Kabler, 1961). The first use of sodium azide to produce selectivity is credited to Hartman in 1936 (Kenner, Clark and Kabler, 1961; Mallmann and Seligmann, 1950).

This compound is used today in England (Houghton, 1961), as well as in this country (Standard Methods, 1965). Further development of the media was attained by contributions from various investigators. Media which are being used today were reported by Slanetz and Bartley in 1957, and Kenner, Clark and Kabler in 1961.

The most recent work concerning fecal streptococci is the identification of species represented in feces of various animals, their occurrence in nature away from the animal host, the survival of various members of the group in water, and their application to the problem of determining the sources of pollution.

Occurrence in Nature

The presence of fecal streptococci in sewage and sewage polluted water is well recognized, and for a number of years they have been suggested as possible indicators of fecal contamination in water supplies (Mallmann and Seligman, 1950). In 1953, Litsky, Mallmann and Fifield reported that the streptococci are present in feces, sewage and contaminated water. Also, that they are not found in potable waters, "virgin" soils, and sites out of contact with animal and human life. These findings were confirmed by Leininger and McClesky (1953); and by Slanetz, Bent and Bartley (1955). Mundt (1964) reported the findings of his study regarding the presence of these organisms in nature. He found S. faecalis and especially S. fecalis variety liquefaciens to be adaptable to "several diverse ecological environments," and on occasion to thrive in nature apart

from the animal hosts. Geldreich, Kenner and Kabler (1964) also reviewed the occurrence of streptococci, as well as coliforms and fecal coliforms, on vegetables and insects. They reported these indicators as present "in very low numbers," with streptococci more numerous than fecal coliforms, however, less numerous than total coliforms. Their data supported the work of Frazer et al. (1956) who reported no definite type predominated on plants or insects.

When a dextrose azide broth was used, Mallmann and Litsky (1951) were unable to isolate enterococci from soils which were not treated with sewage or animal manure.

Bacterial Indicators

The total coliform test has never been recognized as the perfect indicator, and in the early 1900's serious attempts were made to distinguish between the coliform bacteria of lower animals and those from man (Leininger and McClesky, 1953). Additional bacterial indicators of human pathogens were sought by various investigators (Leininger and McClesky, 1953; Croft, 1959). The fecal streptococci have received considerable attention in this respect, particularly in England (Leininger and McClesky, 1953). The need for a test organism other than coliforms for detection of fecal contamination in foods, water, soil, and other materials was reported by Litsky et al. in 1953. These conclusions were drawn because of the coliforms' ubiquity in nature, ability to multiply outside the animal body, and failure to measure sanitary quality.

Leininger and McClesky (1953) stated that the ubiquity of the coliform group in surface waters, apparently free from dangerous contamination, almost nullifies the value of the test when applied to surface waters. The coliforms have been found in uncontaminated soil and water and may be of non-fecal origin. They have been found to persist in these environments for long periods, though no recent contamination exists (Litsky, Mallmann and Fifield, 1953). The use of streptococci as indicators in some phase in the study of fecal pollution in this country has been suggested by many investigators including Mallmann and Seligmann (1950), Litsky, Mallman and Fifield (1953), Barnes (1956b) and Croft (1959).

The sensitivity of the coliform organisms to chlorine has led Mallmann to believe the streptococci have a place in the analysis of swimming pool water (cited by Kjellander, 1960). The coliform test is questionable in "warm, sluggish" streams and perhaps the streptococci test is applicable (Mallmann, 1961). In 1934, Mallmann and Sypien compared the streptococcus and coliform indexes of samples of water taken five feet from the shore of a bathing beach. They found that the streptococcus counts always corresponded with the bathing load while the coliform and total plate counts did not. The streptococci were not found at points free from bathing pollution although the coliform organisms were present in such areas.

Wiebe, Raj and Liston (1961) found the enterococci to be better than the coliforms in indicating the fecal contamination of sea foods. They found more consistent and higher recoveries of enterococci as

compared to coliforms. This area was again studied in 1968, by Slanetz, Bartley and Stanley. In a study of shellfish and shellfish waters an evaluation of the membrane filter technique as well as a comparison of indicators was undertaken. Salmonellae were isolated in samples where neither fecal coliform nor fecal streptococci were detected. The indicators were considered to be comparable. However, the testing of larger volumes of sample and periodic checks for salmonellae were recommended for this area.

Estimate of the Origin of Contamination

Some work was done to divide the streptococci into major groups so that the origin of the contamination might be estimated. Kenner, Clark and Kabler (1960) studied the distribution of streptococci in feces from various animals using four different streptococcal media. They reported that this group of organisms permits a qualitative interpretation of the possible sources of pollution when careful classification is used. In the same year, Bartley and Slanetz (1960) examined feces, sewage and water with similar intentions. Their results indicated also that it appears possible to distinguish between human or animal contamination in certain instances. Isolates of typical Streptococcus fecalis were not found in the feces of domestic animals, and Streptococcus bovis isolates were found only in feces of animals, particularly cows and sheep.

Whittenbury in 1965 worked with two groups based on their physiological and biochemical properties. He reported that

Streptococcus faecalis was more numerous in human feces and Streptococcus faecium more numerous in animal feces, and that these organisms could be used to determine the type of contamination. He stated that when tellurite tolerance and reducing capabilities of the organisms were studied they could be completely differentiated. Barnes (1956a) had previously reported the use of 2,3,5 triphenyl-tetrazolium chloride (TTC) in differentiating these organisms. Mundt (1964) noticed in the literature that considerable importance had been placed upon Streptococcus faecalis and Streptococcus faecium as an element of differentiation between human or animal contamination. He noticed a host preference on the part of species of the enterococci, but also found a widespread occurrence of both species. He reported that a distinction between human or animal pollution is premature considering the information available on this subject. He recommended additional research emphasizing the occurrence of the fecal streptococci in fecal material and in nature.

Recent Studies

Recently, studies have been made with the ratios of the fecal streptococci to the coliform group in a particular water sample. More recently, the ratios have been concerned with fecal streptococci to fecal coliforms. Geldreich (1966) utilized ratios of the fecal coliform and fecal streptococci groups to estimate the source of contamination. Human sources produced a fecal coliform/fecal streptococci ratio of 4 to 1, while in lower animal contamination

the ratio reduced to 0.7 to 1 or less (Geldreich, 1966). In a study in 1955, Litsky, Mallmann and Fifield found the ratio of enterococci to Escherichia coli to be 7:1, rather than Escherichia coli being more numerous as previously reported. They suggested more sanitary significance be given the enterococci group. Ostrolenk and Hunter (cited by Slanetz, Bent and Bartley, 1955) found that in 37 per cent of 51 fecal specimens the enterococci occurred in numbers equal to or greater than numbers of Escherichia coli. Litsky, Mallmann and Fifield (1955) found the fecal streptococci to be almost as numerous as coliforms in certain samples. Slanetz, Bent and Bartley (1955) reported the number of coliforms were generally greater than the numbers of enterococci in water samples tested, however, enterococci were detected in all samples containing coliforms except one, which had a low coliform index.

In 1959, Croft, encouraged by the Standard Methods Committee, studied methods for detecting enterococci in water. He reported that enterococci determinations in water should provide valuable supplementary information concerning pollution.

Application of Membrane Filter

Slanetz, Bent and Bartley (1955) isolated fecal streptococci from feces, sewage and water. Later they published a favorable report concerning the use of membrane filters which said the various types of enterococci or coliforms might be isolated satisfactorily from the mixed populations using the membrane filter technique

(Bartley and Slanetz, 1960). Likewise, Kenner, Clark and Kabler (1961) recommended the membrane filter procedure over the multiple tube procedure whenever applicable. The Standard Methods Committee in 1959, encouraged a study of the media for fecal streptococcal detection using membrane filters (Croft, 1959). The 12th edition published in 1965 included a section regarding this procedure. Gilcreas (1967), in discussing the future of Standard Methods for the examination of water and waste water, stated that the place of the membrane filter method in the bacteriological examination of water has become reasonably stabilized.

The development of improved membrane filter media through the years has greatly aided the usefulness of the procedure. Different individuals have described media which were reported to give the most accurate determinations of the fecal streptococci. In 1957, Slanetz and Bartley formulated their M-enterococcus agar medium, and reported it produced "better results for enterococcus enumeration than any other medium tested to date." The authors claimed this to be a simple and direct method for isolation as 100 per cent of 2,500 isolates were identified as fecal streptococci. Sureau et al. in 1958 (cited by Slanetz and Bartley, 1964) reported favorable results using this medium with the membrane filter procedure. They found it to be more selective for fecal streptococci than other media tested. A year later Croft (1959) reported M-enterococcus agar medium of Slanetz and Bartley had the same order of productivity as a combination of azide dextrose and ethyl violet azide broths.

In 1961, Kenner, Clark and Kabler in studies with streptococci in surface waters devised a new medium, Kenner Fecal (KF) streptococcus medium, which was reported to give a greater variety of Streptococcus strains isolated when compared to other media tested. They found that streptococcal numbers were essentially the same from duplicate samples of human feces when cultured in KF streptococcus medium and in azide dextrose and ethyl violet azide broths. They also noted larger numbers of streptococci were recovered from domestic animal feces when grown in KF streptococcus medium than were obtained by the azide dextrose and ethyl violet azide broths.

Colonial Appearance

The appearance of fecal streptococci on agar media containing TTC has been mentioned by several investigators. The colonial appearance of enterococci on a membrane filter was described by Slanetz, Bent and Bartley in 1955. On the medium used, the colonies were described as flat, light pink, or raised and dark red with a pink periphery. Some colorless colonies of Gram positive bacilli were observed from polluted river water. All pink or red colonies on some filters were cultured and proved to be enterococci. The same authors, in 1957, described fecal streptococcus colonies on M-enterococcus agar medium as pink to dark maroon in color and 0.5 to 3.0 mm in diameter (Slanetz and Bartley, 1957).

Barnes (1956a) reported Streptococcus faecium, Streptococcus bovis and Streptococcus durans were very poor reducers of TTC, while Streptococcus fecalis was reported to be a strong reducer of TTC.

Wiebe, Raj and Liston (1961) also noted colony characteristics on a tetrazolium agar medium. They classified colonies showing an intensely deep red center and a white periphery, or a pink center and a white periphery as type I. Type II were those colonies appearing as white or pale pink throughout. Notably, cultures of Streptococcus bovis and Streptococcus durans were reported as giving white colonies. Tilton and Litsky (1967) seemed to confirm this characteristic for Streptococcus bovis. They stated that the reduction of TTC by this group is sporadic, appearing brick red or colorless at times.

Kenner, Clark and Kabler (1961) reported fecal streptococci colonies on KF streptococcus agar medium to be pink or red in color and 0.3 to 2.0 mm in diameter.

Geldreich (1967) discussed the enumeration of fecal streptococci on an agar medium containing TTC. He said all red or pink colonies should be counted using a magnification of ten to twenty diameters and that orange, yellow, white, or other colored colonies should not be counted since such types have not been observed in fecal examination.

In summary, the fecal streptococci have generally found acceptance as indicators of pathogenic bacteria of fecal origin. The controversy now found in the literature is on the use of these organisms as tracers of pollution. Streptococcus faecalis is considered an indicator of human contamination. Streptococcus bovis and Streptococcus fecium are reported as fecal streptococci found in animal fecal material. Streptococcus bovis is prominent in feces of

ruminants, and has not been reported as part of the normal flora of humans. Geldreich (1966) has shown the correlation of Streptococcus bovis to cattle in a study of a prison dairy farm. Streptococcus fecium has been isolated from human sources, however, it is found in much higher numbers in cattle. Certain investigators have disagreed with those who report the fecal streptococci to be good indicators of the source of pollution. Further studies in this area are needed as well as the development of methods to rapidly distinguish these strains of fecal streptococci.

MATERIALS AND METHODS

Source of Cultures

Known stock cultures of fecal streptococci for this study were obtained from three sources, the American Type Culture Collection (ATCC), Bacteriology Department, South Dakota State University (SDSU), and Mr. H. L. Jeter of the Federal Water Pollution Control (FWPC) laboratory at Cincinnati, Ohio. Streptococcus faecalis and Streptococcus bovis cultures were obtained from the ATCC with code numbers 8043 and 9809 respectively. Cultures of Streptococcus durans, Streptococcus faecalis var. zymogenes (isolated by Raymond Stark, SDSU, from a frozen food sample), Streptococcus fecium and Streptococcus faecalis var. liquefaciens were obtained from the culture collection at SDSU. Known cultures which were obtained from the FWPC laboratory included cultures of Streptococcus faecalis, Streptococcus bovis, Streptococcus equinus and Streptococcus faecalis var. liquefaciens.

Unknown cultures of fecal streptococci for identification were isolated from water samples obtained from three sampling areas. The first sampling location was a lagoon which served as a disposal unit for the dairy herd of South Dakota State University. This lagoon is rectangular, having a surface area of approximately 7500 square feet, and is held at a level of five feet by an over flow outlet. The samples were obtained from a point just prior to the outlet. The samples, from which the isolates were obtained, were collected in August and September of 1967.

The effluent from the waste water treatment facilities of the City of Brookings, South Dakota, was selected as the second source of isolates. The samples were obtained at the point where the constructed outfall-line flows into a ditch leading the effluent south of the city. This facility was serving an approximate population of 12,000. All samples were obtained during September and October of 1967.

The James River, an interstate waterway of eastern South Dakota, was sampled during July and August of 1967. The samples were obtained from stations representing the southern one half of the river's pattern in this state. This section may be briefly described as that area representing the river from Huron to Yankton, South Dakota.

Samples from these sources were obtained in sterile bottles which were immediately transported to the laboratory for isolation of organisms. Samples of the lagoon water and waste water effluent were obtained by direct immersion of the sample bottle. The river water samples were collected at midstream at a depth of 0.5 meter with a Kemmerer water sampler and transferred to a sterile bottle.

Isolation of Cultures

In the laboratory, bacteria from these samples were collected by membrane filtration of 0.5, 1.0 and 2.0 ml of undiluted sample from each source. An exception to this was the waste water effluent samples which were diluted 1:100 with sterile diluent. (Refer to

Section III of Materials and Methods for procedure for filtration and incubation on M-enterococcus agar medium.)

Mature colonies were picked at random from the various plates having fifty or fewer colonies. All colonies were picked on the plates that had fifteen or fewer colonies.

The colonies isolated from the river water and waste water effluent were treated identically in the following manner. The selected colony was inoculated into sterile tubes of azide dextrose (AD) broth which contained 0.02 per cent sodium azide and served as a selective enrichment step. These tubes were incubated at 37° C and checked for growth at 1 and 2 days. Positive AD broth tubes were used to inoculate sterile tubes of ethyl violet azide (EVA) broth. This medium contained 0.04 per cent sodium azide and was used as a "confirmed step" in the isolation of fecal streptococci. Incubation of these tubes was the same as that described for AD broth. Positive tubes of EVA broth were streaked onto M-enterococcus agar plates for isolation. After incubation at 37° C for 2 days, isolated colonies were inoculated into the Streptococcus stock culture medium described below. These tubes were incubated at 37° C for 1 day and stored in the cold room at 6° to 8° C.

Stock Culture Medium

	Gram
dextrose	1.0
tryptose	10.0
yeast extract	5.0
agar	7.0

Add to 1000 ml of distilled water and distribute 8 ml per screw cap tube (13 X 120 mm)

After all the isolates were collected as tested, stored cultures, they were inoculated into brain heart infusion (BHI) and incubated at 37° C for 20 to 24 hours. The presence of pure cultures of streptococci was confirmed with an American Optical dark contrast phase microscope at 1000X using a wet mount procedure.

Colonies isolated from the lagoon water were treated similarly with one exception. EVA broth has been reported to be inhibitory for some strains of Streptococcus bovis, therefore, this EVA broth step was not used in the isolation procedure for fecal streptococci from the dairy lagoon. Examination of the AD broth microscopically for streptococci was added in place of the EVA broth confirmed step.

Physical and Biochemical Characterization

The streptococcus cultures being studied were differentiated into species using a series of selected physiological and biochemical tests. The tests selected were those reported extensively in the literature for the characterization of fecal streptococci. Cooper and Ramaden (1955), Tilton and Litsky (1967) and Iwami (1967) reported similar tests in their characterization of fecal streptococci.

The stored cultures which had been microscopically observed to be streptococci were inoculated into BHI broth and incubated at 37° C for 20 to 24 hours. The storage cultures were then returned to the cold room. One to two drops of the turbid cultures from sterile Pasteur pipettes were used to inoculate the characterization media listed as follows. The first five tests represent five of the six

tests used by Sherman (1937) for the identification of fecal streptococci. All cultures were incubated at 37° C for 2 days. All broth cultures which became turbid were reported as positive, and physical or changes in color were reported in specific tests.

1. Growth at 10° C. Tubes of BHI broth were pretempered to 10° C prior to inoculation, inoculated in small quantities and returned to the 10° C incubator. The tubes were not allowed to warm. Observations for growth were made at 5 and 7 days.
2. Growth at 45° C. Tubes of BHI broth were inoculated with the test organism, incubated at 45° C for 2 days and examined for growth.
3. Sodium chloride tolerance. To test for growth in a high salt concentration, sodium chloride was added to BHI broth to give a final concentration of 6.5 per cent.
4. Growth at pH 9.6. Organisms were tested for growth in BHI broth. The pH was aseptically adjusted to 9.6 with a 38 per cent (wt/vol) of sodium phosphate solution ($Na_3PO_4 \cdot 12\ H_2O$).
5. Growth in 0.1 per cent methylene blue milk. Sufficient 1 per cent sterile solution of methylene blue was added to sterile skim milk to give a final concentration of 0.1 per cent (vol/vol) of methylene blue. Organisms were inoculated and observed at 1, 3, and 7 days.
6. Tolerance test in bile. The isolated organisms were tested for their ability to grow in bile in BHI broth. Forty ml of

sterile 10 per cent oxgall solution was added to 60 ml of BHI broth. Inoculated tubes were observed for growth at 1 and 3 days.

7. Litmus milk reaction. Tubes of sterile litmus milk with 0.75 g Difco litmus per liter of 10 per cent skim milk were inoculated. Cultures were observed at 2 and 7 days for acid production, for curd formation, for proteolysis and for reduction of litmus.

8. Reduction of potassium tellurite. Plates containing pre-sterilized potassium tellurite in BHI agar (1:2500) were prepared and the agar was streaked with the test organisms. The plates were observed at 1, 3, and 7 days for production of black colonies which result from the reduction of potassium tellurite. Test organisms giving negative results on this plate medium were inoculated into a sterile skim milk medium containing potassium tellurite also at a 1:2500 concentration. Darkening of the medium constituted a positive test. The tube cultures were examined at the same intervals, 1, 3, and 7 days, as the tellurite plate procedure.

9. Starch hydrolysis. Organisms which are unable to grow at 10^{o} C are reported by Bergey's Manual (1957) in the Viridans Group. Fecal streptococci in this group are Streptococcus bovis and Streptococcus equinus. These suspected organisms were tested for starch hydrolysis by surface inoculation onto plates of BHI agar containing 1.0 per cent starch.

These standard plates were found to produce very slow growth. Therefore, a tube medium with reduced starch concentration producing the desired criteria was employed. Starch broth containing 1.0 per cent tryptose, 0.2 per cent soluble starch (Fisher), 0.15 per cent KH_2PO_4, 0.25 per cent K_2HPO_4, and 0.3 per cent yeast extract was inoculated. Hydrolysis was tested by placing two drops of the test medium in a depression of a spot plate and adding one drop of Gram's iodine. Positive starch hydrolysis was indicated by the absence of a blue or rust color.

10. Lactose fermentation. Isolates which were selected for testing of starch hydrolysis were also tested for fermentation of lactose. Tubes of 1.0 per cent lactose fermentation medium with a Durham tube and containing brom thymol blue as the pH indicator were used. Production of acid (yellow color) or gas was recorded.

11. Gelatin hydrolysis. The medium used was that described by Iwami (1967).

	Per Cent
Tryptose (Difco)	2.00
NaCl	0.57
K_2HPO_4	0.25
Yeast Extract	0.30
gelatin	0.40
agar	1.50
distilled water	qs to 1000 ml

pH adjusted to 7.2 with 1 N NaOH

A single streak was made across each gelatin plate with each organism to be tested. The cultures were incubated for 2

days at 37° C. Following incubation the plates were flooded with an acidic mercuric chloride solution (mercuric chloride, 15.0 g; concentrated HCl, 20.0 ml; distilled water, 100 ml) which is reported as a gelatin precipitant by the Society of American Bacteriologists (1957). The lack of a white precipitate in the region of growth indicates hydrolysis of the gelatin.

12. Hemolysis. Blood agar plates were prepared by adding sheep's blood to sterile tryptose blood agar base (Difco) at 45° C to give a final concentration of 5.0 per cent by volume. The blood plates were streaked with test organisms and incubated at 37° C with high humidity for 2 days. Recordings were made as: alpha for greening with intact cells in the zone surrounding colonies, beta for complete clearing of the medium and gamma for no apparent hemolysis around the colonies.

Membrane Filter Characterization

A Millipore Standard Hydrosol Filter unit was used for all filtrations in this study. The filters used were commercially sterilized Millipore filters, type HAWG, white, grid marked and 47 mm in diameter. The procedure used in filtering the samples is that described in Standard Methods for the Examination of Water and Waste Water (1965). After filtration, the filters were placed on the surface of 10 ml of M-enterococcus agar medium in a sterile 60 mm

petri plate. This medium is the same as that used in the initial isolation of the fecal streptococci, and is recommended by Standard Methods (APHA, 1965) for the isolation of fecal streptococci. M-enterococcus agar medium was found by Croft (1959) to be of the same order of productivity as the azide dextrose and ethyl violet azide multiple tube method, and received his recommendation to the Standard Methods Committee. Burkwall and Hartman (1964), in a comparison of direct plating media for the isolation and enumeration of enterococci, found this medium to produce acceptable results. This medium was chosen as the isolation medium in this study because it is being used in various Public Health laboratories at present, and will probably continue to find acceptance in this area as water pollution abatement studies continue.

The plates containing the membrane filters were inverted and incubated in an atmosphere maintained at, or very near, 100 per cent relative humidity at a temperature of 37° C for 2 days. Stock cultures or selected isolates to be studied were diluted 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} with sterile dilution blanks. Generally 0.1 and 1.0 ml of the three highest dilutions were filtered.

The colonies obtained on the membrane filters by the above methods were then studied. The colony size, color, surface elevation, internal colony characteristics and any other distinguishing aspects were of interest. An American Optical cycloptic stereoscopic microscope with a magnification of 15 to 20X was used.

Photographs were used to record colonial characteristics. Typical colonies with regard to size and other characteristics were selected for photographs. A Pentax Spotmatic camera (Honeywell) mounted on the stereoscopic microscope with a photographic adapter was used for all photography using Kodachrome X film. A Wolf MG-7 illuminator was modified by shielding the lighted surface except for a 10 cm diameter circle near the center. The stereoscopic microscope, with the base removed, was placed over this opening. Two number one photoflood lamps were placed at each side of the stereoscopic microscope at a distance of 18 inches and an angle of 45 degrees. The flood lamps provided surface lighting to give the correct color of the colony being photographed, while the Wolf illuminator provided transmitted lighting to show the internal characteristics of the colony.

Comparisons were made among all labeled cultures and selected biochemically identified isolates listed in the initial paragraphs of this section. Comparisons of colonies were made on two differential media, M-enterococcus agar and KF streptococcus agar. The KF streptococcus agar medium was included into the study because it had been reported to be less inhibitive to fecal streptococci of animal origin, particularly Streptococcus bovis. By including both media, the inhibitive effects of each could be compared and the results could possibly be used in interpreting the data obtained from isolating fecal streptococci from the surface waters.

RESULTS AND DISCUSSION

Biochemical Characterization

The first portion of this study involves the biochemical identification of fecal *Streptococcus* isolates. The identification of 302 isolates from three types of surface water permitted the reporting of their distribution.

Ninety-five isolates were obtained from the dairy lagoon at South Dakota State University. The distribution of the identified isolates is given in Table 1.

Table 1. The Distribution of Fecal *Streptococcus* Species and Biotypes Isolated From the SDSU Lagoon

Organism	Number of Isolates	Per Cent of Total
S. faecalis	39	41
biotype I	30	33
biotype II	2	2
biotype III	3	3
S. faecalis var. *liquefaciens*	8	8
S. fecium	1	1
S. bovis	7	7
biotype I	2	2
Unidentified	3	3
TOTAL	95	100

Streptococcus faecalis and its biotypes represented 79 per cent of the total number of isolates, compared to 9 per cent for Streptococcus bovis and its biotype. The results indicate that this lagoon could be receiving human wastes as well as the effluent from the dairy operation because a large percentage of Streptococcus faecalis was found. Cooper and Ramadan (1955), Barnes, Ingram and Ingram (1956) and Kjellander (1960) have reported this organism to be of human origin. Bartley and Slanetz (1960) isolated Streptococcus faecalis from human feces, but not from cattle feces.

Streptococcus bovis was reported in high numbers in the feces of ruminants, but was not isolated from human feces (Kenner, Clark and Kabler, 1960). The low numbers of Streptococcus bovis found in this source tend to agree with a similar study conducted at the University of Wisconsin. Iwami (1967) did not isolate this organism from a dairy lagoon being studied there, although the organism was isolated from bovine feces with the same methods. She attempted to explain her findings with an experiment showing the poor survival of this organism in the lagoon environment. Streptococcus fecium, which is not a recognized species of enterococcus, was represented by a single isolate from the lagoon water. This organism is widely distributed in animals, however, and is also found in man (Barnes, Ingram and Ingram, 1956). This species is identified by its reactions to tellurite, tetrazolium and litmus milk. Streptococcus faecalis var. liquefaciens comprised eight per cent of the isolates.

The second source of isolates was the waste water effluent of the City of Brookings, South Dakota. The distribution of the identified isolates from this source is given in Table 2.

Table 2. The Distribution of Fecal Streptococcus Species and Biotypes Isolated From Effluent of the Waste Water Treatment Facilities of the City of Brookings, South Dakota

Organism	Number of Isolates	Per Cent of Total
S. faecalis	54	50
biotype I	32	30
biotype II	1	1
biotype III	5	5
S. faecalis var. liquefaciens	11	10
S. fecium	0	0
S. bovis	0	0
Unidentified	4	4
TOTAL	107	100

One hundred and seven isolates were obtained. The isolates from this source were all identified as Streptococcus faecalis, biotypes of Streptococcus faecalis or Streptococcus faecalis var. liquefaciens. These organisms may all be considered to be human in origin. The fecal streptococci generally associated with fecal contamination from animals, namely Streptococcus fecium and Streptococcus bovis, were not found among these isolates. The James River served as the final

source of fecal streptococci isolates considered for characterization (Table 3). One hundred isolates were obtained with 78 per cent being representatives of Streptococcus faecalis and its biotypes.

Table 3. The Distribution of Fecal Streptococcus Species and Biotypes Isolated From the James River

Organism	Number of Isolates	Per Cent of Total
S. faecalis	61	61
biotype I	14	14
biotype II	3	3
S. faecalis var. liquefaciens	9	9
S. fecium	7	7
S. bovis	0	0
Unidentified	6	6
TOTAL	100	100

(See summary Table 4.)

With this waterway receiving waste water effluent from several sources along its route, it seems probable that Streptococcus faecalis could be well represented. Streptococcus faecalis var. liquefaciens was found to represent nine per cent of the total fecal streptococci isolated. This figure corresponded to eight per cent for this organism reported by Bartley and Slanetz (1960) from river water in a similar study. Streptococcus bovis was not isolated from this source even though contamination by domestic animals seemed possible.

From these results, recent pollution by farm animals would not be suspected. The lack of Streptococcus bovis here, as was found in the dairy lagoon, may be related to its survival outside the intestinal tract of ruminant animals. Since little is known concerning the survival of the various species, this question must await further study.

Seven isolates of Streptococcus fecium were obtained from the river water. These organisms were all isolated from the same sampling site, which was in a rural location downstream from the outfall of the waste water treatment facilities of the City of Huron, South Dakota. Streptococcus fecium has been isolated from human sources, but since this organism was not found in the waste water effluent of Brookings, South Dakota, it might be considered to be of animal origin in this case.

The significance of the entire group is their indication of the possible presence of pathogenic bacteria of fecal origin. The significance of the distribution of the species within this group is the ability of certain species to indicate the type of contamination. Human origin would be indicated by a high percentage of the enterococcus group and fewer numbers of the enterococcus biotypes (Kenner, Clark and Kabler, 1960). These same investigators used the enterococcus group as an indicator of pollution and found it represented 77 per cent of the total streptococci from human sources, 12 per cent from cattle and 10 per cent from swine.

Table 4. The Distribution of Fecal Streptococcus Species and Biotypes Isolated From Selected Surface Waters of South Dakota

Organism	Dairy Lagoon	Waste Water Effluent	James River	Total	
	NO.	NO.	NO.	NO.	Per Cent
S. faecalis (typical)	39	54	61	154	50.9
S. faecalis biotype I	30	32	14	76	25.1
biotype II	2	1	3	6	2.0
biotype III	3	5	0	8	2.7
S. faecalis var. liquefaciens	8	11	9	28	9.3
S. fecium	1	0	7	8	2.7
S. bovis (typical)	7	0	0	7	2.3
S. bovis biotype I	2	0	0	2	0.7
Unidentified streptococci	3	4	6	13	4.3
TOTAL	95	107	100	302	100.0

Discussion of Biochemical Reactions of Fecal Streptococci

As a result of investigations of the fecal streptococci as indicators of pollution many characteristics of the various species are known. Deibel (1964) discussed these organisms in an excellent review of the group D streptococci. Hartman, Reinbold and Saraswat (1966) considered the fecal streptococci to be one of the better characterized bacterial groups. In reviewing the taxonomy of the group, they noted many reports of isolates which gave reactions which would place them in-between established groups. In discussing this further they mentioned additional difficulty in differentiating enterococci isolates which do not fulfill all the Sherman criteria (Sherman, 1937). Kenner, Clark and Kabler (1960) devised an "enterococcus biotype" group to include these strains, and Burkwall and Hartman (1964) in a medium comparison referred to this group as "atypical enterococci." Other workers (Cooper and Ramadan, 1955), in an attempt to subdivide the streptococci from various sources into groups convenient for differential purposes, applied the name "atypical faecalis" to groups I, II, III, IV, and V. These organisms differed from typical Streptococcus faecalis in one, two, or three standard characteristics and was thought to include the "so-called Streptococcus faecalis variants and enterococcus-like organisms recorded by several investigators."

In the present study, isolates were obtained for identification so the distribution in surface waters could be noted. The problem regarding the placement of the various "atypical" organisms was also

encountered. In an attempt to name these groupings, the biotypes I, II, and III were used. The categories represent groups whose members varied from the typical strain by one, two, or three reactions respectively. The tests in which the Streptococcus faecalis biotypes tended to give "atypical" reactions were the reduction of tellurite, reduction of 0.1 per cent methylene blue and growth in 6.5 per cent sodium chloride in BHI broth. The S. bovis biotype reported showed variation regarding growth at pH 9.6, and slight reduction in 0.1 per cent methylene blue milk.

These atypical forms were reported to account for 25 per cent of the strains isolated from human feces (Cooper and Ramadan, 1955). In this study, S. fecalis biotypes represented 30 per cent of the isolates from the three sources. Kenner, Clark and Kabler (1960) found 11 per cent of 1092 isolates to vary in one or more biochemical reactions.

Discussion of Differential Tests

Many of the tests used in this study to differentiate the fecal streptococci are relatively simple tolerance tests. These tests are in common usage in laboratories engaged in the routine identification of these organisms. Other tests are being varied or developed by different individuals who are interested in obtaining more rapid results, or more information regarding the grouping of the fecal streptococci.

The reaction involving the reduction of tellurite is one of these tests. The medium as well as the concentration of tellurite is being varied. Cooper and Ramadan (1955) used a concentration of 1:5000 tellurite in an isolation medium to achieve satisfactory results. A level of 1:2500 tellurite in BHI agar medium is currently recommended as a differential test. (Jeter and Zobel, 1967) In this study, this recommended medium was first used. All isolates which were able to grow well were recorded as tellurite tolerant. All cultures which were unable to produce growth on the agar surface were then subjected to tellurite milk. This medium (Iwami, 1967) contains pre-sterilized potassium tellurite in sterilized skim milk. The final concentration is also 1:2500 as in the agar medium. The possibility that the aerobic environment of the agar plate might have contributed to the negative reaction was reduced by using this liquid medium. A variation in reduction of tellurite was found among the isolates. Some isolates were able to produce the characteristic black colonies on agar in one day, while others would give similar results in three days. Other isolates were able to reduce the tellurite only at the site of the heavy inoculum. The isolates showing questionable reduction of tellurite were inoculated into the tellurite milk medium. Generally these organisms did not produce a complete blackening of the medium. Varying shades of gray and amounts of black precipitate were noted. This latter medium appears to be better suited to quantification of tellurite reduction than the agar medium.

The test for gelatin hydrolysis by these organisms is also of interest in this study. Ordinary gelatin tubes, 12 to 14 per cent gelatin were first used. However, isolates capable of liquefying this amount of gelatin were not found. Isolates suspected to be the Streptococcus faecalis var. liquefaciens on the basis of curd shrinkage in litmus milk were streaked onto gelatin plates containing 0.4 per cent gelatin (Society of American Bacteriologists, 1957). This method gave satisfactory results, and required only a 48-hour incubation period. After addition of acidic $HgCl_2$, clear zones near areas of growth indicate gelatin hydrolysis. This organism, S. fecalis var. liquefaciens, was found in 9 per cent of the isolates.

Another biochemical test worthy of discussion is that of starch hydrolysis. All cultures which failed to grow at 10° C, but grew at 45° C, were tested for their ability to hydrolyze starch. Starch hydrolysis has been suggested as a preliminary screening procedure for S. bovis, and has been used as such in this laboratory for the isolation of this organism from bovine fecal material. In this study of the starch hydrolyzing ability of the isolates, plates of BHI agar containing 1.0 per cent soluble starch were first used. This method was not found to be satisfactory, as growth of the organisms on the agar surface was quite limited. Zones of starch hydrolysis were either lacking or not easily distinguishable; and therefore, another method was sought. A liquid medium (Society of American Bacteriologists, 1957) containing 0.2 per cent starch in BHI broth was employed, and found to be more satisfactory. The inoculated tubes

were incubated at 45° C and tested after two days in an attempt to determine rapid starch hydrolyzers. To test the tubes for starch hydrolysis, a small amount of the broth was drawn aseptically with a Pasteur pipette and two to three drops placed in a depression of a spot plate. Addition of one drop of Gram's iodine produced readable results. Several cultures were capable of complete hydrolysis in two days, however, more were capable of only partial hydrolysis in this time. These cultures were then incubated for longer periods. Several cultures which were not capable of hydrolyzing the starch, although other biochemical tests indicated they were animal isolates, were classified as unidentified.

Membrane Filter Characterization

This portion of the study was conducted to determine if the fecal Streptococcus species being studied could be differentiated on the basis of their colonial characteristics on a membrane filter on an agar medium.

The original stock cultures were first compared on filters on M-enterococcus agar medium. Streptococcus faecalis, ATCC 8043, produced a round, mounded colony approximately 2 mm in diameter. The center one-half of the colony was dark maroon in color with a pink surrounding area appearing as a ring (Figure 1). This organism is apparently a strong reducer of tetrazolium as evidenced by the dark-centered colony. This characteristic has been noted by others interested in differentiating Streptococcus faecalis from Streptococcus fecium (Barnes, 1956a; Whittenbury, 1965).

Streptococcus faecalis var. zymogenes was studied under identical conditions and found to give much smaller colonies in every trial (Figure 2). Typical colonies of this organism are described as round, somewhat flattened with a raised center. The maximum diameter is approximately 1 mm for the largest colonies. The raised center is darker than the pink edge and is a maroon color. These colonies could not be enlarged in size even upon prolonged incubation.

Streptococcus faecalis var. liquefaciens was found to produce the largest colonies of any of the species (Figure 3). They were 2.5 to 2.75 mm in diameter, and appeared as a round, flattened colony. The center, approximately 2.0 mm in diameter, was a dark maroon (like Streptococcus faecalis, ATCC 8043) while the outer ring was pink and colorless at the edge.

The culture of Streptococcus durans used in this study produced round, raised colonies 2.0 mm in diameter. They appeared to be a dark maroon with the color the same throughout, with some colonies lighter near the edge (Figure 4). Several irregular colonies were noted with this organism. Barnes (1956b) describes Streptococcus durans as producing white or very pale pink colonies which is different from the tetrazolium reducing properties of the strain studied.

A culture received as Streptococcus fecium produced colonies which were not distinguishable from those of Streptococcus durans. The culture was compared biochemically with cultures of Streptococcus durans and Streptococcus faecalis, and characteristics were found to

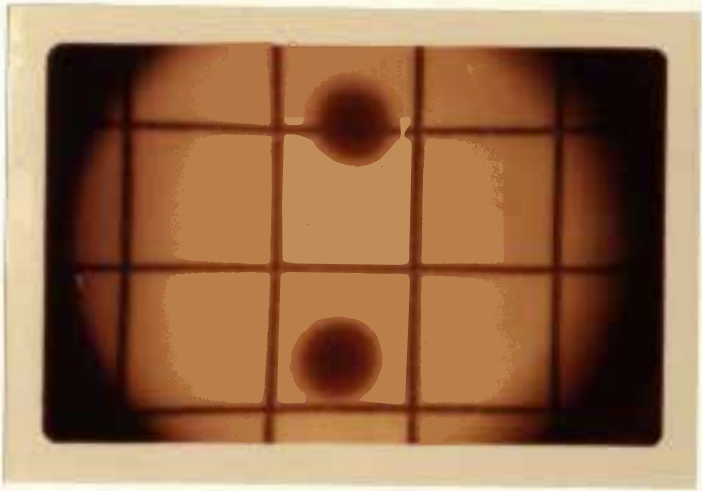

Figure 1. Streptococcus faecalis, ATCC 8043, on membrane filter using M-enterococcus agar medium, 60 hours at 37° C, 15X

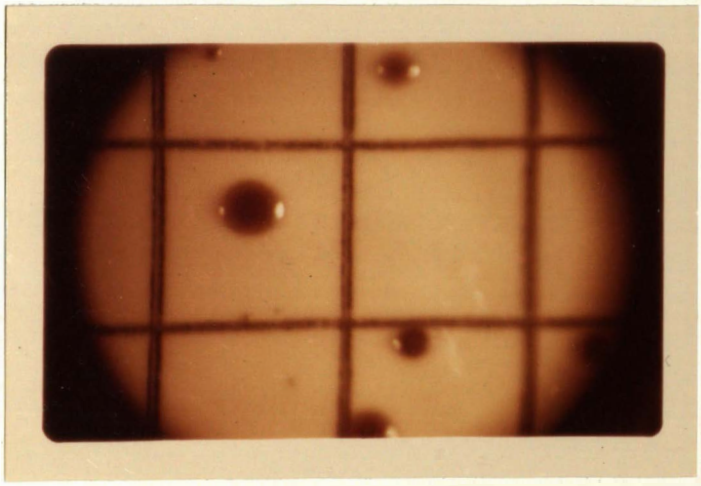

Figure 2. Streptococcus faecalis var. zymogenes on membrane filter using M-enterococcus agar medium, 60 hours at 37° C, 20X

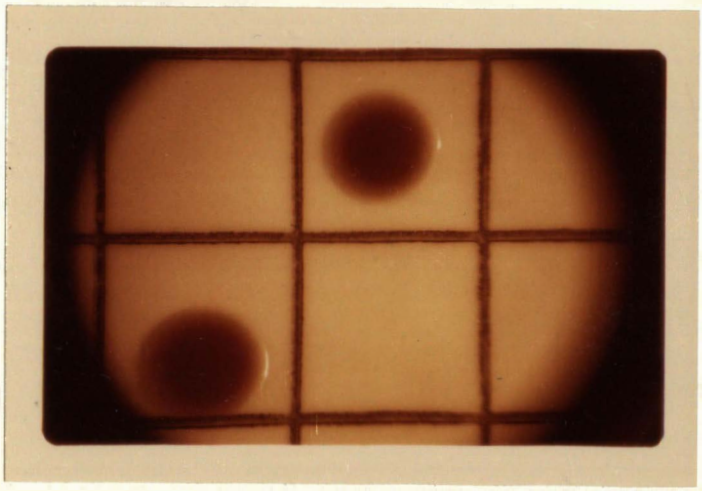

Figure 3. Streptococcus faecalis var. liquefaciens, SDSU Bacteriology Department Stock #B-750, on M-enterococcus agar medium, 60 hours at 37° C, 20X

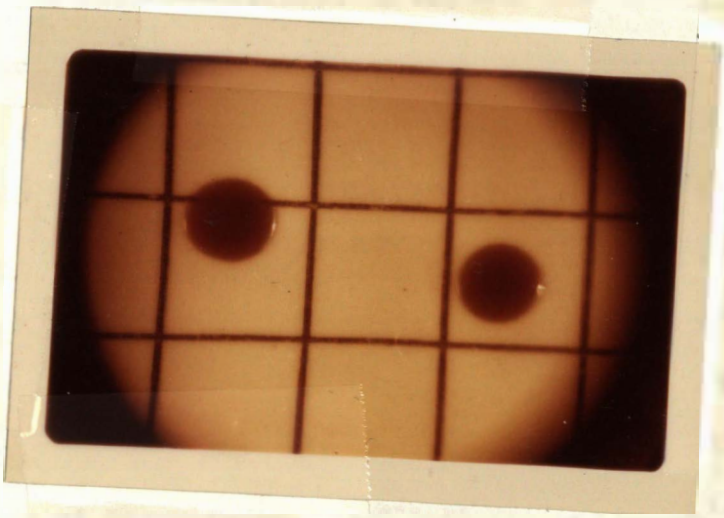

Figure 4. Streptococcus durans, SDSU Bacteriology Department Stock #B-755, on M-enterococcus agar medium, 60 hours at 37° C 15X

be identical to those of Streptococcus durans. The culture was considered to be mislabeled.

Streptococcus bovis, ATCC 9809, was found to be a weak reducer of TTC as light pink colonies were obtained (Figure 5). Some difficulty was experienced in growing this organism on M-enterococcus agar. Colonies were obtained when a ten-fold increase in inoculum was used. The largest colonies obtained were round, mounded and approximately 1.5 mm in diameter. Smaller colonies with similar appearance were also noted.

Streptococcus pyogenes was carried as a control for the selectivity of the medium and produced no growth.

A second comparison was made between these known culture colony characteristics and those of a group of known cultures sent by Mr. Jeter of the FWPC laboratory. A culture labeled Streptococcus fecalis produced colonies similar to those described for this species in the known set of cultures. The biochemical tests of this culture were comparable to the type culture. A second culture labeled Streptococcus faecalis produced colonies which were different from the Streptococcus faecalis, ATCC 8043, already described. The biochemical tests of this organism indicated it was properly labeled, however, with atypical reactions on the reduction of tellurite and TTC.

A culture of Streptococcus faecalis var. liquefaciens was received and checked biochemically. The colonies of this organism on M-enterococcus agar medium were dark maroon throughout, flattened and

approximately 2 mm in diameter. The flattened appearance of these colonies seemed comparable to the known culture with the same label. It is doubtful this variety could be distinguished from typical Streptococcus faecalis by colonial characteristics.

A culture labeled Streptococcus bovis MG-21 was also received. This strain was a rapid hydrolyzer of starch and typical of Streptococcus bovis, ATCC 9809, in other reactions. This culture produced small, pink colonies on M-enterococcus agar medium, and was difficult to grow. These characteristics seem typical of Streptococcus bovis. Another culture of animal origin, Streptococcus equinus, was also received. This culture produced colonies 2.5 mm in diameter which were dark maroon with a lighter pink ring. This culture was not found to hydrolyze starch. Other biochemical tests indicate this culture may be a biotype of Streptococcus faecalis.

Another comparison of interest was made between the known stock cultures and representatives of the biochemically identified types. Isolates #2, #107, and #254 were selected as representatives of 154 isolates identified as Streptococcus faecalis which were obtained from the James River, dairy lagoon, and waste water effluent respectively. Isolates #2 and #254 appeared to be similar and could be compared to the known stock culture of Streptococcus faecalis. Isolate #107 developed colonies similar in size to isolate #2 and #254. However, it appeared a slightly brown color as contrasted to the deep maroon of the known culture and these other two isolates

being compared. The lighter edge found typical in this species was less pronounced in isolate #107.

Biochemically identified isolates of Streptococcus faecalis var. liquefaciens were represented by isolates #39, #199, and #255. These represent 28 isolates from the James River water, dairy lagoon water, and waste water effluent respectively. The colonies produced by these organisms were all similar, however, they were different from the colonies produced by the stock culture representative of the same species. The colonial characteristics obtained here could be considered similar to those found for Streptococcus durans of the known stock used as a reference.

Isolates #46 and #47, both from the James River water, were identified biochemically as Streptococcus fecium. Colonies produced by these cultures were very light pink, small colonies and larger colonies were pink with a light maroon center (Figure 6). The largest colonies were approximately 1.0 mm in diameter.

Isolated cultures of Streptococcus bovis were represented by isolates #113, #241, and #242, all isolated from the dairy lagoon. These cultures all developed similar colonies. Smaller colonies appeared as round, mounded pink colored colonies while colonies larger than 1 mm developed a darker pink or light maroon center.

In the study of colonial characteristics just described, the author pointed out that Streptococcus bovis cultures were difficult to recover on the primary isolation medium. It was found that

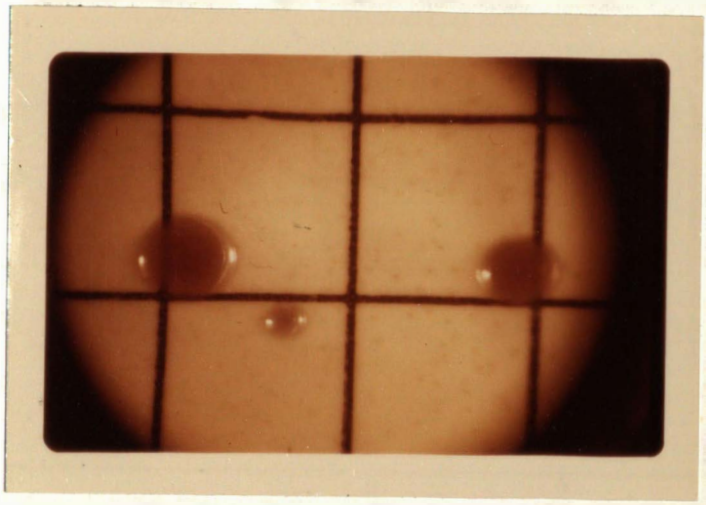

Figure 5. Streptococcus bovis, ATCC 9809, on membrane filter using KF streptococcus agar medium, 60 hours at 37° C, 20X

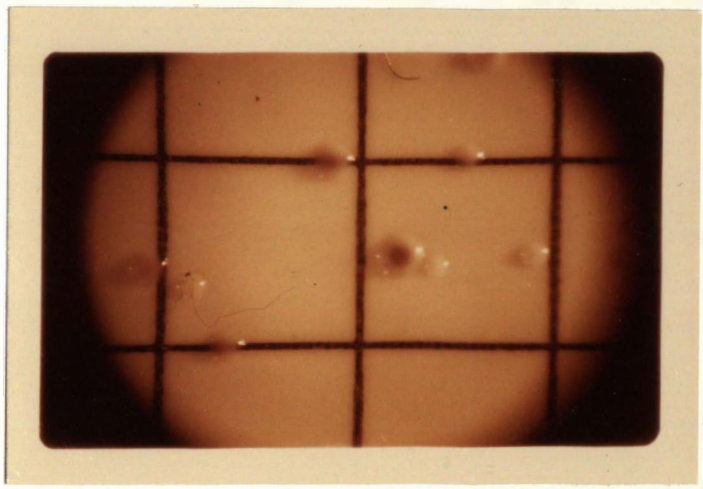

Figure 6. Streptococcus fecium, isolate #47, on membrane filter using M-enterococcus agar medium, 60 hours at 37° C, 20X

Streptococcus bovis ATCC 9809 and certain other strains could be grown on the agar medium on a membrane filter only with dilutions which provided high numbers of organisms. This differed from other species, such as S. fecalis ATCC 8043, where filters with a single colony were observed. An experiment was conducted in which several cultures of S. bovis were grown on media containing all the ingredients of M-enterococcus medium except sodium azide which was added with a gradient in concentration from 0.02 to 0.04 per cent with intervals of 0.05 per cent. Some strains were capable of TTC reduction and produced light to dark pink colonies. Another strain, MG-21, produced growth on all plates when a dilution 10^{-4} was used, and produced visible growth only on the plate with 0.025 per cent sodium azide when dilution 10^{-6} was used. This growth appeared macroscopically as slight pink dots on the membrane filter, and only with a stereoscopic microscope with 25-50 magnification could distinct colonial morphology be seen. These findings could perhaps explain a portion of the problem concerning the judging of minute, slightly pink colonies on membrane filters mentioned in the literature (Croft, 1959).

The results of this initial membrane filter study indicate the fecal streptococci can be rapidly isolated from surface water samples for characterization.

Further studies will be needed to improve the membrane filter characterization of the fecal Streptococcus species and biotypes.

Additional studies of the characterization and survival of Streptococcus bovis are especially recommended. This organism is found in high numbers in domestic animals and is being considered as an indicator of surface water pollution from this source.

CONCLUSIONS

1. Fecal streptococci isolated by selective media from the SDSU dairy lagoon, the waste water effluent of the City of Brookings and the James River were divided by physical and biochemical test into the following species and biotypes:
   A. Typical Streptococcus faecalis, 50.9 per cent of 302 isolates.
   B. Biotypes of Streptococcus faecalis, 29.7 per cent.
   C. Streptococcus faecalis var. liquefaciens, 9.3 per cent.
   D. Streptococcus fecium, 2.7 per cent.
   E. Streptococcus bovis, 3.0 per cent.
2. Streptococcus bovis was isolated only from the dairy lagoon.
3. The membrane filter procedure was found to be a rapid, reproducible method for isolating fecal streptococci from water.
4. Streptococcus fecium and some strains of Streptococcus bovis could be recognized on a membrane filter on TTC medium by the white or pink colonies formed.
5. The enterococcus group could be recognized on a membrane filter on a TTC medium by the dark red or maroon colonies formed.
6. The colony color of each organism was found to be reproducible in separate trials, and in most cases was recognizable after incubation at 37° C for one day.
7. Colonies of fecal streptococci were found to be more distinguishable on M-enterococcus than on KF streptococcus agar, although the two were found comparable for enumeration.

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