A classification according to the mammalian sources of coliform organisms isolated from a remote mountain stream
by Gareth Allen Wilson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
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Abstract:
Our mountain watersheds are undergoing expanding recreational use. Thp waters of these streams have been found at times to carry heavy fecal coliform loads. Concomitant recognition of the presence of enteric pathogens make closer examination of these areas for recreational use essential. Indications are that the current techniques for identifying indicator organisms of fecal pollution may be inadequate. It may also be true that fecal organisms may not be the best pollution indicators for the area in question.

A large number of coliform strains isolated from the water of a mountain stream, droppings of big game from the area it drains, and isolates from human sources were examined to determine if differences between these could be identified. The organisms were first compared physiologically and by numerical taxonomy. Gas chromatography using a Beckman GC-2 instrument with a column containing Ressoflex-LAC-l-R-296 was then performed using simple media to determine what bacterial signatures could be identified and to determine the level of metabolic products formed.

Physiological groupings could be determined to some extent for strains identified primarily as fecal coliforms. It was difficult to identify many of the isolates, particularly of IMViC type -+-+, with named strains.

Numerical analysis revealed similarity values of similar levels for coliforms from all sources. Only in deer could occasional strains be found that appeared to be unique.

No specific bacterial signatures could be obtained from gas chromatography but statistical analysis of the principle products, acetic and lactic acids, revealed that the organisms obtained from the water were comparable to those from elk (.90P>.50). Coliform strains of human origin were found to be generally distinct from these (P=<.01). The data from the limited number of coliform isolates from deer indicate that these too may be unique.

A CLASSIFICATION ACCORDING TO THE MAMMALIAN SOURCES OF COLIFORM ORGANISMS ISOLATED FROM A REMOTE MOUNTAIN STREAM

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GARETH ALLEN WILSON

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Approved:

William G. Walter
Head, Major Department

John W. Suttie
Chairman, Examining Committee

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

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INTRODUCTION

> "To be effective as an indicator bacterium, the indicator must: 1) be present whenever pathogens are present, 2) be present only when the presence of pathogens is an imminent danger, 3) occur in much greater numbers than the pathogen, 4) be more resistant to disinfection and the aqueous environment than the pathogen, 5) grow readily on simple media, 6) yield characteristic and simple reactions ...." Bonde (9).

Until the beginning of the last decade the primary emphasis of sanitary microbiologists was the enumeration of coliform bacteria in public water supplies and what this might mean in terms of potential danger to the community. For this purpose coliforms are defined as "all the aerobic and facultatively anaerobic, gram negative, non spore forming rod shaped bacteria which ferment lactose with gas within 48 hours at 35°C" (2). The water quality standards established by the United States Public Health Service still do not permit the presence of coliforms of any type in water designated for domestic use. The appearance of any microorganisms classed as coliforms is considered evidence of fecal pollution (2).

There are no such restrictions applied to water designated for recreational use. Research (8, 25, 53, 64, 65, 75, 109) has shown that the water of high mountain watersheds are not the ultimate examples of pristine purity they were long considered to be. In fact, high quality water has become difficult to define. Not only are the streams of remote areas found to carry remarkably high coliform

loadings (8, 32, 47, 53, 64, 75, 109), but from bacterial cultures of the water we know that there are enteric pathogens present much of the time (18, 25, 32, 52). Often these areas are used as summer livestock range and certainly the presence of domestic animals can account for many of the Salmonella, Arizona, pathogenic Escherichia coli, Leptospira, and enteric viruses reported. Other investigations (3, 20, 59, 65, 92, 111) have shown that these organisms are also carried endemically in the wild small mammals, bear, and deer populations. Although fish found in remote streams have been implicated as the possible distribution centers of microorganisms, they do not normally carry coliform organisms in their intestinal tract (36, 45, 50).

Most coliforms isolated from water have been found to be of little sanitary significance, being normally found in soil and on vegetation (32, 33, 37, 39, 75). Still other types are associated with the feces of warm blooded animals (34, 70). The latter are the types normally associated with the presence of pathogens. Most recent research has dealt with methods for distinguishing between the two types of coliforms. While some degree of success has been achieved in distinguishing the organisms of no sanitary importance from those that are, no good method yet exists for differentiating organisms obtained from human and other mammalian fecal sources (33, 34, 47).

In view of the numerous occasions in which deer, smaller wild animals, and domestic livestock have been found to be carriers of enteric diseases (25, 65), a means of distinguishing between the coliform organisms originating from their respective body discharges may be superfluous. Rather, any water identified as containing fecal contamination should be considered to be polluted water (32). On the other hand, there is a shortage in the literature to date of references implicating big game such as elk and moose as carriers of enteric disease. If this is true, and since it is difficult to cure many streams of their fecal pollution, being able to distinguish the type and source of pollution would simplify a decision concerning the suitability of a body of water for use. Development of techniques for isolation and identification of coliforms according to the species of animal from which they derive would also make epidemiological studies simpler and facilitate removing a source of pollution.

A review of the coliform types and the means by which those of sanitary significance are separated from those that are not is in order. Also to be included is a short survey of other organisms associated with fecal material and why these are not, as yet, considered to be of use in sanitary procedures.

Identification of Coliform Bacteria

Coliforms have already been described as gram negative, lactose fermenting (generally), aerobic or facultatively anaerobic rods. For sanitary purposes, they have traditionally been identified as a group by using a battery of tests known as the presumptive (ability to produce gas in lactose broth at 35°C in 48 hours), confirmed (can produce gas in brilliant green bile broth in 48 hours), and the completed tests (growth in selective media and morphological identification) as outlined in Standard Methods for the Examination of Water and Wastewater (2).

To this battery has been added another technique, the membrane filter. This procedure allows the identification of colonies demonstrating a metallic sheen over all or any part of its surface as coliforms while at the same time allowing an estimate of the numbers present to be made (2).

For more selective identification there are many suggested biochemical procedures in the literature taking advantage of the action of the organism on peptones, sugars, and alcohols, as well as their ability to grow at elevated temperatures (19, 22, 24, 34, 36, 66, 96). From this large group of tests, two have been selected as giving the most information in the shortest possible time, for routine use in identifying fecal coliforms (2). The best of these, the Eijkman fecal

coliform test, is based on the finding by Eijkman (51) that members of the Escherichia group could cause the production of gas in a glucose medium at 46°C whereas organisms of the Enterobacter (Aerobacter) group could not. The test was modified by Hajna and Perry (50, 86) to include bile, a buffering system, and the substitution of lactose for glucose in the medium. The medium with these modifications constitutes the EC (E. coli) broth recommended for performance of the test (2, 50, 86). A final modification (35, 37) lowered the temperature of incubation to 44.5°C ± 0.2°C for 24 hours in a water bath. This is the procedure now recommended for routine use in distinguishing fecal from non-fecal coliforms (2). The principle of the test takes advantage of the fact that fecal coliforms produce the enzyme, formic hydrogenlyase, at 44.5°C reducing formic acid formed during lactose fermentation to hydrogen gas and carbon dioxide (19).

Also recommended, but much less specific in identifying the fecal origin of the coliforms, is the indol, methyl red, Voges-Proskeuer, and Simmons' citrate (with the mnemonic IMViC) group of tests. Of these tests, the methyl red test is of greatest value (32). Data demonstrating its usefulness in identifying fecal coliforms will be cited as a matter of interest. The IMViC results are expressed in a formula of plus (+) and minus (-) signs to describe the difference

between organisms. By use of the formula sixteen strains, ranging from ++++ through ++-- and --++ to ----, all of which have been found, are possible. The IMViC test series can be used separately or in conjunction with the EC test in an attempt to identify sources of fecal pollution. For this purpose the most important types will be described.

The groups ++-- (E. coli var. I), -+-- (E. coli var. II), and +--- (uncommon) are considered to be Escherichia species (2) and are the most frequent indicators of fecal pollution since they are found to occur universally in the gut of nearly all warm blooded animals in all sections of the world (2, 10, 33, 34, 103). These strains contribute the majority of positive fecal coliform (EC) tests, roughly 90% depending on the author, and are referred to as fecal coliforms (34, 39, 70). It must be noted that type -+-- is EC positive far less frequently than ++--, 22% and 93% respectively, again depending on the author (34, 70). A common complaint concerning the use of the ++-- type as fecal indicator is its relatively lower survival rate in an aquatic environment (30). Among the pathogens, -+-- is also the type attributed to Shigella sp. (10, 22) and accounts for the Shigella-like organisms reported in pollution surveys (47).

Types -+-+ and ++-+ are considered to be Escherichia (Citrobacter) freundii types I and II respectively (2), and are principally

of non-fecal origin (10). Strains with the formula --++ (Enterobacter aerogenes var. I), --+- (E. aerogenes var. II), and ---+ constitute the Enterobacter group (2). For reasons to be discussed the enterobacters cause more confusion in the interpretation of fecal pollution than does any other type.

The widest diversification of IMViC types is found in human stools; eleven have been found with the following six predominating: ++--, -+--, --++, -+-+, ++-+, and +++- (34). The ++-- group occurs 87.5% of the time with --++ next most common at 5.4%. Mishra et al. (70) conducted a survey in India and reported that 52.4% of the Enterobacter group --++ responded positively to the EC test. In all, the EC procedure correctly identified 96.4% of all colonies isolated as being of fecal origin. E. coli var. I (++--) type, of course, gives maximum correlation with EC positive tests (34, 38, 70). It is interesting that the methyl red test successfully identified 94.7% of all fecal strains (33).

From the droppings of livestock and other animals, Geldreich et al. (33, 34) isolated six coliform types with the following three predominating: ++--, +++-, and ++-+. Of these 96.2% were identified by the EC test and 99.9% by the methyl red test. From these non-human sources the ++-- type again predominated (95.6% in animals, 97.9% in poultry). The strain --++ was found in 3.3% of animal

droppings surveyed in this country (34) but comprised 12.3% of all organisms found by Mishra and his group (70) in India.

In unpolluted soils the prevailing types are -+-+ (48.0% - 52.0% vs. 0.5% - 1.1% in feces) and --++ (18.8%) (33, 37). Correlating results of EC tests are usually negative and showed fecal coliforms to be usually absent or at a low level in undisturbed soils. The numbers of bacteria per gram of unpolluted soil are also low (28). The number of types of coliforms as well as the number of organisms per gram rises sharply with the degree of pollution in disturbed soils along with the number of positive confirming elevated temperature tests (37).

A similar situation exists on plants, where the types --++ and -+-+ are also found but the type ++++ (24.2%) predominates (39). The type ++-- was found to be present nearly 11% of the time and usually was confirmed by means of the EC test as showing the presence of fecal pollution (39).

It is the soil microorganisms that constitute the principle source of stream contamination during surface runoff (64, 75, 114). The types that are isolated from the waters of many streams correspond most readily to the types found in the soil with few of the types indicative of those found on vegetation being present (33). Fecal coliform numbers in polluted water rise in direct proportion to the amount of

soil contamination by animal wastes. This is true not only in those areas put to use by man for himself and his animals, but also in the most remote high mountain streams draining watersheds extensively used by wild animals (8, 25, 47). Goodrich et al. (47) have demonstrated a high percentage of positive fecal coliform tests performed on microorganisms isolated from a closed mountain watershed used almost exclusively by big game. Research conducted simultaneously on an adjacent watershed open to recreation where big game did not congregate actually demonstrated a lower level of coliform organisms (109). The land area in the closed watershed was extensively covered by big game and some horse droppings, from which debris continuously washed into the stream during surface runoff.

That better methods are required in distinguishing fecal coliforms is demonstrated by the number of IMViC type --++ (Enterobacter sp.) giving positive reactions to the elevated temperature test. This group has commonly been reported isolated from stool specimens as well as from soil. Mishra et al. (70) found that 52.0% of Enterobacter types found in human feces gave positive elevated temperature tests while 43.6% from soil also gave positive reactions. Hendricks (51) reports finding positive EC reactions with a number of Enterobacter isolated from river water in Georgia. Since he compares these with an EC negative "wild type" he obtained from the American

Type Culture Collection, supposedly isolated from a fecal source, he maintains that those EC positive Enterobacter found in the "natural environment" are of non-fecal origin. Dufour and Cabelli (21), in a study comparing EC results to assigned designations of organisms identified by other means, identify the EC negative Enterobacter (96.0% of the organisms in this study) to be of non-fecal origin, while the EC positive organisms overwhelmingly fit the description of Klebsiella and are of fecal origin.

Also to be considered are the 2.0-6.0% of ++-- coliforms of fecal origin that do not give positive fecal coliform tests. This could mean that an equal percentage of water in which they are found could be declared unpolluted when such is not the case (70).

For the reasons discussed, the separation of coliforms into fecal and non-fecal sources is inadequate when based on the IMViC tests which are time consuming and require pure cultures to perform. Also, a not insignificant number of fecal coliform negative samples from stool cultures using the EC test make this test somewhat unreliable as well.

As yet, then, there is no completely satisfactory method available for providing reliable information for determining whether an organism is of sanitary significance let alone any idea of its mammalian origin. However, significant progress has been made in this

direction using several techniques.

E. coli serotyping has been successfully used to identify these microorganisms among those found contaminating streams. By isolating the same types at several sampling points along a water course, the probable source of pollution has been identified (8, 44, 45, 87). There are disadvantages here as well. The majority of organisms isolated that are successfully typed do not appear often enough in a particular study for relationships to be established. The E. coli typing sera used are those prepared commercially against largely human strains of the bacteria and consequently, at times, have little antigenic relationship to those found in the environment. A study by Bissonnette et al. (8), using commercial antisera, found common serotypes of E. coli in both water and fecal samples from big game. Cross reactions among antisera with the bacteria were common and a great many more (approximately 50.0%) did not agglutinate at all. Glantz (43) found that many E. coli strains believed associated with livestock diseases were untypable with commonly used commercial typing sera. By producing antisera against these untyped strains, he was able to establish serologic relationships to disease-causing organisms as well as finding the same organisms in polluted water.

A second serological procedure successfully tried is the use of fluorescent antibody staining of the colonies on a membrane filter.

This technique provided specific identification of the colonies fluorescing as the result of contact with known antisera as well as a method of enumerating the bacteria present since known dilutions of the water sample could be used and multiplied by the number of fluorescing colonies seen on the filter. The colonies are counted with a dissecting microscope and ultraviolet illumination (49).

Serological procedures in general have the advantage of high specificity but preparation of the antisera used has the disadvantage of requiring large areas for rabbit maintenance in addition to the amount of laboratory equipment needed to accomplish the serological procedures themselves. In research projects in which large numbers of microbial strains have been isolated, the outlay in space, equipment, and personnel is great indeed in order to adequately survey even a single watershed.

A relatively simple tool that has been put into use since 1963 (1) in the taxonomic identification of bacterial strains is gas chromatography. This method takes advantage of the fact that lipids, carbohydrates, amines, and metabolic by-products of the bacterial species are different to a degree and have been successfully used in identifying specific microorganisms in a number of instances. Since this method is important to the completion of this research project, it will be separately described below.

Fecal Streptococci as Pollution Indicators

As has been pointed out, the use of coliform bacteria as indicators of pollution has many shortcomings. Most coliforms have been shown to originate from plants and soil and are found to be innocuous in nature. Those that are regarded as positive identifiers of fecal pollution are found inconsistently in polluted waters. Worse, the ++-- type confirmed to be the principal fecal coliform has been discovered to have a shorter survival time in an aquatic environment than many pathogens. At times then, pathogens may go undetected when tests for fecal coliforms are negative (31, 52, 105). The type regarded as representing non-fecal pollutants, --++, is rather frequently found in fecal sources and give every positive reaction of fecal microorganisms. The possible differences in the genera of this type have been described but are far too complicated for the average public health personnel to give them every day consideration.

Large numbers of other genera are found in the gut of animals and many of these have been examined in the attempt to better define fecal pollution. Of these, the streptococci, are found to occur in the droppings of all warm blooded animals and are consistently isolated from the water receiving their discharges (2, 40). In cases where the water contamination was known to be recent or continuing, streptococcal counts, when considered simultaneously with fecal

coliform data derived from the same water, could be used to identify the source of polluting discharges (2, 32, 40). Fecal streptococci are found to be present in similar or greater concentrations than coliforms in fecal discharges of farm animals, other domestic animals, and various wild game. In humans, coliforms occur in numbers four times greater than do the streptococci (64). Also, studies have found that Streptococcus bovis and S. equinus are isolated only from warm-blooded, non-human animal populations, which is a particularly useful characteristic in defining pollution sources (32, 40, 58).

It was also noted that fecal streptococcus numbers increased greatly in storm water runoff. This increase is due to the presence of S. faecalis and S. faecalis var. liquifaciens (40). These organisms are ubiquitous in the environment and are of no value as pollution indicators; these organisms are rarely found in feces of warm blooded animals. These strains show greater survival (13, 32) in an aqueous environment than those from the alimentary tracts of warm blooded animals. Membrane filter cultures from streams heavily contaminated with these strains mask the growth of the known fecal strains. Such large numbers of common streptococci make identification of smaller numbers of significant fecal streptococci difficult even if the latter are present. This fact makes interpretation of results from fecal streptococcus testing programs difficult and risky,

thus making the use of streptococci as fecal identifiers of limited value (32, 40).

Other Microorganisms as Pollution Indicators

A discussion of the difficulties of specific pollution identification using fecal microorganisms must include the preference stated by some for growing the pathogens directly while ignoring the conventional organisms already discussed (2, 31). No authority, including Standard Methods (2) recommends the use of this procedure in lieu of those methods known to be effective for coliforms and streptococci; but in certain situations such findings are definitely significant and methods and media have been developed whereby this can be readily accomplished (2, 52, 104).

Arguments in favor of such procedures center around the apparent occasional ineffectiveness of the standard procedures for the detection of pathogens (31). Fecal coliform counts may be low or non-existent when salmonellae may be at a maximum (48, 95). Research has shown that some salmonellae and possibly even some shigellae (31, 81, 111) have a greater survival rate under adverse conditions than does E. coli. Generally, however, it has been shown that a minimal number of fecal coliforms must be present for infectious levels of pathogens to occur (25). Also, the release of the pathogens themselves are known to be so sporadic that an outbreak would likely have

occurred by the time the organisms could be grown in the laboratory. For this reason a constant check of the level of fecal coliforms is much preferred.

More practically, in Europe, especially, the use of other organisms occurring in the intestinal tract has been considered as fecal pollution indicators (9). Foremost among these are the sulfite-reducing anaerobes such as Clostridium perfringens. Not only is their survival much better than that of E. coli, sulfite reducers are a much more homogenous group than coliforms and do not multiply in the soil. They can be used to estimate the recentness and extent of an instance of pollution, their numbers varying in direct relation to the distance from the source of pollution (9). Objections to the method are based on the ubiquitousness of C. perfringens (115) and other sulfite reducing spore formers - i.e., C. sporogenes - in the environment. However, C. perfringens is the principal sulfite reducing organism found in the majority of pollution cases (9). Significant increases in numbers of these organisms over those originally present can be easily determined by establishing background counts (or "background pollution") for these organisms in soil and water (9).

Two remaining organisms also have received consideration as pollution indicators. Staphylococcus aureus is normally found on human skin and mucous membranes and is more resistant to halogen

disinfectants than coliforms. Pseudomonas aeruginosa is found in human otitis externa and other infections. Demonstration of the absence of either of these organisms would be of immediate value in determining the safety of recreational waters, especially those used for swimming (2).

It is obvious that a variety of methods is being recommended for identifying specifically fecal pollution, other than identifying fecal coliforms. Such a method may be readily established by use of the system discussed in the next section.

Gas Chromatography

In the 20 years since James and Martin (61) first reported a successful separation by gas-liquid chromatography many applications have been discovered for it. Continuing improvements in gas chromatography (GC) columns and detector systems have made possible increasingly sensitive determinations. It has proven a rapid, reliable, sensitive, qualitative method of separating complex mixtures of volatile compounds. Any mixture of volatile compounds or ones that may be rendered volatile by the simple methods now available (29, 54, 77, 80, 87, 110) may be successfully separated on a GC column at a cost that need be no more than that of a research microscope. Examples of its use are the ease and reliability with which pesticide studies may be made in environmental suitability surveys of suspect watersheds

or the elucidation of the fatty acids in butterfat. In pre-GC days, butterfat was thought to contain 11 fatty acids; today over 140 components are known (54).

Abel, DeSchmertzing, and Peterson (1) in 1962 first successfully applied this system to microbiology. On the theory that differences in chemical composition of bacteria would be governed by natural evolutionary relationships if the organisms were grown under defined conditions, they methylated and analyzed cell lipids. They were unable to distinguish between genera of Enterobacteriaceae using the method but were able to find differences in the lipid profiles of selected families of the class Schizomycetes.

Since this primary effort, gas-liquid chromatography (GLC) has been applied not only to distinguishing species and strains of bacteria in pure culture (72, 73, 76, 79, 80, 90), but also when their presence has been suspected in pathological situations (27, 74).

Most chemical compounds, lipids, carbohydrates, and amines found in a bacterial cell have now been volatilized (28, 29, 78) and the data obtained have provided much information in clarifying taxonomic relationships among microorganisms. Several major analytical approaches have been used. The first of these is the examination of pyrolyzed products of whole bacterial cells. This is a complicated procedure because of the difficulty encountered in standardizing the pyrolysis

of whole cells due to the critical nature of time and temperature, composition of the medium used for growing the cells, and age of cells (16). A commonly used procedure is the extraction of specific chemical compounds directly from the cells. These compounds are then volatilized (28, 29, 80) and the resultant chromatograms examined for the presence or absence of designated peaks, i.e., "signatures" or patterns characteristic of a given organism. Even if the peaks obtained are all similar, a situation often encountered in distinguishing between the strains of a species, the peak area may be calculated and strain differences may often be noted statistically (54, 87).

A method used as widely as the extraction and volatilization of specific chemical compounds from the cells, is the use of media extracts (54, 72, 83, 110) or head space gas (106) from metabolic products synthesized by the organisms during growth. This is probably the simplest of all the recommended procedures (54, 74, 93, 110); no special techniques are required, the products are easily extracted and injected directly into the machine. All bacteria synthesize many volatile compounds during their growth cycles. Those compounds present as non-volatile metabolic products may be easily converted by a simple methylation procedure (110). Together they provide valuable information concerning the identification of bacteria.

Bowdon and Basset were the first to distinguish cultures of E. coli and E. aerogenes using head space analysis (106) of the volatile metabolic products of these organisms. More sensitive detectors have since made the detection of specific types of bacterial growth possible at very early stages (71, 72, 73, 74). The only limits on the procedure would seem to be the sensitivity of the column and detector.

Columns adequate for determining all the volatile compounds of an organism are difficulty to achieve. The metabolic products, volatile and non-volatile, are polar compounds and by the use of a corresponding polar stationary phase column, most difficulties may be overcome and a reasonably broad range of elutions may be expected (69).

The choice of a detector is somewhat simpler. Flame ionization (FID) is the most popular method giving good response with a broad range of compounds (69). It also has the advantage of being water insensitive thus making extraction procedures easier. It is also insensitive to formic acid during the analysis of volatile lipids (those lipids containing 1-8 carbon atoms).

The electron capture detector (ECD), extremely sensitive in the nano and pico gram range, has been used in highly sophisticated procedures, often in combination with FID, for early detection and identification of microbial growth. It is quite expensive and has

the added liability of being virtually insensitive to alcohols, common by-products of microbial metabolism (69).

The thermal conductivity detector (TCD) has also been used in a number of procedures; it detects formic acid readily (55, 62, 93, 104) and, at a temperature of 125°C, recommended for the analysis of C_{1-8} volatile fatty acids, is thought to have a greater sensitivity for these compounds (12). It is generally less sensitive than FID or ECD and has the added disadvantage of trailing water peaks at the beginning of a chromatogram. This requires careful extraction from the parent solution of the compound to be volatilized (110). It has proven quite useful for simple, low cost identification of the anaerobes which produce a variety of volatile and non-volatile acids as a result of their metabolism (110).

A range of volatile fatty acids and non-volatile compounds similar to those found in the method recommended for anaerobe identification using a TC detector is found in the mixed acid fermentation of the methyl red-Voges Proskauer tests of the IMViC series. Fermentation of glucose by *E. coli* for the methyl red test is typified by a high yield of acetic, lactic, formic, succinic acids, and ethyl alcohol. The aerogenes type of fermentation, the basis of the Voges-Proskauer test, is typified by the formation of neutral products such as 2,3-butanediol and ethyl alcohol; other volatile and non-volatile compounds are

present in lesser amounts (19, 102).

It would be a simple procedure to adopt the methods of anaerobic microbial identification in an attempt to elucidate differences in strains of coliforms producing these products. Before attempting such a procedure, a thorough analysis using as many biochemical tests as practical should be carried out in order to group the organisms as nearly as possible. The source of a strain, whether from water, soil, or the droppings of a particular species of livestock or wild game should receive primary consideration. The use of similarity values (88, 97, 101) from numerical taxonomy would prove useful in placing strains in groups from which organisms could be selected for determining the relation of the group to other groups or even an individual organism if these prove physiologically distinct.

Statement of Experimental Purpose

The work by Stuart et al. (personal communication) on the Mystic watershed above Bozeman, Montana yielded a collection of a number of pure strains of coliform bacteria. The organisms were isolated from the stream water and from the feces of elk, deer, bear, and horses deposited on the ground drained by the stream.

As many of the coliform cultures as possible were selected for full biochemical evaluation followed by grouping of those strains with the same reactions. From these, strains were selected for

further study in a gas chromatograph using a thermal conductivity detector. It was expected on the basis of the components of the mixed acid reaction that the chromatograms would show similar peaks. Determination of strain differences were done by measuring the relative areas of the peaks formed and their statistical comparison to peak areas from other groups of organisms.

Testing emphasis was primarily on those organisms of IMViC formula -+-- and ++-- with accompanying positive EC tests since these are defined as fecal coliforms. Other IMViC types were included for comparative purposes.

MATERIALS AND METHODS

Selection of the Test Microorganisms

Selection of coliforms for further testing from those isolated by Stuart and his group was based on the following criteria: (1) the remoteness of the region from which an organism had originally been isolated and (2) the reactions originally shown on EMB agar, the IMViC group, the EC test, and lactose fermentation.

To ensure the organisms selected were indeed from regions with minimal contamination from humans or their livestock, selections were made on the basis of the map of the area and the location (8) from which the organisms had been isolated. Only coliforms from the most remote sections showing few indications of use other than by wild game were chosen. Since the site was also used to a limited extent by horses, droppings of this species were included in the study group to discover their relationship to isolates from wild game.

Organisms were selected for study so that large numbers of fecal strains would be included. It may be seen in Appendix A that well over half of all strains chosen fit the description of fecal organisms as explained in the discussion. EC tests had been done on only those organisms isolated from water. Fecal coliforms from animal droppings were selected on the basis of a metallic green reaction on EMB, typical of *E. coli* (2), and the IMViC patterns. EC test results were also considered in the selection of water strains. Strains typical of

E. coli var. I constituted over one half of those chosen. Isolates resembling E. coli var. II (-+--) and large numbers of IMViC types -+-+ and --++ were included to discover the relationship, if any, of these other common types to fecal flora. Coliforms isolated from deer, in particular, lacked the typical coliform pattern, showing predominantly types -+-+ and --++. As a matter of interest, small numbers of other types were included in rough approximation to the proportion in which they appeared to occur in the original tests.

Also selected for identification were strains showing typical Salmonella-like or Shigella-like reactions on MacConkey agar, in the IMViC tests, and on Kligler's iron agar (KIA) when the latter had been done. This was done to determine if there were any pathogens being washed into the stream along with the coliforms.

The number, types, and sources of the strains ultimately chosen are listed in Table XIV. Those with laboratory numbers beginning with 2 are from water; those beginning with 6 from wild game or horses. The letter following the number designates the species from which it was isolated (see Appendix A key). Strains from elk ultimately predominated among those selected because the area is one inhabited by an elk herd. Horses were next most numerous followed by deer and bear. The moose sample was included for interest.

Ten E. coli strains isolated from human stool samples were received from the Montana Department of Health Laboratory in Helena. These were handled in the same manner as the other organisms for later comparison by numerical taxonomic methods and in the gas chromatograph.

All organisms were reisolated on EMB and MacConkey agar to ensure continued viability of the organisms and purity of the strains. Individual colonies were inoculated to fresh stock culture slants, incubated for 24 hours, then stored at 4°C until use. Those cultures chosen that could not be reisolated in this way from the original stock culture slants were heavily inoculated into trypticase soy with 2.0% yeast extract broth (TSY) and reincubated. If after 48 hours, no growth appeared, 5.0 ml of broth was placed in the culture tube and the tube incubated for seven days with transfers to fresh TSY broth at two day intervals. If at the end of the designated time, no growth appeared, another culture was selected from the same general group.

Control Microorganisms

To be certain that the media used in the biochemical investigations were reacting properly and to provide a source of known reactions by various organisms for comparison purposes, a number of known strains of bacteria were used.

On hand at Montana State University were strains of Escherichia coli, Enterobacter (Aerobacter) aerogenes, Shigella flexneri, Salmonella typhosa, Enterobacter cloacae, Enterobacter alvei, Proteus rettgeri, Providencia alcalifaciens, Salmonella enteriditis bioserotype D, Salmonella paratyphi B (S. enteriditis bioserotype paratyphi B), Shigella dysentariae, and Arizona sp.. From the Montana State Department of Health Laboratory in Helena were obtained Flavobacterium sp., Alcaligenes denitrificans, Herbicola-lathyri group, Edwardsiella tarda, Aeromonas hydrophyla, Pseudomonas stutzeri, Pseudomonas diminuta, Pseudomonas putrifaciens, Alcaligenes faecalis, Proteus morganii, Mima polymorpha, Shigella sonnei, Proteus mirabilis and an indole-negative Escherichia coli.

Classification

Classification of Enterobacteriaceae was based on that of Ewing (23, 66, 96). This system appears to supersede that found in the current edition of Bergey's Manual (10) according to a majority of modern texts (66, 96) and papers (24, 116). For the non-enteric types isolated, Bergey's Manual (10) and Hugh (57) served as references.

Preliminary identification of organisms was by means of the Elizabeth King (Bartlett, personal communication) classification system for gram negative microorganisms. The procedure takes advantage of the fermentation of 1% glucose by Enterobacteriaceae in

the oxidation-fermentation (OF) medium of Hugh and Leifson (57) as well as their oxidase negative characteristic in Kovac's (63) oxidase reaction. These reactions were used to separate the Enterobacteriaceae from the other gram negative bacilli.

Following preliminary separation by use of the King system, subsequent identification of all strains was done by a battery of tests selected from Edwards and Ewing (24), Ewing (22), and Martin (66).

Culture Media and Reagents

The tests, media, and references to procedures employed are as follows: Levines EMB agar (BBL), MacConkey agar (Difco), Kligler's iron agar (KIA, Difco) were prepared as directed (7, 85, 108), growth and reaction on each of these were recorded after 24 hours incubation. In the event of clear colorless (non-lactose fermenting) colonies on MacConkey agar, the plates were held for another 3 days to watch for slow lactose fermenting organisms and consequent changes in the indicator. All growth observed on the media and reactions during the biochemical tests were recorded as shown in the key to Appendix A.

For the OF reaction, two 13 x 100 ml tubes containing 3 ml of OF basal medium (Difco) with 1% glucose added were lightly inoculated and one overlaid with about 5 mm (0.5 ml) of sterile paraffin oil. The tubes were then incubated and observed daily for up to four days for

acid formation (57, 108). Acid formation in both the open and sealed tubes was recorded as a fermentative reaction, acid formation in only the open tube was recorded as an oxidative reaction, and failure to form acid in either tube indicates failure of the organism to utilize dextrose by either method.

The oxidase test was modified as directed by Paik (84). For this, 1% dimethyl-p-phenylenediamine was prepared fresh and a drop placed on the surface of the culture plate. Color changes of pink-maroon-black, usually seen within five minutes, indicated a positive reaction.

Indole tests involved incubating 5 ml of inoculated peptone water (1.5% tryptose in water) for 48 hours. One-half ml of Kovac's reagent (84) was added to the culture followed by gently shaking the tube. A deep red color collecting at the surface of the broth was considered positive.

For the methyl red-Voges Proskauer test, two tubes containing 5 ml of MR-VP broth (Difco) were inoculated and incubated for 48 hours. After this period, 2 ml of modified O'Meara's reagent (for the detection of acetoin) (84) was added to one of the tubes, the tube shaken and reincubated for 4 hours with hourly observation. A positive Voges-Proskauer test was indicated by the development of a red band at the top of the broth culture. If results were difficult

to read, the test was repeated with incubation of the culture at room temperature.

The second tube, for the methyl red test, was reincubated for 2 more days. This four day incubation period was suggested by Paik (84). After this time, four drops of methyl red indicator (84) was added to the tube. A red color was formed if the test was positive.

MR-VP broth used for the analysis of metabolic products by gas chromatography was prepared as for the MR-VP test, then tubed in 10 ml amounts in 18 x 142 mm anaerobic culture tubes (Bellco #1803-S1). Further treatment of this medium will be discussed under gas chromatography.

Simmons' citrate agar (BBL) was prepared and incubated as directed. A blue coloration of the medium after 24 hours or more was taken as positive. The tubes, if negative, were observed for seven days since this is the time indicated for some Providencia strains to react (7).

Urea broth (Difco), motility agar (Difco), KCN broth base (Difco) with 15 ml of 0.5% KCN solution added, phenylalanine agar (Difco), gelatin medium (Difco) were all prepared and used according to directions. The KCN broth must be cold when the KCN solution is added. The 10% ferric chloride used in the phenylalanine reaction was prepared only once and stored at room temperature. Reactions

were recorded as described in Appendix A.

The Falkow (26) decarboxylase broth was prepared in four portions. To two portions 0.5% lysine and arginine, respectively, were added; to a third 1% DL-ornithine (the pH of this portion usually required readjusting to 6.8) (66). The fourth portion of the base was retained as a control and contained no amino acids. For each organism a set of 4 tubes containing 4 ml of the respective broths was inoculated and overlaid with about 4 mm (0.7 ml) of sterile mineral oil. Non positive tubes were allowed to incubate for up to 4 days. Reactions were recorded as negative if only an acid reaction resulted. Positive reactions were recorded for alkaline (purple) reactions. If the indicator in the control tube remained unchanged (blue) the organisms did not ferment glucose and the proper conditions for the reaction could not be obtained (21). These too were recorded as negative.

Organic acid media and purple broth base were both prepared from individual reagents as directed by Vera and Dumoff (108). One percent mucic (galactaric) acid was added to the organic acid broth base and boiled until the acid dissolved. The pH was readjusted to 7.4 with 10% NaOH and 4 ml of the medium dispensed into 13 x 100 mm test tubes and autoclaved. A lemon yellow (acid) reaction in the medium, usually occurring within 1-4 days, was recorded as positive.

The tubes were incubated for a total of 15 days as recommended.

Purple broth base was used as the base for the carbohydrate fermentation reactions. One percent sucrose, glucose, lactose, and mannitol or 0.5% dulcitol, salicin, adonitol, or inositol (22) were used. All except sucrose and lactose were weighed and placed directly in the flasks at the time the media were made. The broth was dispensed in 4 ml amounts to test tubes, 50 mm durham tubes added, the tubes capped and autoclaved. Sucrose and lactose were prepared as 10% solutions, filtered through a millipore membrane filter, and dispensed in 0.4 ml amounts to tubes containing 4 ml of autoclaved broth base, giving a final lactose and sucrose concentration of about 1%. Uninoculated controls as well as controls containing organisms known to utilize each respective sugar were run with each batch of test sugars. The cultures were incubated for up to four days and the time of carbohydrate utilization by the organisms recorded.

EC medium (Difco), prepared and used as recommended in Standard Methods (2) was incubated after inoculation from a 24 hour TSY broth culture at 44.5°C in a water bath with gentle shaking for 24 hours. A positive reaction was recorded if gas was formed at the end of that time.

Trypticase soy broth (Difco), used for providing viable cultures for the EC test and inoculating the MR-VP broth employed in the gas