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A New Method for the Rapid Isolation of Streptococcus Bovis from Water

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A NEW METHOD FOR THE RAPID ISOLATION OF STREPTOCOCCUS BOVIS FROM WATER

BY

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A NEW METHOD FOR THE RAPID ISOLATION

OF *STREPTOCOCCUS BOVIS* FROM WATER

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor / Date

Head, Microbiology Department / Date

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INTRODUCTION

The importance of the fecal streptococci as indicators of water pollution has come under increasing scrutiny in the past decade. This has been due in part to improved methods of detection of fecal streptococci. Also, the fecal coliform and total coliform tests and their significance have been questioned (20,21). The fecal streptococci may furnish a more reliable means of identifying sources of pollution (23).

The fecal streptococci are those gram-positive cocci which occur in the intestinal tract of man and animals. The Sherman criteria for identification of the fecal streptococci are used in Bergey's Manual of Determinative Bacteriology to differentiate the viridans and enterococci from the other streptococci (5,32). The enterococcus group includes Streptococcus faecalis, S. faecium, and S. faecium var. durans (9). The viridans group includes two species of interest in this study, Streptococcus bovis and S. equinus. All of the above mentioned species of Streptococcus contain Lancefield's group D antigen and occur in nature in the intestinal tract of man and animals. These two facts warrant placing S. bovis and S. equinus in a general group with the enterococci and referring to them as fecal streptococci (9,26).

Like the fecal coliforms, the fecal streptococci are indicators of pollution in water. The characteristics of an indicator include: (1) the organism must be found in feces and sewage, (2) the organism

is found in polluted water, (3) the organism is not found in pure water sites away from man and animals, and (4) the organism does not multiply outside the host in water or soil (7).

The usefulness of the fecal streptococci does not end here, however. The fecal streptococci can be used to identify either human or an animal source of pollution. This is illustrated by the fact that S. faecalis strains are found predominantly in the normal flora of the human intestinal tract. In contrast, S. equinus and S. bovis are found predominantly in the caecum of horses and the rumen of cattle, respectively (12).

Although S. bovis has been isolated, identified, and reported by various authors, an improved method for isolating this particular species was not devised until Koupal (19) modified existing methods of isolating fecal streptococci. Koupal's method of isolating S. bovis was relatively crude, in that it requires many steps and a refined substrate was never utilized.

It is the purpose of this investigation to develop a new method of isolating S. bovis and to evaluate the usability of these improvements in tracing ruminant water pollution (23). These improvements would include a rapid method for detection and enumeration of S. bovis for quantitation of this organism from surface waters as an indicator of bovine fecal pollution.

LITERATURE REVIEW

Classification

Classification of the streptococci dates back to 1906 when Andrews and Horder (2) established seven different groups of streptococci based on morphology, fermentations, and growth characteristics of milk.

Earlier reports came from Thiercelin in 1899 as reported by Deibel (9,36). He used the term enterococcus to define gram-positive diplococci of intestinal origin.

Orla-Jensen (25) made the next contribution to streptococcal classification. He based his scheme on the tolerance of cells to temperature. Classifications based on growth temperatures included: (1) Streptococcus faecium and (2) S. glycerinceus and S. liquefaciens. Orla-Jensen (25) also described a Streptococcus which occurred in bovine feces and appeared to have characteristics differing from other fecal streptococci. He termed this organism Streptococcus bovis.

Sherman (31) made the next contribution to the streptococcal classification in 1937. He proposed four different divisions based on growth at 10 C and 45 C. These groups included the pyogenic (no growth at 10 C or 45 C), the viridans (no growth at 10 C, growth at 45 C), the enterococcus (growth at 10 C and 45 C), and the lactic (growth at 10 C, but not at 45 C). Sherman (32) put S. bovis and

S. equinus in the viridans group because of their inability to grow at 10 C or to grow in 6.5% (w/v) sodium chloride medium.

It was later found that all organisms of the enterococcus group contained Lancefield's group D antigen (31). It was also found that S. bovis and S. equinus contained the group D antigen which could be demonstrated with special techniques of analysis (30). Henceforth, all the organisms containing the group D antigen are referred to as the fecal streptococci (9,15).

Despite their similarities, S. bovis and S. equinus may be separated biochemically. Hartman, Reinbold, and Saraswat (15) reported in 1968 that S. equinus characteristically does not ferment lactose and has no apparent reaction in litmus milk, whereas S. bovis does ferment lactose and produces acid in litmus milk.

Origin and Significance

Streptococcus bovis was probably first isolated from the fecal material of cattle by Thiercelin in 1899 (9,36). It was Orla-Jensen (25) who isolated and identified S. bovis from cattle in 1919. Isolation was also accomplished by Cooper and Ramadam in 1955 (6) when they reported that typical S. faecalis seems to indicate a human origin of contamination and a starch hydrolyzing S. bovis indicates pollution of animal origin. They suggested that this information may be useful in tracing pollution in water, milk, and other foodstuffs.

It is well known that S. bovis is highly amylolytic (10,29). S. equinus, which is closely related to S. bovis, is a weaker starch

hydrolyzer and only under special conditions (10). Starch hydrolysis has in the past been used as a diagnostic test to separate S. bovis from the enterococcus group (27). Because of its occurrence in large numbers in the rumen of cattle and sheep (12,22), S. bovis appears to be an ideal indicator of pollution from ruminant animals (23,35).

Nutrition

Streptococcus bovis has received wide attention because of its ability to hydrolyze starch. Other reasons for its usefulness include its ability to synthesize a dextran from sucrose (8). It is the only member of the genus Streptococcus that can utilize ammonium salts as the sole source of nitrogen (38). Niven, Washburn, and White (24) report that S. bovis can grow well on simplified media.

Niven et al. (24) reported on the nutrition of S. bovis in 1948. They concluded that biotin was required for initiation of growth and that thiamine stimulated growth. The growth response to pantothenic acid and riboflavin was variable depending on the strain. They also concluded that no single amino acid was required for growth and that inorganic sulfur in the form of sodium sulfide could be utilized.

Ten years later, Ford et al. (11) while studying the nutritional requirements of lactic acid bacteria reported that of the 26 strains of S. bovis studied none had an essential requirement for a specific vitamin or growth factor when cultured anaerobically. When S. bovis cultures were grown aerobically, a requirement for nicotinic acid,

biotin, and thiamine was observed in various combinations with the various strains tested.

In a study on the starch hydrolyzing capabilities of various fecal streptococci, it was found that 97% of the S. bovis strains tested gave a reading of (+++) or complete hydrolysis on 0.2% starch plates at 24 hours (27). This contrasts with earlier work done by Koupal (19) in 1970 using a 1.0% starch concentration for detection of S. bovis. It was also found that as the starch concentration was increased time was not a factor in affecting hydrolysis. Thus, it was concluded that 0.2% starch was the optimum concentration for solid media and 24 hours was sufficient time for incubation (27).

Isolation Techniques

Streptococcus bovis is a common inhabitant of the rumen and also a dominant Streptococcus species of the fecal material of ruminants (9,12). A rapid method of isolating S. bovis was not developed until 1970 when Koupal (19) modified an existing method to select for S. bovis. Until this time, it could only be generally assumed that S. bovis was being cultivated by the Most Probable Number (MPN) method in the use of azide dextrose and the confirmatory ethyl violet azide (EVA) broth used for enumeration of fecal streptococci (1). It has been found that EVA inhibits growth of many strains of S. bovis (17). The MPN does not necessarily indicate either animal or human contamination. This is illustrated by the fact that S. faecalis, a common inhabitant of the human alimentary tract, may also be cultivated by the MPN method (3). It may also be noted that

the MPN method does not provide actual enumeration of the bacteria in a water source, but rather an index of the number of bacteria which are most likely to give the obtained results (1).

The membrane filter technique in recent years has become increasingly reliable for actual estimation of bacteria in water, as it provides a direct method for counting bacteria (1). An earlier study of isolation methods for fecal streptococci was done by Cooper and Ramadam in 1955 (6). They reported the tellurite method is 97.6% effective for bovine feces. Results from thallium acetate medium are very similar to the tellurite method. Tetrathionate broth proved to be useful in isolating human fecal streptococci, however, yields from sheep and cattle feces were quite low.

Another commonly used medium is SF medium developed by Hajna and Perry in 1943 (14) which was modified by Hajna in 1951 (13) and became known as BAGG broth.

KF Streptococcus agar medium has been proven useful in isolating fecal streptococci (18). The other most commonly used medium to enumerate fecal streptococci by membrane filtration is M-Enterococcus medium (33).

Five different media used for isolating fecal streptococci were evaluated by Pavlova, Brezenskii, and Litsky in 1972 (26). They conclude that of the five media, KF Streptococcus agar and Pfizer Selective Enterococcus agar yielded the highest counts of fecal streptococci and the lowest counts of nonfecal streptococci. M-Enterococcus agar and azide sorbitol agar had the lowest counts

of fecal streptococci. Thallium acetate agar had somewhat higher counts of fecal streptococci, but also had the lowest selectivity.

This information supports the work of Slanetz and Bartley (34) who in 1964 reported KF Streptococcus agar giving higher counts than M-Enterococcus agar when sewage was tested. It was noted, however, that KF Streptococcus agar was not suitable for isolating fecal streptococci from sea foods or sea water and that KF Streptococcus agar did not support good growth of S. bovis on membrane filters.

Starch Hydrolyzing Capabilities

There are two major groups of starch hydrolyzing enzymes. They are the alpha and beta amylases. Hydrolysis by beta amylase yields a sugar, maltose, and a limited dextrin. Beta amylase removes two glucose units at a time until, in the case of amylopectin, it comes to an alpha 1:6 linkage. Alpha amylase on the other hand degrades starch in a random manner. That is, it attacks the polysaccharide chain at random points throughout the chain.

There are a number of different methods for determining amylase activity. The most commonly used methods employ the following changes in substrate: (1) increase in reducing power, (2) change in iodine color, (3) decrease in viscosity (4), and (4) colorimetrically, employing a soluble starch-dye complex (28).

Investigation into the starch hydrolyzing capabilities of S. bovis is scanty at best. It was generally assumed that S. bovis produced an amylase until 1952 when Hobson and MacPherson (16) studied the enzymes produced by the microorganisms in the rumen of

a sheep. They obtained several strains of streptococcus which they said were probably related to S. bovis. The enzyme produced was an extracellular, constituitive enzyme identified as alpha amylase.

Hobson and MacPherson (16) have accurately described the action of S. bovis alpha amylase and the products formed by its action which are maltose, maltotriose, and glucose. They also provide an accurate description of methods used to obtain these data.

Rinkderknecht et al. (28) innovated a method of employing a dye labelled starch complex to determine alpha amylase activity colorimetrically. This method lends itself to the detection of amylase activity by a bacterial culture. The use of the dye labelled starch complex differs from the iodine method, in that the dye is solubilized and a clearing of the broth medium is observed. Therefore, an amylolytic bacterium such as S. bovis could possibly be detected providing a suitable method of culturing could be developed.

In this study, modifications of the Koupal (19) method of detection of S. bovis and the Rinderknecht (28) method of detecting starch hydrolysis will be used to develop a new method for rapid detection and enumeration of S. bovis from surface waters as an indicator of bovine pollution.

MATERIALS AND METHODS

Source of Cultures

The known *Streptococcus bovis* cultures used throughout this investigation were obtained from the American Type Culture Collection (ATCC). They were designated *S. bovis* 9809 and *S. bovis* 15351. Isolates of *S. bovis* were obtained from the rumen of dairy cattle and from sites on the Big Sioux River.

Media and Culture Preparation

Because of the conflicting reports in the literature concerning which isolation medium gives the best results for fecal streptococci, it was decided that a comparison should be made of the commercially available M-Enterococcus (ME) medium, KF Streptococcus (KF) medium, and the modified media prepared in our laboratory.

Growth curves were done with broth media containing the same constituents as the media used for plate counts, but without the agar, amylose azure, or the 2,3,5-triphenyl tetrazolium chloride (TTC). Growth studies were carried out in 300 ml flasks equipped with 12 mm side arm tubes and clean out ports (No. 5771 Bellco Glassware Co.). Anaerobic conditions were maintained by using a Vacutainer stopper on the clean out port through which an 18 gauge needle can be inserted. The flask was evacuated and flushed three times with nitrogen. The flask was then refilled with an atmosphere of 75% nitrogen and 25% carbon dioxide. A slight negative pressure was left in the flask to allow for fermentation gases.

Cultures were incubated at 37 C on a New Brunswick Model G-25 gyrotory shaker at 225 rpm to achieve good gas exchange with the medium.

Growth curves were determined turbidimetrically using a Bausch and Lomb Spectronic 20 spectrophotometer set at 620 nm. Absorbency was determined at 2 hr intervals. An uninoculated medium was used to adjust the spectrophotometer for 100% transmittance.

Stock Cultures. Preparation of the stock cultures followed the same procedure for all experiments. A 3 mm loop was used to transfer S. bovis cells from maintenance medium (37) to 10 ml of sterile Brain Heart Infusion (BHI) broth. The inoculated BHI was incubated for 18 hr at 37 C in an anaerobic atmosphere of 75% nitrogen and 25% carbon dioxide. A Torbal jar (Model AJ-2, The Torsion Balance Co.) equipped with a pressure gauge and needle valves was used to maintain the desired atmosphere. This procedure produced a cell concentration of approximately 2.1×10^9 organism/ml when compared with McFarland nephelometer barium sulfate standards. The appropriate dilutions of inoculum were made in 99 ml sterile dilution blanks.

Plate Count Medium. All media used for plate counts contained filter sterilized 2,3,5-triphenyl tetrazolium chloride (TTC). TTC was added at a concentration of 1 ml 1.0% TTC per 100 ml of medium. One percent agar and 0.2% amylose azure were also used in the plate count medium. All media used for growth studies and plate count procedures were autoclaved at 121 C and 15 psi for 10 min.

Iodine Concentration. Preliminary investigation into the use of Koupal's method of detecting S. bovis involved the use of several different iodine concentrations. Iodine solutions of 1.0, 0.5, 0.25, and 0.1% and Gram's iodine were prepared with twice the amounts of potassium iodide as the amount of iodine per 100 ml distilled water.

Preparation of Starch-Dye Complex .

In 1967, Rinderknecht et al. (28) reported on a new method for detecting starch hydrolysis employing a dye labelled starch as the substrate. The dye used for labelling was Remazolbrilliant Blue R (RBB) (Calbiochem #55435, 1972). The labelled starch molecule is given in the following formula:

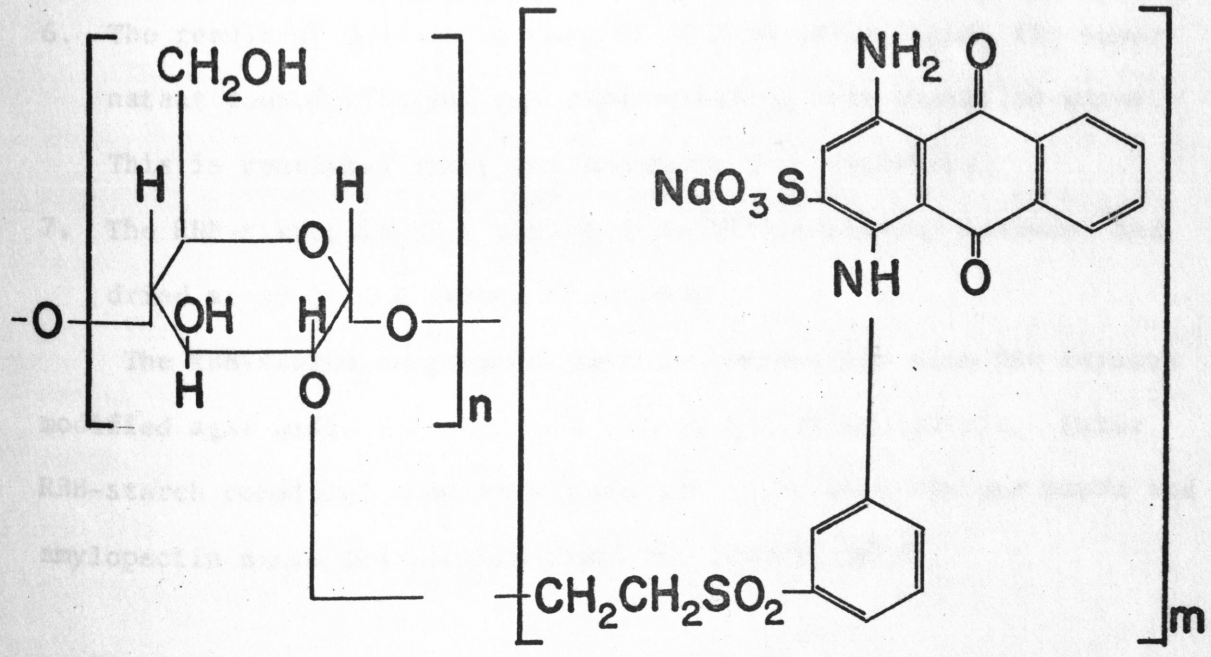

Figure 1. STARCH REMAZOLBRILLIANT BLUE

The starch was labelled in the following manner:

1. Five gm of Soluble Starch Merck (Merck and Co. 09903) are added to 200 ml of distilled water, mixed, and heated to 50 C with constant stirring.
2. Two and one half gm of RBB are added to 200 ml of distilled water and heated to 50 C with constant stirring.
3. The RBB solution is added to the starch solution with constant stirring.
4. Four gm of sodium sulfite are added to the RBB-starch solution in small portions over a period of an hr. Stirring is continued at 50 C.
5. After 1 hr, the RBB-starch solution is treated with two gm of Na_3PO_4 in 20 ml distilled water. Heating at 50 C and stirring is continued for 7 hr.
6. The resultant dye-starch complex is then centrifuged, the supernatant poured off, and the complex washed with distilled water. This is continued until the supernatant is colorless.
7. The RBB-starch is then washed twice with anhydrous methanol and dried at 50 C in a vacuum dessicator.

The RBB-starch complex was used in conjunction with the various modified agar media for the detection of starch hydrolysis. Other RBB-starch complexes used throughout the study were amylose azure and amylopectin azure (Calbiochem 17266 and 172678, 1972).

Isolation Media

The media used in this investigation were not commercially available with the exception of M-Enterococcus (ME) medium and KF Streptococcus (KF) medium. In an endeavor to find an optimum nutritional substrate for S. bovis, plate counts and growth curves were done with various modifications of these two media.

Koupal (19) described a modification of the ME Medium which had a reduced sodium azide content and raffinose substituted for dextrose. The medium was described in the following manner:

Modified M-Enterococcus Medium

Bacto tryptose	20.0 g
Yeast extract	5.0 g
Dipotassium phosphate	4.0 g
Raffinose	2.0 g
Sodium azide	0.2 g
Bacto agar	10.0 g
TTC	10.0 ml
Water	1000.0 ml

The raffinose substitution was designed to make the medium more selective for S. bovis and the reduced sodium azide to decrease inhibition of S. bovis and still inhibit the growth of gram-negative bacteria and spore formers.

Because KF medium had been indicated as a better isolation medium for streptococci (26), the suitability of this medium for isolating S. bovis was studied. The first modification of KF medium was the reduction of the sodium azide content and elimination of the Bacto-brom cresol purple. This was designated as KF-1 and is given in the following formula:

KF-1 Medium

Bacto proteose peptone No. 3	10.0 g
Yeast extract	10.0 g
Sodium chloride	5.0 g
Sodium glycerophosphate	10.0 g
Maltose	20.0 g
Lactose	1.0 g
Amylose azure	2.0 g
Sodium azide	0.2 g
Bacto agar	10.0 g
TTC	10.0 ml
Water	1000.0 ml

The toxicity of the chloride ion and its effect on the growth of S. bovis was studied. A growth medium containing a reduced amount of NaCl was prepared. It was designated as KF-2 and had the same constituents of KF-1 with the exception of the NaCl content which was reduced from 5 gm to 1 gm per liter.

The substitution of raffinose for dextrose aided in the selectivity of modified ME medium because S. faecalis could not ferment raffinose and S. bovis can ferment this sugar. The same reasoning applies to the substitution of raffinose for maltose in KF medium. This substitution was made and the resultant medium was designated as KF-3. KF-3 is described in the following formula:

KF-3 Medium

Bacto proteose peptone No. 3	10.0 g
Yeast extract	10.0 g
Sodium chloride	5.0 g
Sodium glycerophosphate	10.0 g
Raffinose	20.0 g
Lactose	1.0 g
Amylose azure	2.0 g
Sodium azide	0.2 g
Bacto agar	10.0 g
TTC	10.0 ml
Water	1000.0 ml

A combination of the modifications described by KF-1, KF-2, and KF-3 was designated as KF-4 and is given in the following formula:

KF-4 Medium

Bacto proteose peptone No. 3	10.0 g
Yeast extract	10.0 g
Sodium chloride	1.0 g
Sodium glycerophosphate	10.0 g
Raffinose	20.0 g
Lactose	1.0 g
Amylose azure	2.0 g
Sodium azide	0.2 g
Bacto agar	10.0 g
TTC	10.0 ml
Water	1000.0 ml

Koupal (19) had shown that raffinose acted as a selective agent in ME medium. Growth studies were done with varied amounts of raffinose to determine an optimum level of this sugar for growth of S. bovis. KF modification with one half the amount of raffinose (10 g) was designated as KF-5. KF modification with one fourth the amount of raffinose (5 g) was designated as KF-6. KF-7 medium did not contain raffinose and served as a control.

Plating Technique

Because a different procedure of plating and incubating a membrane filter was used, a detailed description of the new technique developed for this study will be described.

The standard procedure for use of membrane filters involves layering the filter on the agar medium after the sample has been filtered. The procedure developed in the course of this investigation takes place in the following steps:

1. The membrane filter apparatus is initially sterilized by autoclaving for 15 min at 121 C and 15 psi.
2. Two tenths of a milliliter of sterile distilled water is placed in the bottom of half of sterile plastic 60 mm petri dishes (Falcon Plastics #1007 Petri dish).
3. The desired volume of water sample is filtered and the filter aseptically transferred to the moistened petri plate. The moisture will diffuse throughout the filter providing an adhesive force between the filter and the bottom of the petri plate.
4. The agar medium to be used is cooled to 55 C. Sterile wide mouth pipettes which had been pretempered at 55 C are used to layer 3.0 ml of medium on the filter.
5. The agar medium is allowed to solidify and the plates which are not inverted are incubated at 37 C in an atmosphere of 75% nitrogen and 25% carbon dioxide. A Torbal jar is used to maintain the desired atmosphere.
6. The plates are examined at 24 and 48 hr. Colonies are counted with the aid of an American Optical binocular stereoscope at a magnification of 30X.

Identification Procedures

Unknown isolates from rumen and water samples were identified by selected biochemical tests, as set forth by Sherman (32) and Hartman et al. (15). These tests included growth at 10 C, growth at 45 C, bile sensitivity, salt tolerance, starch hydrolysis, and lactose fermentation.

All isolates were picked from the colonies with the aid of an American Optical binocular stereoscope at a magnification of 30X. Photographs were taken to demonstrate zones of starch hydrolysis. A Pentax Spotmatic camera (Honeywell) with a 1.5X closeup lens was placed on a tripod and illumination provided by four Westinghouse 75 watt reflector spotlamps.

The isolate was picked from a zone of hydrolysis with a needle and transferred to a tube of BHI broth and incubated at 37 C for 24 hr. After a pure culture was confirmed, the isolate was transferred to maintenance medium as described by Tiede (37).

Maintenance Medium

Bacto tryptose	20.0 g
Yeast extract	5.0 g
Dextrose	1.0 g
Dipotassium phosphate	4.0 g
Bacto agar	10.0 g
Water	1000.0 ml

Biochemical tests were performed on cultures confirmed as streptococci. Biochemical media were inoculated using a 3 mm loop and incubated at 37 C. The following procedures and media were used for identification of streptococcus cultures:

1. Growth at 10 C. Pretempered tubes of BHI were incubated for 5 days and examined for turbidity.
2. Growth at 45 C. Pretempered tubes containing BHI were incubated for 48 hr and examined for turbidity.
3. Bile sensitivity. Bile medium containing 40 ml of 10% oxgall and 60 ml BHI broth was inoculated and examined for growth at 48 hr.

4. Salt tolerance. A 6.5% sodium chloride and BHI broth was inoculated and incubated for 48 hr. Growth indicated a tolerance of salt.
5. Starch hydrolysis. BHI broth contained 0.1% soluble starch. After 24 hr of incubation, a drop of inoculated starch medium was added to a drop of Gram's iodine. The absence of a blue color indicated starch hydrolysis.
6. Lactose fermentation. One percent lactose with a methyl red indicator was incubated for 48 hr. A yellow color indicated acid production from lactose.

Bacteria which grew at 45 C, but not at 10 C, grew in bile, but not in salt, hydrolyzed starch, and fermented lactose were considered to be S. bovis.

RESULTS AND DISCUSSION

The purpose of this investigation, as stated in the introduction, was carried out in two phases of study. The first phase involved the development of a suitable growth medium for Streptococcus bovis using various modifications of the available commercial media. The second phase was to find a rapid and improved method of detecting the starch hydrolysis by S. bovis.

Growth Medium Development

As an indicator of starch hydrolysis Koupal (19) had used a 1% starch agar overlay technique and visualized the zones with a 1% iodine solution. More recent studies indicate the use of 0.2% soluble starch concentration using agar medium is best (26). Starting from this premise a method was worked out which employed a 0.2% dye labelled soluble starch. This greatly simplified the detection of S. bovis.

Further study of Koupal's (19) method revealed some basic problems. First, the nutrient medium employed was M-Enterococcus (ME) medium (33). ME medium was developed for the isolation of enterococci and did not isolate S. bovis. The sodium azide level of ME medium is 0.04%. Koupal (19) reduced the sodium azide level to 0.02% and also substituted raffinose for dextrose. A review of the literature showed KF Streptococcus (KF) medium to be more selective for enterococci and also to provide higher counts of S. bovis from fecal samples (26). The S. bovis counts may be questionable, however, in

that KF medium also contains 0.04% sodium azide which has been shown to be inhibitory to S. bovis (19).

Because of conflicting evidence in the literature concerning the best isolation medium for S. bovis, investigation into a suitable substrate for S. bovis was given priority. Various modifications of KF and ME media were used for growth determinations of S. bovis.

Comparison of M-Enterococcus and Modified M-Enterococcus Media

Contrary to previous work (19), growth studies done in broth culture indicate modified ME is not as good a growth medium for S. bovis as is ME (Fig. 2). When these two media were used in conjunction with the membrane filter technique, it was found that modified ME provided slightly better results than ME (Table 1). It is interesting to note that ME has twice the sodium azide content as modified ME and yet allowed more than twice the amount of growth as modified ME in broth culture. This suggests that the sodium azide is not as critical a factor in growth of S. bovis as has been speculated. The only other difference in the two media is the sugars contained in them. ME contains dextrose as the carbon source and modified ME contains raffinose as the carbon source. Koupal (19) reported that S. bovis could utilize these two sugars almost equally. Growth curves done in duplicate in our laboratory do not confirm this. On the contrary, raffinose is unsatisfactory in the growth of S. bovis. This is probably due to raffinose being a

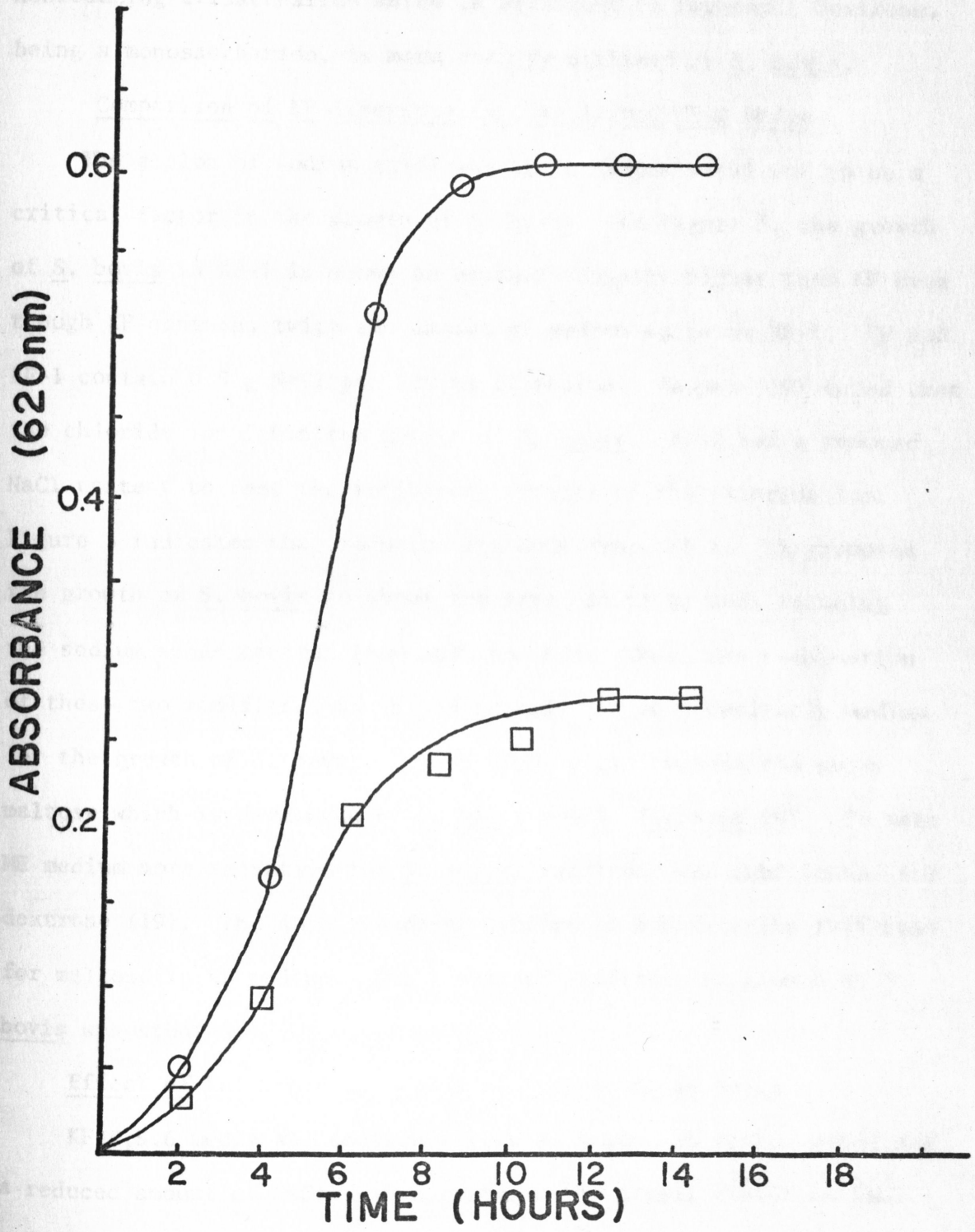

Figure 2. Growth curves of Streptococcus bovis in M-Enterococcus (ME) medium and modified M-Enterococcus (MME) medium. Symbols: ○, ME; □, MME.

nonreducing trisaccharide which is difficult to ferment. Dextrose, being a monosaccharide, is more readily utilized by S. bovis.

Comparison of KF Streptococcus, KF-1, and KF-2 Media

The action of sodium azide was again demonstrated not to be a critical factor in the growth of S. bovis. In Figure 3, the growth of S. bovis in KF-1 is shown to be only slightly higher than KF even though KF contains twice the amount of sodium azide as KF-1. KF and KF-1 contain 0.5 g NaCl per 100 ml of medium. Koupal (19) found that the chloride ion inhibited growth of S. bovis. KF-2 had a reduced NaCl content to test the inhibitory effects of the chloride ion. Figure 3 indicates that reducing the NaCl from .5% to .1% promotes the growth of S. bovis to about the same extent as does reducing the sodium azide content from .04% to .02%. Thus, the combination of these two modifications should provide the most desirable medium for the growth of S. bovis. These three media contain the sugar maltose which is fermented by S. bovis and S. faecalis (5). To make ME medium more selective for S. bovis, raffinose was substituted for dextrose (19). The same reasoning applies to substituting raffinose for maltose in KF medium. The effect of raffinose on growth of S. bovis was studied.

Effect of Raffinose on Growth of Streptococcus Bovis

KF-4,5,6 media all contain a reduced amount of sodium azide and a reduced amount of NaCl. KF-3 contains the normal amount of NaCl and a reduced amount of sodium azide. The growth curve of S. bovis in KF-4 (Fig. 4) indicates that raffinose in place of maltose limits

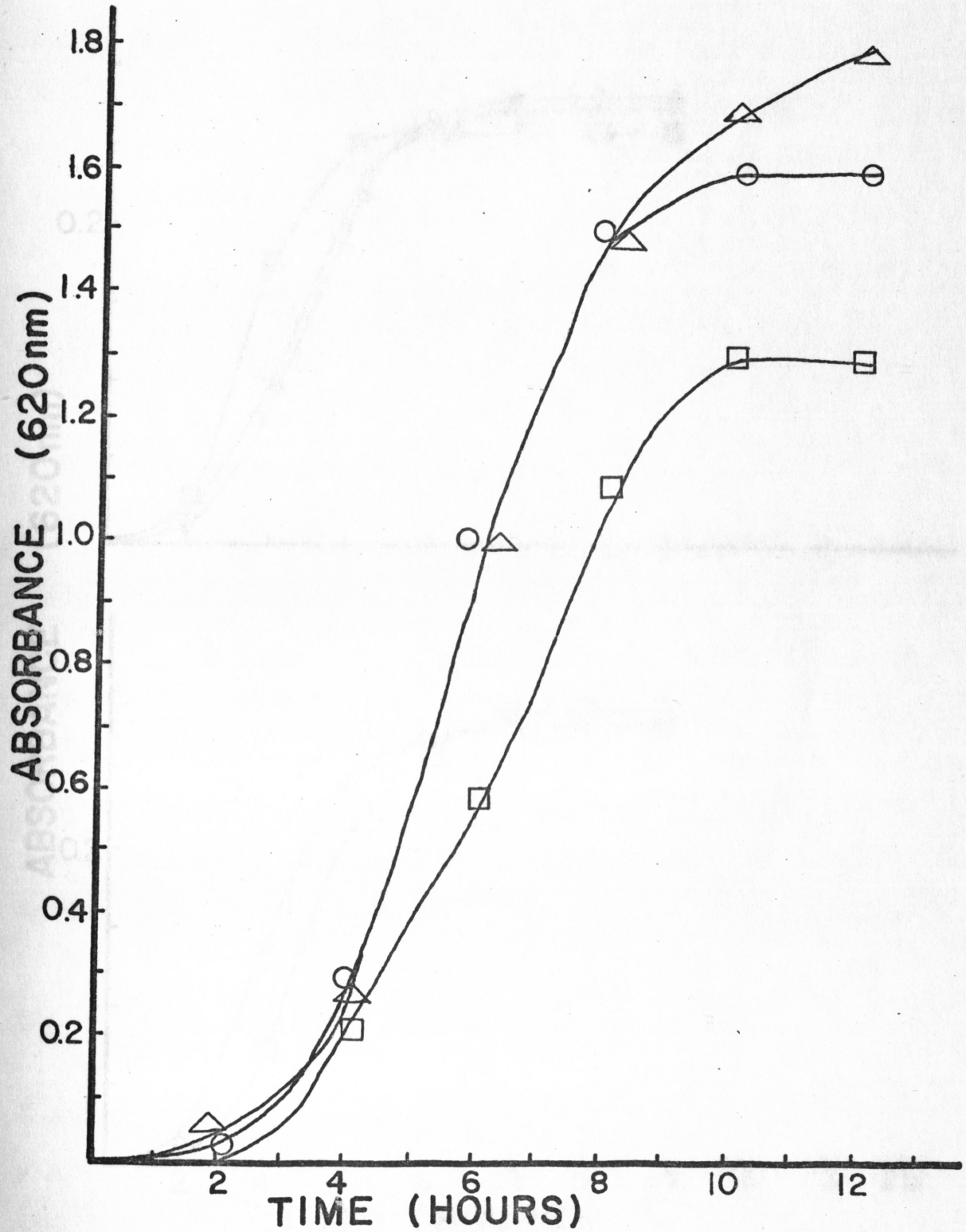

Figure 3. Growth curves of Streptococcus bovis in KF Streptococcus (KF) medium and two modifications of KF. Symbols: □, KF; ○, KF-1; △, KF-2.

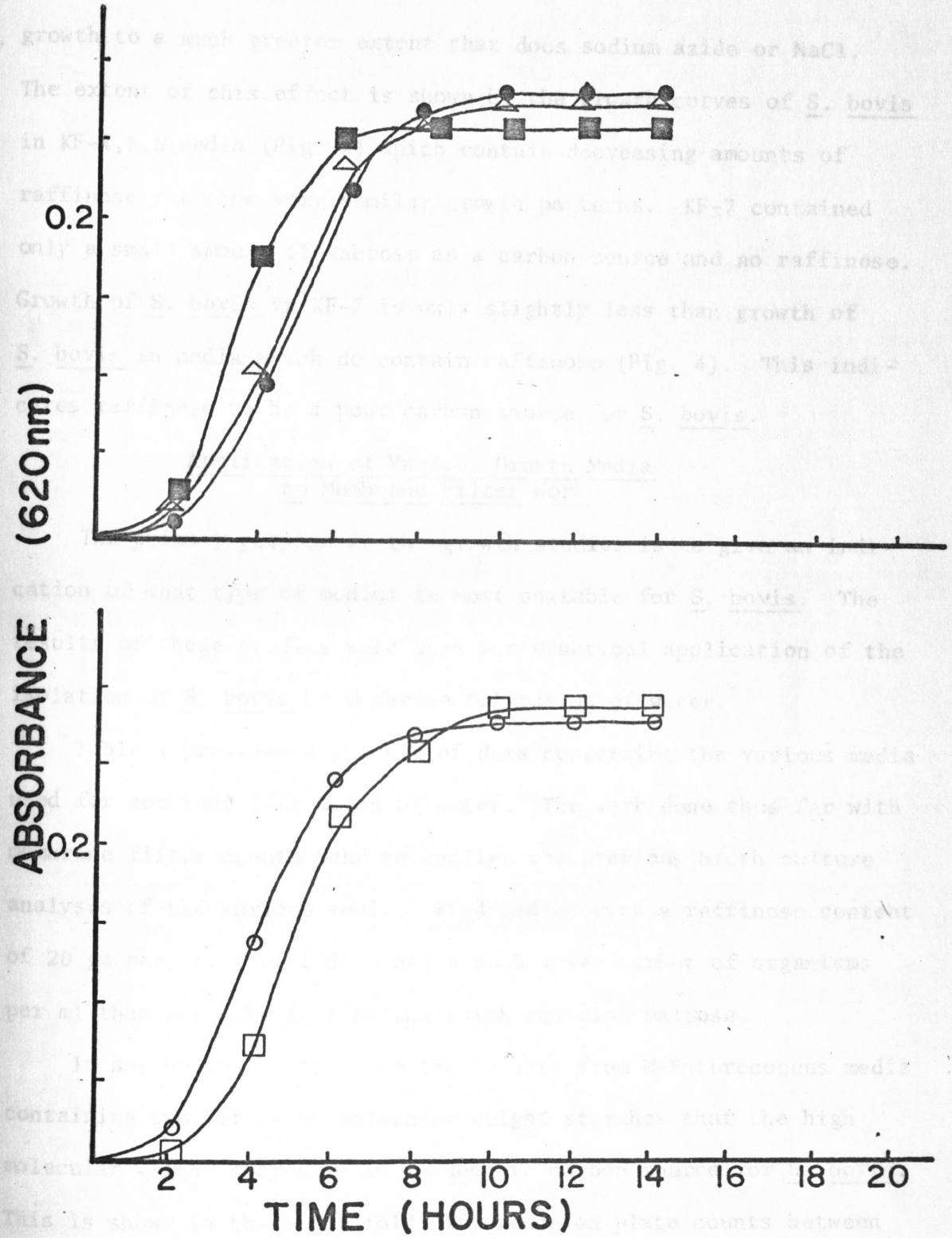

Figure 4. Growth curves of Streptococcus bovis in various modifications of KF Streptococcus medium. Symbols: □, KF-3; ○, KF-4; △, KF-5; ●, KF-6; ■, KF-7.

growth to a much greater extent that does sodium azide or NaCl. The extent of this effect is shown by the growth curves of S. bovis in KF-4,5,6 media (Fig. 4) which contain decreasing amounts of raffinose yet show very similar growth patterns. KF-7 contained only a small amount of lactose as a carbon source and no raffinose. Growth of S. bovis in KF-7 is only slightly less than growth of S. bovis in media which do contain raffinose (Fig. 4). This indicates raffinose to be a poor carbon source for S. bovis.

Application of Various Growth Media to Membrane Filter Work

The primary purpose of the growth studies is to give an indication of what type of medium is most suitable for S. bovis. The results of these studies were used for practical application of the isolation of S. bovis by membrane filtration of water.

Table 1 provides a summary of data concerning the various media used for membrane filtration of water. The work done thus far with membrane filter counts tend to confirm the previous broth culture analyses of the various media. KF-4 medium with a raffinose content of 20 gm per liter of medium has a much lower number of organisms per ml than does the KF-1 medium which contains maltose.

It may be speculated from the results from M-Enterococcus media containing two different molecular weight starches that the high molecular weight starch is an inadequate carbon source for S. bovis. This is shown in the eight fold difference on plate counts between the starch-agar overlay and the amylopectin-azure complex.

Table 1. Summary of plate counts of Streptococcus bovis on various media. Incubation temperature, 37 C; atmosphere, 75% N_2, 25% CO_2; incubation period, 24 hr with exception of modified M-Enterococcus which was 48 hr. [2]INA = Information Not Available.

Medium	Sample Source	Type of Hydrolysis Indicator	Sugar (%)	NaCl Concentration (%)	No. of Bacteria/ml X 10^5
M-Enterococcus	Known S. bovis	Starch-agar overlay	Dextrose 0.2	0	40
M-Enterococcus	Known S. bovis	Amylopectin azure	Dextrose 0.2	0	5
Modified M-Enterococcus	Known S. bovis	Starch-agar overlay	Raffinose 0.2	0	42
KF Streptococcus	Known S. bovis	Starch-agar overlay	Maltose 2.0	0.5	23
KF-1	Known S. bovis	Amylose azure	Maltose 2.0	0.5	180
KF-1	Rumen Fluid	Amylose azure	Maltose 2.0	0.5	4
KF-2	Rumen Fluid	Amylose azure	Maltose 2.0	0.1	7
KF-3	Rumen Fluid	Amylose azure	Raffinose 2.0	0.5	INA
KF-4	Known S. bovis	Merck azure	Raffinose 2.0	0.1	2.4

The effect of the reduced NaCl content is confirmed by the plate counts with KF-1 and KF-2 media. KF-2 contains the reduced amount of NaCl and shows a slight increase in number of organisms per ml.

Comparison of Iodine Concentrations

In the investigation of methods of visualizing zones of hydrolysis, it was decided that visualization with iodine was an inadequate and temporary method. In an effort to improve visualization or hydrolysis zones, an investigation was undertaken into the effects of varying iodine concentrations.

Previous methods employed the use of a 1.0% iodine solution (19). Although the 1.0% iodine permitted visualization of large zones of hydrolysis quickly and distinctly, it was found that the smaller zones of incomplete hydrolysis were obliterated by the dark blue starch-iodine complex. With this in mind, different concentrations of iodine were tested. The concentrations used were 1.0, 0.5, 0.25, and 0.1% and Gram's iodine. The comparative results of this investigation are in Table 2.

Table 2. Comparison of different iodine concentrations used for visualization of starch hydrolysis zones by Streptococcus bovis.*

Iodine Concentrations	Time of Visualization	Zone Sharpness	Length of Zone Visualization
1.0%	++++	++++	++++
0.5%	++++	++++	++++
Gram's I_2	++	+++	++
0.25%	++	+++	++
0.1%	+	+	+

*A rating of (+) is the lowest rating and (++++) the highest rating.

The 0.5% solution of iodine was as good an indicator as the 1.0% iodine. However, since, there is less iodine giving the same reaction it is consequently more efficient. Also, the starch-iodine complex was not as dark as the 1.0% giving a better indication of incompletely hydrolyzed zones.

The Gram's iodine and the 0.25% gave the same moderately sharp zones. Using these solutions, development of the color took longer than the 0.5% and the zones once visible faded much quicker. The 0.1% iodine concentration was unacceptable.

Starch-Dye Complex

Koupal's method of visualizing zones of starch hydrolysis is a relatively difficult technique. Also, once the plates are flooded with iodine, growth studies can no longer be continued with the colonies on them. Fading of the iodine color also presents a problem in that it is impossible to store a plate for further reference to a particular colony.

A method was sought in which the zones of hydrolysis could be seen without an extra visualization step and one which would not be toxic to the bacteria being cultivated.

Trial runs with Remazolbrilliant Blue R (RBB) and alpha amylase showed hydrolysis zones developed in a crude starch-dye complex in agar. It appeared that this system could be used to detect starch hydrolysis by S. bovis.

The three starch-dye complexes described in the Materials and Methods section were utilized in varying concentrations. Starch-dye concentrations used were 0.2% and 0.3%. It was the purpose of an increased starch-dye concentration to obtain more zone sharpness. However, it was obvious from the results with the 0.3% concentration that zone sharpness was not going to improve with higher concentrations. The only variations shown by starch concentration were time of visualization and zone size. Table 3 presents starch-dye complexes with zone size, visualization time and zone sharpness.

Table 3. Zones produced by Streptococcus bovis with various starch-dye complexes evaluated according to zone size, time of visualization, and zone sharpness.

Starch-dye Complex	Starch-dye Concentration	Zone Size	Time Visualization	Zone Sharpness*
Amylopectin azure	0.2%	1-2 mm	48 hr	+++
Amylose azure	0.2%	4-6 mm	24 hr	++
Merck azure	0.2%	1-3 mm	24 hr	+++
Amylose azure	0.3%	1-2 mm	48 hr	++

*(+) indicates lowest rating; (++++) indicates highest rating

The limiting factor concerning starch hydrolysis is the molecular weight of the starch. The amylopectin being of high molecular weight cannot be broken down as readily as a low molecular weight starch, and thus took longer to develop a zone as shown in Table 2. Zone sharpness with amylopectin-azure was better than amylose-azure.

Amylose-azure is the substrate most susceptible to the enzyme alpha amylase as demonstrated by the larger zones and the shorter time of zone development. The zones in Merck-azure were not as large as the zones demonstrated in amylose-azure, but rather gave an intermediate size zone between amylopectin-azure and amylose-azure. Typical zones produced by S. bovis in a Remazolbrilliant Blue R-amylose substrate are shown in Figure 5.

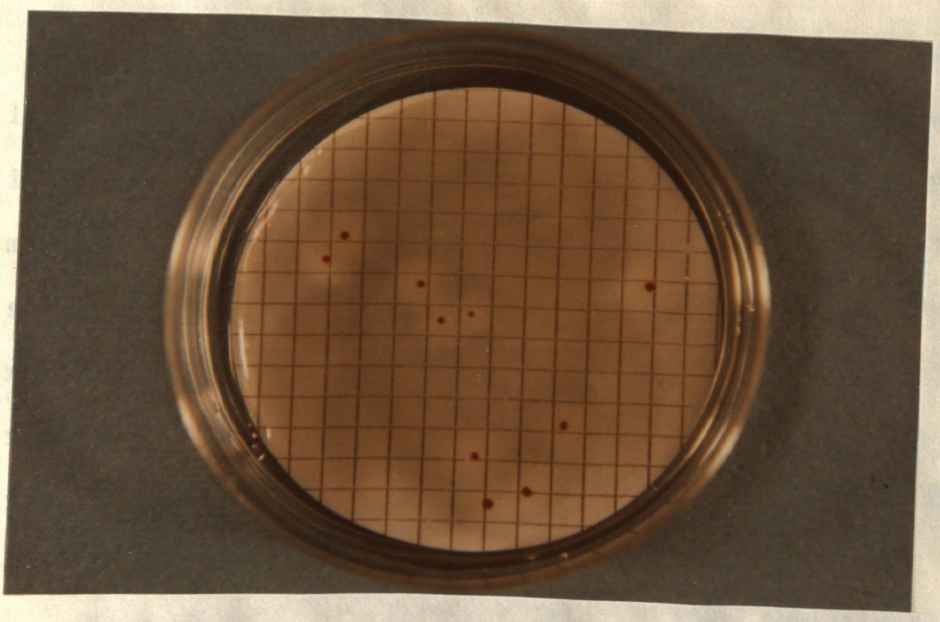

Figure 5. Zones of starch hydrolysis by S. bovis in a Remazolbrilliant Blue R-amylose substrate.

Amylopectin-azure and amylose-azure were commerically prepared. The Merck-azure complex prepared in the laboratory is the substrate of choice because it is cheaper to prepare the complex from available laboratory materials and because it yields zones as rapidly as the amylose-azure. It should be noted that the method of labelling the starch with RBB may be used for any soluble or insoluble starch (28).

General Observations

The essential point of this thesis is not simply detecting another indicator of pollution, but rather the development of a method to rapidly detect S. bovis which is indicative of bovine or ovine contamination in water.

In light of the data of this thesis, the best method for isolating S. bovis should include using the plating technique described in the Materials and Methods section and modified KF Streptococcus medium with an amylose-azure indicator. Modifications of the medium should include reducing the sodium azide content from 0.04% to 0.02%, reducing the NaCl content from 0.5% to 0.1% and substituting raffinose for maltose in equal proportions. The plates should be incubated at 37 C in an atmosphere of 75% nitrogen and 25% carbon dioxide for 24 hr.

Even though the primary purpose of this investigation has been fulfilled with the development of a new method for rapid detection of S. bovis, there are several possibilities for further study.

It should be understood by the reader that the research reported in this thesis is a part of ongoing research in our laboratory. Work being done at the present time includes comparing S. bovis alpha amylase with an alpha amylase of known activity from Bacillus subtilus. It is hoped that alpha amylase activity will eventually be used to characterize S. bovis by its correlation with zone size in a substrate containing the Remazolbrilliant Blue-starch complex. This would greatly aid in detection and enumeration of S. bovis and may eliminate the need for biochemical tests with an isolate in order to identify it.

Very simply it would answer the question, "Would a zone diameter of 'x' mm identify the colony as being S. bovis?" It may be that this would be a distinguishing factor between S. bovis and the atypical Streptococcus faecalis reported by Koupal (19) which can also hydrolyze starch. Enzyme analysis and the use of other fecal streptococci in conjunction with S. bovis may give more information pertaining to the selectivity needed for enumeration of S. bovis from water.

CONCLUSIONS

1. A modified KF Streptococcus medium containing 0.02% sodium azide, 0.1% NaCl, and raffinose will select for Streptococcus bovis.
2. A dye-starch complex using Remazolbrilliant Blue R and a low molecular weight starch such as amylose can be used to detect starch hydrolysis by S. bovis.
3. The use of raffinose in place of maltose or dextrose does not promote the good growth of S. bovis.
4. An NaCl concentration of 0.1% in KF Streptococcus medium is more suited for growth of S. bovis than a concentration of 0.5%.
5. An incubation period of 24 hr is sufficient time to obtain detectable zones of starch hydrolysis by S. bovis under the conditions tested.
6. An iodine concentration of 0.5% is better suited for Koupal's method of detecting starch hydrolysis zones than the 1.0% concentration.
7. Modified M-Enterococcus medium does not provide better growth of S. bovis in broth culture, but allows better selection of S. bovis than M-Enterococcus medium in membrane filter work.

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