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A Study of the Identification of
Medically Important Streptococci

by

Margaret Antoinette Hardy

B.S., Western Michigan University, 1971

Thesis

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Date:

Approved:

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Advisor, Chairman of Graduate Committee

April 24, 1977



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APPROVED



Chairman, MCV Graduate Council, Dean, School of Basic Sciences

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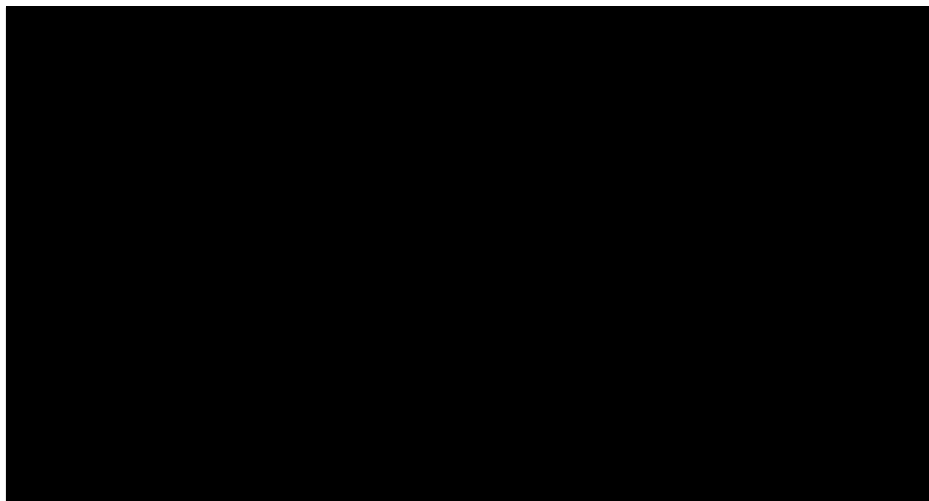


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I. INTRODUCTION

A Review of the Development of the Study of the Streptococci

The world of microbes has existed invisible and unknown to man throughout the ages. As late as the middle of the nineteenth century disease and death brought about by germs was still a mystery and thought by many to be due to malignant spirits. It was only with the development and proper use of the microscope that it was possible for man to be certain of the presence of such minute creatures and thus to begin intelligently his battle to study and control them.

Essentially it can be said that it all started in the mid-seventeenth century with a Delft, Holland shopkeeper by the name of Antony von Leeuwenhoek. It was his guarded methods of grinding lenses to perfection followed by his relentless observations through them that culminated in the literal founding of the field that would eventually come to be known as microbiology (80,82). It took this obscure Dutchman huddled over his lens and making copious notes on the little "animalcules" he discovered through its power to make the members of the prestigious Royal Society of London acknowledge and laud the fact that indeed such creatures did exist. The magnitude of his accomplishment was such that it was not until two years after he reported the existence of small, rapidly moving animals in rain water that Robert Hooke, the most famous microscopist of the time, could confirm that observation (82). For the first time in history, Leeuwenhoek was seeing the unseeable.

The question may arise as to what place, if any, this Dutch shopkeeper holds in the study of the streptococci per se. It would be very

dramatic, albeit ludicrous, to even attempt the suggestion that Leeuwenhoek one day looked through his lens at a small string of spheres and rose from his chair in exhilaration at having discovered the streptococci. Nevertheless, it was he who opened the eyes and minds of the world upon an unknown realm and from that point on man's natural and unsatiated quest for knowledge remains to be satisfied these three hundred years later. And this initiation of microbiological study has very much to do with the streptococci.

The roll call of microbiological greats who followed after Lœuwenhoek includes such well known names as Pasteur, Koch, Behring, Roux, as well as many others. Even if the precise accomplishments of these gentlemen are not exactly clear in the minds of men today, they are memorialized over the entrances to scientific institutions or in the stationery titleheads of prominent pharmaceutical houses. To the serious streptococcologist though some personages of lesser repute are of note.

The word "streptococcus" (from two Greek words, "streptos" meaning twisted or chain, and "kokkus" meaning berry or seed) was first used in this form by Billroth in 1868 (82). His use of the term though was purely of a descriptive nature and was not an announcement of the discovery of a new genus of bacteria. He used the word "streptococcus" to describe a certain phase of his coccobacteria which was a universal genus into which he placed all microbes, considering their activity in disease secondary to the diseases themselves. No one recognized streptococcus as a separate genus until much later. To be sure there were reports by several observers of chain cocci but these were

included in the genus "Micrococcus", members of which were described as occurring in many diseases. In fact almost every disease was thought to be due to a micrococcus. There is no doubt that others saw them in many disease states but no good differential descriptions were given and their causal relationship to disease was debated heatedly.

The first clear cut description of streptococcus as a genus was given by Rosenbach in 1884 (37,105). The first species name given by him was Streptococcus pyogenes to cocci that grew in chains which had been isolated from suppurative lesions in man.

Frederich Fehleisen isolated streptococci from a case of erysipelas in 1882 (37,105) and was the first to grow them in pure culture (more or less easily since no other organisms were present in the lesion). He clearly described these chain cocci and called them "Erysipelaskokken", the specific name Streptococcus erysipelatis not being given to cocci from erysipelas until after Rosenbach's publication (1884). Other reports slowly followed of the discoveries of "new species" of streptococci.

It is not difficult to imagine the confusion of this early period when few facts were known that might help to differentiate or correlate the strains, and investigators were often quick in deciding that characteristics were fixed and determinable enough to be considered species characteristics as well as in concluding how they were related to disease. The use of stains, pure cultures, and microphotography was just beginning. Surmises with wholesale application were published in detail and reams of words were rushed into print with very little founda-

tion in fact. After Rosenbach's demonstration of a reasonably lasting morphologic generic distinction among cocci, new species of streptococci were created by giving them the name of the disease in which they were found and of which they were supposed to be the specific cause. Thus, there was a S.pyogenes, a S.scarlatinae, a S.erysipelatis, a S.diphtheriae, and so on.

Not all nineteenth century scientists agreed with what was then still just a theory that microbes caused diseases. The postulate of the nonbelievers was that the bacteria only accompanied the diseases or were the result of the disease processes. The very diseases that were proved later to be due to streptococci were among the first to be subjected to the most rigorous investigation and disputes. The reason for this was simply because they were so prevalent and harmful.

In spite of the number of species names being given to the streptococci there were some investigators who believed that all those considered pathogenic for man were essentially the same, only varying in virulence and in the power to adapt themselves to the tissues in which they grew thus causing the different symptoms. Just before the turn of the century, the argument advanced in favor of the close relationship of the pathogenic streptococci was given impetus with the introduction of antistreptococcal serum which was obtained by injecting horses with several strains of streptococci obtained from different diseases. The initial success of this serum in the therapy of a variety of streptococcal diseases was considered by some a point too apparent to be allowed any misinterpretation. However, as always there were many who were not so conveniently convinced. Their skepticism proved justified as reports began filtering in of the total ineffectiveness of the

serum in cases where its supposedly unlimited powers were expected to produce miraculous results. This suggested that there were various species of streptococci at work and it was only by chance that the serum had been produced by immunization with the same organism which happened to cause the disease.

Up to this point in time there had been no satisfactory criteria for judging the specific characteristics of the various streptococci found in disease. This is not to say that minds had been idle on the problem. One of the first assessments was, of course, one of the most obvious: morphology. However the size of the individual cocci were soon found to vary too little to be of any practical use. The eye straining demands of such an examination would have no doubt prompted the search for a simpler criterion anyway. More promising seemed to be the lengths of the chains formed. In 1891 von Lingelsheim (1) suggested the terms "streptococcus brevis" for the short chained forms which rendered the broth they grew in uniformly turbid, and "streptococcus longus" for the long chained forms which would form a precipitate on the bottom of the broth tube leaving the supernatant clear. He further showed that the majority of the virulent forms belonged to the latter type and the avirulent to the former. In spite of the initial inclination to totally disregard such a simplistic approach there undoubtedly was a certain validity to the point. Even the nomenclature had been refined to include a complete range of terms beginning with "brevissimus" (diplococci) and "brevis" (4-8 cocci chain) and progressing to end with "longus" and "longissimus" (1,3) which essentially meant long and even longer respectively. The value of such an often subjective

approach was soon realized to be of very little consequence however when chains of mixed length were found to be in the same culture and opinions as to the instability of the cohesiveness between the individual cocci were brought to the fore.

Staining properties were also soon found to be inherently valueless. Researchers discovered that all of the streptococci took to the different dyes in the same manner and it was not long before it became apparent that, relatively, one streptococcus looked as good as another.

The argument for and against the division of streptococci into species continued and was hotly contested with little new evidence in favor of either side until Schottmüller in 1903 gave his definitive demonstration of the blood agar plating method of the division of the streptococci into broad groups according to the effects of the growth on red blood cells (37,105). What Schottmüller found was that most of the streptococci which were isolated from severe pathologic conditions possessed the ability to cause the lysis of red blood cells. These he referred to as the hemolytic streptococci. The streptococci which he found associated with less serious pathologic states caused only a slight hemolysis resulting in a greenish discoloration of the blood. These were put into a group which came to be known as the viridans streptococci. (This inadequately described designation has plagued classifiers ever since Schottmüller first employed it, even progressing to the point of being used as a species designation i.e. S.viridans.)

Schottmüller also distinguished two other groups of streptococci at this time. The first was based on the propensity of a group of streptococci to grow in large mucoid colonies on blood agar plates; and

in his last group he placed the well known "pneumococci".

This new evidence separating the pathogenic from the non-pathogenic strains based on hemolytic reactions was once again hailed by those favoring this theory that all pathogenic strains from different sources were alike. In many ways a loose interpretation of this broad separation holds true in the minds of many even to today. The point of divergence in thought however occurred when investigators discovered that speciation was not quite as simple as that and the mere presence of hemolysis of one sort or another did not exclude, or include, the possibility of a pathogenic strain being present.

It was not until 1908 that Mandelbaum (105) added a third group of streptococci to this classification, namely those strains which caused no hemolytic effect whatsoever on red blood cells. These came to be known as anhemolytic or saprophytic streptococci and their relationship to human disease at this time was not considered to be very great.

The whole question of red blood cell hemolysis by the streptococci was effectively pulled together by Brown in 1919 (13). It is he who brought into general usage the Greek terms "beta" hemolysis depicting total dissolution of the red blood cells, "alpha" hemolysis for partial dissolution and analagous to Schottmüllers' viridans group, and "gamma" hemolysis in reference to the non-hemolytic strains. This work contributed this familiar nomenclature which has persisted to the present day. Although there is a definite trend for modern investigators to disregard "gamma hemolysis" as an anachronism, and rightly so since it describes a negative result, there are still many who cling to the term for

convenience sake. The preferable designation for these strains would be non-hemolytic, or non-reactive but recent publications demonstrate the inclination to describe all hemolysis in terms of "reactions" and thus the nomenclature alludes to a beta reaction, an alpha reaction, or a gamma reaction (26). In some instances "hemolytic" is reserved for any reference to beta hemolysis and this is then used in conjunction with the aforementioned alpha and gamma reaction (15). As always, change comes slowly but optimistically true semantics will ultimately prevail in the least confusing manner.

It had been discovered very early that the streptococci were capable of fermenting carbohydrates. Nocard and Mollereau recorded in 1887(105) a series of tests made on strains of streptococci isolated from cows with inflamed udders. Their use of three sugars and two alcohols did not differentiate their strains from others, but their work achieves importance in that it set an initial foundation for future investigators.

It was eighteen years later, in 1905, in the atmosphere of the debate as to whether more than one species of streptococci existed that Gordon set up a system for the preliminary classification of the streptococci based on biochemical reactions (41). His main purpose was to illustrate the fact that streptococcal individuality was real, not just apparent, and to set to rest those arguments which opposed these distinctions. It would not be too ambitious to state that his battery of nine tests did in fact lay the foundation for all future work on streptococcal classification.

The following year, 1906, Andrews and Horder published their ex-

tensive report making use of, and giving legitimacy to, the principals set forth by Gordon (1). One of the main motives behind the Andrews and Horder study was to emphasize the paramount importance of recognizing the type of streptococcus concerned in a disease process so that correct and specific antistreptococcal serum therapy could ensue. They argued against the use of multivalent sera and in favor of a horse for every strain to insure specificity. With the application of Gordons' carbohydrate tests extensively and comparatively, plus some of their own criteria, they were able to ascertain fairly reliably the type of streptococcus involved and hence form an intelligent judgment as to prognosis and treatment.

Following the publication of the notable studies of Schottmüller, Gordon, and Andrews and Horder, the state of the art of classification diverged with investigators using their own methods depending upon either morphology, blood hemolysis, fermentation reactions, or animal pathogenicity, and in only a few instances upon a random combination of these. The general lack of correlation rendered most reports valueless to other researchers. Holman (1916) (48) and Blake (1917) (10) were among the first to attempt to bring order out of the confusion by simplifying and standardizing techniques which had heretofore been used with little uniformity among investigators. Their work also emphasized for the first time the importance of the conjunctive use of hemolytic reactions on blood agar with carbohydrate fermentations in order to obtain a consistent and stable analytical scheme of identification. Brown (1919) adopted and elaborated upon these two schemes and this very important work proved to be the new basis for streptococcal classi-

fication, with very little alteration by others in the ensuing years(13).

Work on streptococcal identification continued but by far the most important contribution to the methods of classification of the streptococci occurred in 1933 with the publication of the serological technique of Lancefield dividing the hemolytic streptococci into groups by means of a precipitin reaction, based on the presence in these organisms of a group specific polysaccharide (60). The presence of this "C" substance closely parallels those species lines which had been previously known but with greater specificity and accuracy. The Lancefield method complemented and extended rather than disrupted previous works while at the same time it pointed out the location of new groups or species of which bacteriologists had been unaware. The specific soluble substance upon which the serological grouping is based was first found by Hitchcock(1924) as a "residue antigen" and was believed by him to be common to practically all hemolytic streptococci (47). This view prevailed until Lancefield(1933) discovered its group specific nature. The singular brilliance of this addition to streptococcal classification has yet to attain an equal.

After Lancefield's publication, studies on the identification of the streptococci experienced a momentary flourish culminating in what can be alluded to as one of the classic reviews of the genus Streptococcus which was written by Sherman in 1937 (88). Realizing the limited scope of any attempt of this kind he nevertheless went on to present a system of classification which was the most methodical and standardized encompassing hemolytic reactions, Lancefield groupings, and biochemical reactions. Needless to say his system has since been modified, exten-

ded, and improved upon over the years. This however does not detract from its comprehensiveness at the time it was written, nor its apparent worth today. Sherman's work has stood the classic test of time and in many ways the validity of his work still holds where the author had anticipated impending antiquity.

By the end of the 1930's it was apparent that the Lancefield grouping technique of the streptococci was becoming an extremely important tool for systematic significance as well as of great practical import. Until this time evidence had indicated that most human pathogens belonged to group A. (Group D was also granted recognition as having limited importance though in no way comparable to that of group A.) Brown in 1938 (14) and Hare in 1940 (45) reported most emphatically that even though there had been sporadic reports of cases of infection by other streptococcal groups (44,46), for man these were to be considered suspect as opportunist pathogens of little or no epidemiological significance. There was to be a short lived echo of this type of thinking (86) but the 1940's served as the decade in which this belief was shown to have little foundation. There are probably three primary factors which contributed to this: (i) the simplification of grouping techniques combined with the greater availability of the grouping sera permitted the wider study of the epidemiology of the streptococci; (ii) bacteriologists were turning their attention more to the non-respiratory streptococci where group A no longer predominated; and (iii) the advent of sulfonamide therapy prompted the determination of the serologic group of a streptococcus, especially one outside the respiratory tract, in order that a definitive course of treatment

could be adhered to. Rantz and Kirby(1942) issued one of the first reports which showed that only 25% of streptococci from sources other than the respiratory tract were group A (87). Soon thereafter publications appeared which substantiated their observations (22,36,83,84,103). The time had come when group A was no longer viewed as having a monopoly on being able to cause disease; however, in the minds of most investigators this one species still ranked as the foremost enemy to conquer among the streptococci.

Following the establishment of the "unusual" groups of streptococci as legitimate agents of infection, there followed a literary drought on the streptococci in relation to disease. The explanation for this is not readily available which leaves speculation as the primary source of reasoning. There are however several factors which seem to be the most apparent. The first would be the introduction and wide spread usage of penicillin and other antibiotics which led to a sense of false security as to the continued importance of the streptococcus as a disease causing agent. The second would be the introduction by Maxted in 1953 (71) of the use of the bacitracin disc sensitivity test for the fairly reliable identification of group A streptococcus (the pathogen still considered by most to be of primary importance). Lastly there was the emphasis placed by physicians themselves on the group A infections and, more predominantly, the postinfectious sequelae of glomerulonephritis and rheumatic fever, as well as their increasing concern with streptococcal endocarditis. In the midst of this paucity of publications however, occasional case reports did filter out citing single or small collections of infections due to non-group A

or D (57,69,108). With the entry into the 1960's there was also a limited yet increased interest in noting that group B infections were not infrequent (28,50,70). It was not though until 1965 that Reinarz and Sanford reported on cases of infection due to streptococci other than group A or D emphasizing the clinical entities in which these isolates were involved (90). This publication was soon followed by one from Feingold et al. (34) which focused on the etiologic role of the extrapulmonary streptococci. Both of these studies attributed the majority of infections to groups A,B,C,D, and G with groups B and C obtaining a new predominance.

Consequent to these surveys, Duma et al. released a most comprehensive bacteriologic and clinical study of streptococcal bacteremias. The objective of these investigators of calling attention to the many serious infections in man caused by the streptococci was successfully achieved. Aside from aptly illustrating the spectrum of streptococcal groups that can be involved in infectious syndromes, it sought to stress that serologic grouping of streptococci has more than academic merit in that such information can be of significant value to the clinician in his attempt to understand and thus combat the infectious diseases confronting him.

Interest in determining the distribution of the streptococci in clinical specimens appears to have heightened once again as demonstrated by recent publication (2,12,19,51,79). Concomitant with this is the renewed efforts to obtain simpler, more direct methods of identification of streptococci adaptable to the working clinical laboratory with emphasis on biochemical reactions as well as serologic groupings. It

is with the aim of accentuating the importance of speciating the streptococci which are found in infectious disease that all such work must be directed if all is not to be for naught.

A Review of Streptococcal Classification and Identification
Methodology

The haphazard growth of bacteriology from investigations of micro-organisms in relation to plants and animals not unnaturally resulted in a comparative neglect of their relations to one another. Linnaeus, the eighteenth century Swedish naturalist, was detailed in his classification of bacteria; however, he placed all bacteria into one genus "Chaos" (107). This descriptive title is pointedly indicative of man's understanding of microbes at that time. It should be noted here that at that time Leeuwenhoek's descriptions of bacteria were less than a hundred years old, and it was not until a hundred years later that Pasteur reported a function for several of these animalcules.

The beginnings of a formalized manual for classification purposes began with the publications by Chester in 1901 (107), and progressed until continued attempts were eventually embodied in the successive editions of Bergey's Manual of Determinative Bacteriology, the first edition of which appeared in 1923.

Bacterial structures offer few distinguishing characters, and the bacteriologist has come to rely on physiological, antigenic and other characters for differential criteria. Also, bacteriologists primarily interested in the role of bacteria in disease naturally adopted pathogenicity, as elicited in the study of experimental infections, as an important criterion. Unlike plant or animal systems, bacterial systems are largely arbitrary and do not necessarily reflect, except by accident, genetic or evolutionary relationships between the different groups of bacteria (106). The concept of bacterial "species", however often

vague, is nevertheless composed of fairly definitive characteristics which have developed as accepted and convenient forms of identification.

For classification the greatest weight is given to differences that establish an identity with a minimum number of tests. As many characters as possible need to be determined. Not all strains within a group will have a given character, but from the frequency with which different characters occur, and from the association of characters in the strains under consideration, a number of similarities are established which justify recognition.

Most of the strains of streptococci which cause frequent disease in man, or his domestic animals, have been extensively studied and can be characterized quite accurately. Those strains which cause infrequent problems and/or constitute part of the normal body flora are still only vaguely characterized. Thus, though there is little difficulty in accounting for several well defined species of streptococci it is often difficult to provide a logical subdivision of the whole.

Progress in the identification and classification of the streptococci has been made only as methods have been developed, improved, and brought to bear on these problems. The feasibility of the widespread application of a certain test is also of significance if it is to gain ready acceptance. Undoubtedly in any identification scheme some characters will be given more weight than others. However, it is still the summation and overall pattern of characteristics that will eventually dominate in successful speciation.

Early attempts at streptococcal identification involved the application of known biochemical tests which had been used with other

organisms. It is not possible to give credit to specific workers for methods such as the liquefaction of gelatin, reducing properties, action on milk, plus others, as these tests had long been in use for the study of bacteria and it was a natural development that they should be applied to the streptococci. Basically, three general characters have up to now served for most purposes of streptococcal identification: (i) hemolysis production on blood agar, (ii) biochemical and physiologic tests, and (iii) serological grouping. None by themselves have proved to be entirely satisfactory.

Action on blood agar as described by Schottmüller in 1903 (37,105) was among the first of the unique characteristics to be specifically applied to the streptococci. The hemolysins which are produced are either oxygen labile (streptolysin O) or oxygen stable (streptolysin S) and need different conditions for their production. Factors such as variations in the culture media, method of inoculation, pH, oxygen tension, and to some extent the concentration of glucose play roles as to the type of reaction which will be produced by the streptococcus on blood agar (38,39,53,85).

The species of animal blood used in the culture media was also recognized early to be of practical significance. Becker(1916) was one of the first to observe that strains of streptococci might be hemolytic on one kind of blood but not on others and emphasized the need for a standardized blood agar plate (9). Brown(1919) based his beta, alpha, gamma designations on the appearance of streptococcal colonies on horse blood agar (13). He also compared several strains of beta hemolytic streptococci in horse, human, and rabbit blood agar and

found that variations in appearance on these blood agars were quite insignificant. The appearance of alpha strains, however, was so dependent on the type of blood used that certain strains resembled the beta type on one kind of blood but not on another. It was consequent to his work that horse blood became the generally accepted standard in many laboratories. Horse blood was also found satisfactory and recommended as a result of additional investigations by others but at the same time the suitability of other blood sources was not discounted (20, 40, 49, 78, 92). Although horse blood is most commonly used in the United Kingdom and Europe, in the United States the advent of serological grouping and the increasing attention given to the importance of the recognition of group A streptococcus established sheep as the most useful of the animal species as a source of blood. Krumweide and Kuttner(1938) reported that the problem of differentiating beta hemolytic streptococci from Haemophilus hemolyticus was resolved when the use of sheep blood was found to inhibit the latter group but not the former (59). Feller and Stevens(1952) advocated sheep blood when they discovered that it negated the situation of recovering beta hemolytic strains that were not serologically groupable (35). Neussle et al.(1955) studied tonsillitis and pharyngitis patients and observed a marked increase in the recovery of group A streptococcus from sheep blood agar plates versus human blood agar plates (76). As a consequence of these reports, coupled with the emphatic need which was felt to emphasize the recovery of respiratory group A streptococcus, sheep blood agar became the media of choice in most laboratories.

It is not uncommon for investigators to relate beta hemolysis

with virulence and/or pathogenicity. In addition, many also equate "hemolytic streptococci" with group A streptococci only, disregarding the other species which are also beta hemolytic. This type of reasoning has been proved to be erroneous as the ability to produce beta hemolysis is not confined to group A streptococci (26,34,36,38). It should be emphasized that a beta reaction does not necessarily imply pathogenicity, nor does an alpha or non-reaction exclude it.

The second criterion used is biochemical and other physiologic tests. Gordon(1905) initiated study in this direction with his spectrum of nine test for the differentiation of the streptococci (41). Included in his scheme were milk clotting, reducing ability, and fermentation tests. Thermal resistance was studied by Ayers and Johnson in 1914 (4) and this ushered in the utilization of heat tolerance as a significant physiological characteristic in streptococcal differentiation (5). At this same time the final pH attained in glucose broth cultures proved to be of substantial significance and was also used for many years thereafter.

The ability of certain streptococci to hydrolyze sodium hippurate was discovered in 1922 by Ayers and Rupp (7). The value of this trait in distinguishing group B streptococci has only recently attained its greatest significance (11,33,52) as increasing emphasis is placed on the importance of recognizing this pathogen (8,16,104). The recent development of newer methods, such as pigment production (72), and the CAMP reaction (24) also illustrate the new felt expediency of identifying this group. Although promising, it must be cautioned that these latter tests have yet to establish themselves as to their relative

merit. This should not be construed as cynacism and an implication of any deficiencies in the methods themselves, but merely that resultant data as to their effectivity in routine situations is still awaited before general acceptance will be accomplished.

The production of ammonia from peptone by streptococci was recommended by Ayers, Rupp, and Mudge in 1921 as a very useful differential test (6). Although it was successfully and extensively applied by its developers, this method was not widely adopted by bacteriologists and as a consequence its utility was not fully established. It was not until 1943 that Niven et al. determined that arginine was the substance responsible for the ammonia production (75), and it is only most recently that the substantial merit of arginine hydrolysis as a differential tool has been reiterated once again (42).

The tolerance of some streptococci for bile had been recognized fairly early though the origin of this discovery cannot be established. As early as 1912 bile was used as a selective medium for fecal streptococci in milk (58) and soon thereafter (1916) it was observed that the mastitis streptococci (group B) also exhibited considerable tolerance for bile (91). Weissenbach(1918) observed the tolerance of the enterococci for bile and subsequently recommended a bile medium for the differentiation of these streptococci (93). In 1926 Meyer and Schonfeld incorporated esculin into the bile media and regarded this as the best single differential test for the enterococci (32,95). Their results were substantiated by Weatherall and Dible(1929) with a similar media (102). Sherman(1937) however paid scant attention to this association considering it obvious that the ability to attack esculin and

the ability to grow in bile were two distinct traits (93). He dismissed as a curious phenomenon the compulsion of some workers to combine the two reactions. The situation lay dormant until Swan in 1954 resurrected the use of a bile-esculin medium as a single reliable and simple method for the recognition of the group D streptococci (95) and its utility was further established by Facklam and Moody in 1970 (30,32) and Wasilauskas and Ellener in 1971 (99).

It has already been commented on as to the early recognition of the ability of the streptococci to ferment carbohydrates. The overall application of fermentation tests is well established in the study of bacterial identification. Sherman's general scheme (93) placed considerable emphasis on a streptococcus' ability or inability to ferment a particular substrate. The inclusion of additional substrates, or reemphasis of known ones, into classification and identification schemes coincided with efforts to definitively speciate the streptococci (17,25,42,43). There is no reason to doubt that this association will continue as long as interest in streptococci remains.

In this particular category of streptococcal identification methods fall what are perhaps the three most widely accepted methods of recognition and differentiation in use today, namely catalase production (or in this instance, the absence of it), bacitracin sensitivity, and optochin sensitivity. Nearly all of the streptococci give a negative catalase reaction due to their lack of a cytochrome system. There are exceptions though as some members of group D have been found to be catalase positive (55,61) but this occurrence is so rare as to be negligible.

The disc test for sensitivity to bacitracin was developed by

Maxted in 1953 (71). It is a valuable screening test for S.pyogenes. The difference in sensitivity between this organism and other hemolytic streptococci is not great, so the potency of the discs must be carefully standardized. Bacitracin sensitivity is not a unique character of the group A streptococci. Nevertheless, the tendency in the past, and in many cases the present, has been to interpret its results as gospel. In the last decade cautionary notes have come to the fore guarding against overinterpretation as false positive reactions have not been found uncommon (18,33,34,63,79). Bacitracin sensitivity can occur and usually does in a number of non-group A streptococci, particularly in groups B,C, and G. False positive results obtained with the bacitracin test generally range from 2-8% (18,58,71,79). The significance of this has its greatest impact in the erroneous classification of those strains which are not group A. In instances though where the information desired is just to detect group A per se, then this test remains a most valuable tool.

As early as 1911 Morgenroth and Levy (106) noted the sensitivity of pneumococci to optochin (ethylhydrocupreine) and Moore in 1915 (73) made use of this property to distinguish them from other streptococci. As with the bacitracin sensitivity test, it is attractive in its simplicity with the utilization of paper discs impregnated with a standardized concentration of optochin. The method has been shown to be reliable as pneumococci are killed by a concentration of 1/500,000, whereas most other streptococci require 1/5000 or stronger (74). Lund(1959) concluded that the optochin test was more reliable than the bile solubility test on the ground that all pneumococci were sensitive

to optochin, whereas some rough pneumococci gave equivocal results in the bile solubility test (67). Recently the ability of the pneumococci to produce beta hemolysis under controlled conditions has been investigated by Lorian et al. (65,66). Lorian and Markovits subsequently developed a methicillin disc sensitivity test coupling the sensitivity of the pneumococci to this antibiotic disc with its characteristic of producing beta hemolysis anaerobically (64). The comparison of this method with the optochin test, or its advantage over it, has not yet been established.

The final distinction in streptococcal identification is the presence of group specific serologically active polysaccharide antigens i.e., Lancefield grouping. From the 1930's to the 1960's classification was increasingly based on Lancefield's grouping techniques and it is still often considered the sine qua non of streptococcal identification. Certainly its value cannot be debated. Initially from 106 strains of hemolytic streptococci studied, Lancefield recognized five antigenically distinct streptococcal groups and designated them A,B,C,D, and E. Since this early work was accomplished, the number of streptococcal groups identified has steadily increased and currently includes 18, A through H and K through T. There are those species of streptococci though which defy definitive characterization by this method and must be identified through the use of other criteria. Many routine laboratories did not undertake Lancefield grouping because of cumbersome methods plus the time and expense involved. The questionable value of doing such a procedure, other than for academic reasons, has until now also been a factor. However, extraction methods are becoming more

simplified and evidence is accumulating as to the significance of definitive grouping which may serve as an impetus for more widespread usage.

The last decade has brought about better techniques which have provided the information which then formed the basis for new analytical methods. Many bacteriologists have almost come full circle in their thinking and often serology is no longer regarded as the final arbiter in streptococcal taxonomy. It is often now looked upon as just one more character along with morphology, physiology, biochemistry, cell wall components, and genetic information. Whether it be considered the cause or the effect, this type of thinking has no doubt been an important factor in the increasing respectability being given to the non-group A streptococci. In the final analysis though it appears that Lancefield grouping will retain its place in streptococcal identification but perhaps on a more limited and selective basis.

The most widely accepted general classification of streptococci is that which was established by Sherman in 1937 (93). His primary purpose in establishing his four divisions was to put related streptococci together in manageable groups. Sherman's four groupings consisted of: (i) the pyogenic streptococci which are usually beta hemolytic, have a polysaccharide group antigen, are not heat resistant, do not grow at extreme ranges of temperature and pH, and will usually hydrolyze arginine; (ii) the "viridans" streptococci which are not beta hemolytic, usually grow at 45°C, and do not hydrolyze arginine; (iii) the enterococci which can be beta, alpha, or non-hemolytic, have the group D antigen, are generally heat resistant, and hydrolyze arginine; and (iv) the

lactic streptococci which grow at low temperatures but are rather less tolerant of other extremes of temperatures. (As the lactic streptococci are of more concern to the dairy industry and their role in humans negligible, they are not of significance for the purposes of this discussion other than to acknowledge the fact that they exist.)

The general outline of Sherman's classification is well accepted though different organisms are placed in some of the groups. The group polysaccharides characteristic of many of the pyogenic streptococci may occasionally be found in unrelated organisms, and conversely (21). It is now known that there are group D streptococci which are not enterococci. The designation S. viridans is being phased out of existence to be replaced by a category of "viridans streptococci" or simply "alpha streptococci" that have little in common except for a series of negative characters. This designation will no doubt increasingly lose its significance as speciation methods improve for those which were included in this group. And finally there is the addition of the "pneumococci" as a separate and well defined group of streptococci (15). The most recent edition of Bergey's Manual (16) omits for the first time the broad designations of Sherman's divisions on the basis that newly recognized species may cut across those divisional lines Sherman had established. Nevertheless the growth and tolerance tests as proposed by Sherman for the separation of his divisions still retain some usefulness.

II. THESIS OBJECTIVE

In view of the past discussion it should be somewhat apparent that the speciation of the streptococci obtained from various clinical material is of no small import in the successful understanding and ultimate conquest of the infectious diseases in which they are involved. That the term "strep infection" will often conjure up the concept of a single homogeneous entity as the infecting agent is a demonstration of a massive oversimplification of the realities involved. Any importance placed on illustrating the different species of streptococci which are involved in disease processes can only serve to dispel such a fallacy. An alternative would be to broaden the concept by bringing into usage the term "streptococcicosis" which would accentuate the many different streptococcal species which possess pathogenic potential. It is with the goal of accentuating streptococcal speciation by investigating microbiological patterns in clinical specimens from various sources, as well as endeavoring to evaluate, simplify, and perhaps improve upon, present techniques and schemes of streptococcal identification that this present work has been entered upon.

III. MATERIALS AND METHODS

Organisms. Streptococcal isolants were obtained from clinical specimens of varying sources over a five month interval, from June 1976 through October 1976. All strains were Gram positive cocci, catalase negative. Isolants were streaked on sheep blood agar plates to determine purity of culture. Stock cultures were maintained in a 4:1 solution of Todd Hewitt Broth (THB, Difco): glycerol and kept frozen at -70°C . Unless indicated otherwise, an 18 hour growth of the organism in THB was used to inoculate all test media, and all cultures were incubated aerobically at 35°C .

Physiologic Methods. The determination of a streptococcus species was divided into a two part scheme, based on the progressive method of bacterial identification as advocated by Cowan and Steel (1974) (23). The physiologic tests used in Stage I served as a means for presumptive identification of all of the streptococcal isolants. Dependent on the results obtained in Stage I, the confirmatory identification of an organism was accomplished by a second group of tests, either Stage II^a or Stage II^b. The general identification schema was as follows:

Stage I

Determination of hemolysis on:

Sheep blood agar plates (SBAP)

Horse blood agar plates (HBAP)

Rabbit blood agar plates (RBAP)

Bacitracin Sensitivity

Optochin (Ethylhydrocuprein) Sensitivity

Arabinose (acid production)

Esculin hydrolysis in the presence of 40% bile (BEM)

Growth in 6.5% NaCl

Arginine hydrolysis

Glycerol (acid production)

Stage I cont.

- *Hippurate hydrolysis
- *Pigment production in Columbia agar
- *CAMP test

*Hippurate hydrolysis was determined on all of the beta hemolytic isolants; however production of pigment on Columbia agar and the CAMP test were performed on only those organisms which were beta hemolytic, hippurate positive, bacitracin negative.

Stage II^a

(All isolants from Stage I which were BEM negative)

Growth on media containing: 10% bile
40% bile

Carbohydrates, acid from:

- lactose
- mannitol
- salicin
- sorbitol
- trehalose
- inulin

Esculin hydrolysis

Nitrofurazone sensitivity

Serologic grouping

Stage II^b

(All isolants from Stage I which were BEM positive)

Citrate as a carbon source

Carbohydrates, acid from:

- lactose
- mannitol
- raffinose
- salicin
- sorbitol
- trehalose
- sucrose
- sorbose

Serologic grouping

Hemolysis production. Sheep, horse, and rabbit blood agar plates

(Trypticase Soy Agar, Difco, plus 5% blood, Granite Diagnostics) were inoculated by streaking a loopful of the 18 hour THB culture onto the agar surface as well as stabbing into the media. Hemolysis was

recorded as β , beta (clear, colorless zones surrounding colonies), α , alpha (green zones surrounding colonies), or NR(-), no reaction (red cells around colonies intact).

Bacitracin Sensitivity. Noted as a zone of growth inhibition around a paper disc containing 3 units bacitracin (Difco) on a streaked SBAP. All zones of inhibition were measured.

Optochin Sensitivity. Noted as a zone of growth inhibition around a paper disc containing 400ug ethylhydrocuprein (Difco) when placed on a streaked SBAP.

Esculin hydrolysis in the presence of 40% bile. Two drops of an 18 hour THB culture were inoculated onto bile esculin medium (BEM) agar slants (Difco) omitting the horse serum.

Growth in 6.5% NaCl. Two drops of 18 hour THB culture were inoculated into salt broth (Brain Heart Infusion Broth, Difco, plus 6% NaCl w/v) and observed for growth after a 24 hour incubation.

Arginine hydrolysis. Arginine broth (Niven et al., 1942, as in Cowan and Steel's Manual for the Identification of Medical Bacteria, 1974) (23) was inoculated with two drops of 18 hour THB culture. After an 18-24 hour incubation, 0.25ml of Nessler's Reagent (Cowan and Steel, Alternate Formula, 1974) (23) were added. The hydrolysis of arginine was indicated by the development of a bright orange color and precipitate that settled on the bottom of the tube. A negative reaction yielded a yellow (bright yellow to yellow-gray) color and precipitate.

Esculin hydrolysis. Esculin broth (Cowan and Steel, 1974) (23) was inoculated with two drops of 18 hour THB culture and observed for a blackening of the medium. All tests were examined daily for 7 days

before discarding as negative.

Hippurate hydrolysis. Two methods of sodium hippurate hydrolysis were

used: (1) Two drops of 18 hour THB culture were inoculated into a tube containing 5ml of a 10% sodium hippurate broth (Brain Infusion Broth, Difco, plus 10% w/v sodium hippurate, Sigma), incubated for 48 hours, and tested for the production of benzoates according to the method outlined by Facklam et al., 1974 (33).

- (2) The rapid hippurate hydrolysis method of Hwang and Ederer (52) was used with the following modifications: (i) The initial 2 hour incubation period of the test was lengthened resulting in an initial test incubation period of 4-6 hours. (ii) A 37°C aerobic incubator was used instead of a heating block for the incubation of the test system.

Both of these methods were run concurrently on the appropriate isolants obtained during the first 2½ months of this study. Thereafter only the rapid hippurate hydrolysis method was used.

Pigment production in Columbia agar. Columbia agar medium (BBL) was prepared according to directions of the manufacturer, and dispensed, inoculated, and incubated as recommended by Merritt et al., 1976 (72) and observed for a yellow to orange pigment along the line(s) of inoculation. (It was often found that three parallel stabs into a single agar tube often permitted easier observation of the lightly pigmented strains.

CAMP test. The method for the CAMP test was that described by Darling, 1974 (24) using SBAP. The staphylococcal indicator strain was obtained

from H.J. Welshimer, Dept. of Microbiology, Medical College of Virginia, designated "strain A".

Nitrofurazone Sensitivity. Nitrofurazone (Sigma) was incorporated into the media of 3% SBAP in a concentration of 10ug/ml. The plates were inoculated by streaking a loopful of 18 hour THB culture onto the agar surface and observed for growth after a 24 hour incubation.

Growth on 10% bile or 40% bile agars. The medium was prepared by adding 10% or 40% w/v Bacto-Oxgall (Difco) plus 3% v/v sheep blood (Granite Diagnostics) to TSA base (Difco). The medium was dispensed into petri dishes. The plates were inoculated by streaking one loopful of 18 hour THB culture onto the agar surface. Growth was determined as positive (+) or negative (-) after three days incubation.

Production of acid from carbohydrates. With Purple Broth Base (Difco) as the basal medium, test carbohydrates in a concentration of 1% were added and dissolved by heating. The medium was dispensed into tubes in 5ml amounts before sterilization in an autoclave for 15 minutes at 121°C. Each carbohydrate was inoculated with two drops of 18 hour THB culture, incubated, and acid production noted as a change in the color of the medium from purple to yellow. All tubes were examined daily for 7 days before discarding as negative.

Citrate utilization. Two drops of 18 hour THB culture were streaked over the surface of Simmons' citrate (Difco) agar slants. All tests were examined daily for growth and a color change (green to blue) for 7 days before discarding as negative.

Serologic grouping. Grouping antisera was obtained from Burroughs, Wellcome and Co. for groups A,B,C,D,G,H,K,M, and O. Antigens of

isolants obtained during the first two months of the study were extracted using the method of Rantz and Randall, 1955 (88). Antigenic extraction for the remainder of the study was performed using the nonautoclave technique of Watson et al., 1975(101). In both cases the group reactions were determined utilizing microprecipitin tests as described by Swift et al. (96).

IV. RESULTS

343 streptococci were isolated. Tables 1,2, and 3 show the reactions used to speciate the isolants. These tables were compiled from various publications (15,23,30,42,106). For a strain to be placed into a particular species ideally all of its reactions would have had to agree with those listed in Tables 1,2, and 3. However, recognizing that there are many variants within a species, to best place an organism into a particular species a spectrum of reactions was used.

The streptococci were separated initially into one of three divisions based on reactions obtained in Stage I: Division I= hemolytic streptococci i.e. those producing a beta reaction on blood agar, not group D; Division II= alpha streptococci, i.e. those producing an alpha or no reaction on blood agar, not group D; and Division III= group D streptococci. Tables 4,5, and 6 show the species represented within each division as well as the number of isolants and the reactions obtained for them during this study.

Speciation by physiological tests

The tests in Stage I were chosen for their convenience and ability to presumptively identify a species as accurately as possible in the shortest amount of time with the fewest number of tests. Given a pure culture of an unknown strain with which to inoculate the test media, this could usually be accomplished after an overnight incubation period. It should be apparent from the tables that not every test in Stage I was applicable to the identification of every species, or sometimes even for separation into a particular division; but in order to achieve the

most expedient method of division, every strain was tested in this manner.

The observation of hemolysis production on blood agar using three different sources of blood (sheep, horse, rabbit) was undertaken in order to determine what, if any, variations there might be among the three by singular strains within each species.

Among the strains in Division I, hemolysis production was comparable on all three blood agars. Of the hemolytic streptococci studied, a single variation occurred with one strain of S. agalactiae in which an alpha reaction on sheep blood agar (SBA) produced no reaction when on agars in which horse (HBA) or rabbit (RBA) blood were used.

Hemolysis production among the alpha streptococci of Division II also proved fairly comparable among the three sources. Among S. mitis strains, 5/98(5.1%) which produced alpha reactions on SBA and HBA were non-reactive on RBA. Of the 15 S. salivarius strains, 11(73.3%) were non-reactive and 4(7.1%) were alpha reacting on all agars.

The greatest variation in hemolysis production was seen with S. faecalis strains within Division III. Of 106 strains which produced an alpha reaction on SBA, 28/106(26.4%) produced a beta reaction and 1/106(0.9%) no reaction on HBA. Similarly, 28/106(26.4%) produced a beta reaction as well as 5/106(4.7%) producing no reaction on RBA. The only other variation within Division III occurred with a single S. faecium strain producing no reaction on RBA but an alpha reaction on SBA and HBA.

The presumptive identification of group A streptococci through the

use of the bacitracin disc resulted in 30/30(100%) strains being correctly identified with no false negative results being obtained for any of the S.pyogenes isolants. The zones of inhibition varied from 9-16mm. One group B strain (2.9%) and 2/3(66.7%) of the group G strains were found to give false positive results. One S.faecalis isolant (0.9%), 1/14(6.7%) S.salivarius isolants, and 5/98(5.1%) S.mitis isolants also resulted in false positive reactions. However, all of these strains produced strong alpha reactions on blood agar and consequently would not be likely to be misidentified.

In contrast, sensitivity to optochin was demonstrated only by the S.pneumoniae species with all strains of every other species resistant.

The main group of five biochemical tests, i.e. arabinose, bile esculin medium(BEM), growth in 6.5% NaCl, arginine hydrolysis, and glycerol were either singly or in some combination pivotal in some way for species identification within all three divisions.

The importance of BEM was such that it readily allowed for the separation of group D streptococci (Division III) from the non-group D streptococci (Divisions I and II). In no instance in this study did this test result in a false positive or a false negative, i.e. all group D species were positive and all non-group D species were negative.

Arginine hydrolysis was found to be most significant in differentiating among the species included in Divisions II and III. Among the alpha streptococci, arginine hydrolysis proved to be the main point of distinction between S.sanguis (arginine positive) and S.salivarius (arginine negative). Within group D, the enterococci

(S.faecalis, S.faecium) were readily distinguished from the non-enterococci (S.bovis, S.avium) with the former proving to be arginine positive and the latter arginine negative. Of all the group D strains isolated, only 1/111(0.9%) strains of S.faecalis gave an opposing result. All of the species included within Division I proved to be arginine positive with the exception of a single negative result occurring with a strain of S.agalactiae (1/34, 2.9%). Although arginine hydrolysis did not prove useful in differentiating among the species within this division, the test was nevertheless useful in so much that a negative result was indicative of exclusion from this division altogether.

Growth in 6.5% NaCl and the production of acid from arabinose were of importance in distinguishing among the species within Division III. Among the group D isolations, growth in 6.5% NaCl distinguished S.faecalis, S.faecium, and S.avium (all strains positive) from S.bovis (all strains negative). The production of acid from arabinose singled out S.faecium within the enterococci and S.avium within the nonenterococci. Of the 6 S.faecium strains isolated, all were arabinose positive. Also, 4/5(80%) of the S.avium strains reacted with a positive result. The significance of these tests for speciation within Divisions I and II was negligible since, with the exception of a few variants, these strains reacted negatively in both cases.

The production of acid from glycerol was useful within Divisions I and III. Among the hemolytic streptococci, glycerol proved most significant in singling out group B with 33/34(97.1%) strains giving a positive result within 24-48 hours. One half of the group C strains were positive after a 24 hour incubation period with the remaining strains reac-

ting after 72 hours. All of the group G strains were also positive with the reactions apparent after 48-72 hours.

Hippurate hydrolysis, pigment production on Columbia agar and the CAMP test were utilized in a comparison study in order to assess the merit of each in the presumptive identification of S.agalactiae (group B). Hippurate hydrolysis was first used as the screening method by which all of the hemolytic streptococcal isolants were tested. No false positives and no false negatives were obtained i.e. all group B strains gave a positive reaction and all non-group B isolants gave a negative reaction. Of the 34 group B strains, 10(29.4%) failed to produce pigment on the Columbia agar. All (34/34) group B strains provided a positive CAMP test.

The tests which were included in Stage II of the identification scheme were chosen to provide a spectrum of reactions by which to confirm, or in some cases elicit, a species identification. The relative merits of each test will not be dwelt on as all are not of equal import and information concerning them can be obtained satisfactorily from the tables; however there are singular tests which are worthy of mention.

Most of the tests included in Stage II proved to be of minor import for further differentiation of the hemolytic streptococci. A solitary exception seemed to occur though for the differentiation between S.pyogenes and strains of group G which were bacitracin sensitive. Two thirds of the group G strains were incorrectly presumptively identified as group A as a result of these strains exhibiting bacitracin sensitivity. In Stage II it was found that 30/30(100%) of group A

strains did not hydrolyze esculin whereas 3/3(100%) of the group G strains did hydrolyze esculin. The disparity between the number of strains tested in each case is recognized and as such the results must be viewed with this in mind. Nevertheless, the possible plausability of this distinction being of significance deserves recognition.

For the species in Division III, the tests of potential distinction included citrate utilization and production of acid from sorbose. S.faecalis proved to be the only group D species capable of utilizing citrate for growth. However, this fact must be tempered with the observation that only 10/111(9%) of the strains studied provided results after a 24 hour incubation period whereas 97/111(87.4%) required an average of 72 hours incubation time to produce an observable result.

The use of sorbose for the confirmative identification of S.avium proved most satisfactory providing definitive positive results in 24 hours for all S.avium strains isolated. No other group D species in this study resulted in a positive reaction with this carbohydrate.

The division of the alpha streptococci was most aided by the tests in Stage II. S.mutans was singled out as the only species capable of producing acid from mannitol and sorbitol (6/6,100%), all other strains negative. Positive inulin and esculin hydrolysis reactions were confirmatory for S.sanguis and S.salivarius. Approximately 80% of all S.salivarius strains produced positive results for both. (As was previously noted, the distinction between these two would be obtained in Stage I with S.sanguis arginine positive and S.salivarius arginine negative.) S.mitis was distinguished in Stage

II by its lack of, or very erratic pattern of, reaction. It was very characteristic of this species to be lactose positive and negative for every other test.

Sensitivity to nitrofurazone proved not to be the ideal method for differentiation that it had appeared to be as tabulated by Colman and Williams (21) and Topley and Wilson(106). It proved to be approximately 50% accurate at its best. Varying the conditions of the test such as length of incubation, atmosphere, inoculum size, proved fruitless. It was with reluctance that failure had to be admitted in obtaining meaningful results.

Serologic grouping was performed on all hemolytic isolants and resulted in confirmation of the identification of each of the strains in Division I. A spot check of 25/142(17.6%) of all alpha streptococcal isolants was also performed. Of these, 17/25(68%) proved to be nongroupable with the antisera used. One of three of the S.sanguis isolants tested reacted with group H antisera and 4/10(28.6%) of the S.mitis strains tested reacted with group O antisera. Within Division III, 30/126(23.8%) isolants were grouped including all S.bovis, S.avium, and S.faecium strains plus 15 S.faecalis strains. All were found to react with group D antisera. Group D isolants were not routinely serologically grouped as their identification and differentiation is dependent of physiological tests.

Antibiotic sensitivity. Chloromycetin appeared to be the most effective antibiotic among those tested with 314/343(92%) of isolants sensitive to it (Table 7). Five out of seventy-five(6.7%) isolants in Division I, 12/142(8.5%) in Division II, and 12/126(9.5%) of the iso-

lants in Division III displayed resistance to it. After chloromycetin, the most effective antibiotics in vitro were cephalothin, ampicillin, and penicillin. Most of the streptococci resistant to these three agents belonged to group D. The surprisingly low relative effectivity of penicillin can be attributed in great part to the enterococci which accounted for the 64/126 (50.8%) group D streptococci resistant to it. Streptomycin, gentamicin, and kanamycin when tested in vitro appeared to be the least effective drugs. Although the number of resistant strains in all three divisions was relatively high when tested against these three antibiotics, again the greatest number belonged to group D.

As might be expected, group D isolants exhibited the broadest patterns of resistance. Of the 126 isolants, 11 (8.7%) proved to be totally resistant to three or more antibiotics. Of these multi-resistant group D streptococci, 5/11 (45.5%) belonged to the S. avium species with the remaining evenly divided among the S. faecalis, S. faecium, and S. bovis species (2/11, 18.2%). These multiresistant streptococci represented isolations from every clinical source except CSF: 4/11 (36.4%) from urines, 3/11 (27.3%) from wounds and from blood each, and 1/11 (9.1%) from the respiratory tract. With three antibiotics (methicillin, streptomycin, clindamycin) a degree of effectivity was noted in 6% or less of the group D isolated. Seventy-five per cent of the S. bovis strains were sensitive to methicillin but only 2/69 (2.9%) of S. faecalis strains displayed sensitivity to this drug and all S. avium and S. faecium isolated proved resistant. S. faecalis was the species which displayed the most resistance to

clindamycin (1/111,0.9% sensitive) and streptomycin (3/111,2.7% sensitive). S.faecium was equally sensitive to both (1/6,16.7%) as was S.bovis (1/4,25%). Sixty per cent of S.avium strains were sensitive to clindamycin but less than half (2/5,40%) were sensitive to streptomycin.

Among the alpha streptococci, S.mutans, with relatively few exceptions, proved to be consistently more resistant to the antibiotics used in this in vitro test system. The same holds true also for S.agalactiae in comparison with the other hemolytic streptococci of Division II. In both cases the strains possessing the greatest resistance to the drugs tested were obtained from urinary infections.

Streptococcal isolation from clinical specimens

The streptococcal isolants are tabulated by clinical source in Table 8. All of the isolants listed for wounds, urines, CSF(cerebrospinal fluid), and blood are from specimens in which they represent presumptive clinical infection. The respiratory isolants (throat, sputum, tracheal washings) were studied with no judgment being made as to their clinical significance.

Overall, the alpha streptococci comprised the largest number of isolants, 142/343(41.4%), with S.mitis accounting for the majority of these, 98/142(69%). Almost one half, 48/98(49%), of the S.mitis strains were obtained from the respiratory tract where their significance is probably minimal as a possible cause of infection. However, the significance of this species cannot be totally overlooked as it accounted for 21/119(17.7%) of all miscellaneous infections, 23/114(20.2%) of all possible urine infections, and 6/15(40%) of all

blood isolants.

S. sanguis and S. salivarius were isolated with relatively similar frequency. S. sanguis accounted for 14/343(4.1%) of all streptococcal isolants and 14/142(9.9%) of all alpha streptococcal isolations. S. salivarius was found in 15/343(4.4%) of all cultures and comprised 15/142(10.6%) of all alpha streptococci. S. sanguis was detected only in respiratory, 8/14(57.1%), and miscellaneous, 6/14(42.9%), cultures whereas S. salivarius was isolated not only from respiratory, 7/15(46.7%), and wound, 3/15(20%), cultures, but also from urine, 3/15(20%), and blood, 2/15(13.3%), cultures.

S. pneumoniae was found in 9/343(2.6%) of all cultures and comprised 9/142(6.3%) of the alpha streptococcal isolants. As could be expected, 6/9(66.7%) were found in the respiratory tract with 3/9(33.3%) occurring in wound infections. No isolations were obtained from urine or blood cultures.

Relatively few S. mutans isolations occurred overall, 6/343(1.8%). Of these, 4/6(66.7%) were possible causes of urine infections with single isolations, 1/6(16.7%), being obtained from blood and respiratory cultures.

The group D streptococci made up the second largest number of isolations, 126/343(36.7%). Not surprisingly S. faecalis was the most common species isolated with 111/126(88.1%) of all group D isolants and 69/114(60.5%) of the total number of urine isolants, 40/119(33.6%) of total wound isolations, and 2/15(13.3%) of all blood isolations.

S. faecium, although nowhere near in numbers to S. faecalis, occurred as the second most common group D species isolated, 6/343(1.8%), and

contributing 6/126(4.8%) of all group D species. Of these, 4/6(66.7%) were obtained from wound infections with single isolations, 1/6(16.7%) being obtained from urine and blood cultures.

Occurring with similar frequency were S.bovis, 4/343(1.2%) overall and 4/126(3.2%) of all group D, and S.avium, 5/343(1.5%) overall and 5/126(4%) of all group D. Forty per cent of S.avium isolations occurred in both urine and wound infections with 1/5(20%) being found in the respiratory tract. One half of the S.bovis strains were found in blood cultures with 1/4(25%) recovered from urine and wound infections.

No S.faecium var durans or S.equinus isolations were obtained.

In total number of isolations, the beta hemolytic streptococci were found least often, 75/343(21.8%). It would be expected that within this division the S.pyogenes (group A) species would predominate but such was not the case. S.agalactiae (group B) made up 34/75(45.3%) of all hemolytic streptococcal isolations with S.pyogenes second in frequency, 30/75(40%). S.anginosus and S.equisimilis(group C) were both found in 4/75(5.3%) of all hemolytic streptococcal isolations, and group G in 3/75(4%).

S.pyogenes was isolated most frequently from miscellaneous infections, 18/30(60%), most of which were from wound infections. The second most frequent source was the respiratory tract, 12/30(40%). No isolations of S.pyogenes were obtained from any other source.

S.agalactiae was recovered from every clinical source: 16/34(47.1%) from miscellaneous cultures (wounds, genito-urinary tract umbilicus, placenta), 11/34(32.4%) from urines, 5/34(14.8%) from the respiratory tract, and 1/34(2.9%) from blood, as well as accounting

for the single isolation, 1/34(2.9%) from CSF.

As with S.pyogenes, the remaining hemolytic streptococci were recovered only from wound infections and the respiratory tract. S.equisimilis was obtained in the same frequency, 2/4(50%), from both sources. S.anginosus was found only once, 1/4(25%) in a wound infection with the remaining isolants, 3/4(75%), being recovered from the respiratory tract. Two thirds (66.7%) of the group G isolations occurred in wound infections and 1/3(33.3%) in the respiratory tract.

Results are recorded in Table 9 as to the age and sex of 253 patients, accounting for an equal number of streptococcal isolants, of which 121 were males and 132 were females. No age group was spared. Patients in the 0-10 year range accounted for 52/253(20.6%) of all these isolations. S.faecalis, 18/52(19.2%), S.mitis, 11/52(21.2%), and S.pyogenes, 10/52(19.2%) were the species most frequently encountered in this age group. As might be anticipated, 17/18(94.4%) of the S.faecalis isolants were obtained from the urinary tract. Forty per cent S.pyogenes isolations were obtained from the respiratory tract with 6/10(60%) being obtained from wound cultures. Although the majority of S.mitis strains would be expected to have been cultured from the respiratory tract, only 2/11(18.2%) were from this source. Six of eleven (54.6%) were cultured from wounds and 3/11(27.3%) from urine infections.

There is an increase in incidence in the sixth and seventh decades of life which was due mostly to the greater number of group D infections found in these patients. Of the 41 group D isolants found in this age span, 36/41(87.8%) were S.faecalis 23/36(63.9%) of

these were obtained from urine infections.

It may be of interest to note that of the S. agalactiae strains recorded only 1/22(4.6%) was obtained from a newborn. Of the cluster of group B infections found in the third to sixth decades, 12/16(75%) involved females with 4/16(25%) a result of urogenital infections.

V. DISCUSSION

Due to the many facets which were involved in this study of the streptococci, the following discussion will not deal with the work as a whole. Rather, in order to achieve a simpler and more ordered presentation, each division will be discussed individually.

Hemolysis

Since Schottmüller first brought the subject up, many investigators have attempted to find the ideal method for determining streptococcal hemolysis. Brown's definitions of hemolysis were made on the subsurface growth of streptococci in horse blood agar. These definitions have not changed to date but cannot be applied equally to surface growth hemolysis because of the number of extraneous factors which effect the expression of hemolysis. Unfortunately, the ideal method i.e., pour-plate preparations, is not always practical in a laboratory situation. Brown advocated the use of 5% blood as the optimum concentration of defibrinated blood in agar for determining streptococcal hemolysis and is the accepted norm today. Concentrations ranging between 3% and 10% have been used. These concentrations affect only the size of the hemolytic zone and not the definition. A lower blood concentration makes it more difficult to distinguish between alpha and beta hemolysis on streak plates. A higher blood concentration makes some beta hemolytic strains appear non-hemolytic. The use of anaerobic cultures negates both these problems (38) as does the suggestion of the American Heart Association to cut or stab the agar with the wire loop used to streak the surface of the plate (98).

The rabbit vs. horse vs. human vs. sheep blood controversy regarding use in agar plates had been studied with sheep blood being declared the ultimate "winner" for most laboratory use in this country. Sheep blood is recommended as the blood of choice for the detection of beta hemolytic streptococci, particularly S.pyogenes, from throat cultures (76, 98). Sheep blood was found to contain a factor that inhibits the growth of H.hemolyticus, the colonies of which appear identical to beta hemolytic streptococci on the surface of blood agar. Horse and rabbit blood do not. In this study, the source of blood had very little effect on the final expression of hemolysis for any of the species except S.faecalis. Updyke also found only the enterococci affected by the kind of animal blood used (31) although Feller and Stevens reported that horse blood proved more effective for determining hemolysis of groups B,C,F, and G (35). Aside from the other considerations, the tendency of S.faecalis to exhibit an alpha reaction on sheep blood is an advantage in itself in the initial distinction of this species from the hemolytic species. While the observation of streptococcal hemolysis is not an infallible criterion on which to judge an isolant, it nevertheless serves an important function as an initial process in the scheme of identification.

Division I: The Hemolytic Streptococci

S.pyogenes has always been considered the main cause of streptococcal disease in man. It is responsible for a variety of respiratory and septic infections and for the important sequelae of rheumatic fever and glomerulonephritis. As would be expected from previous studies, the majority of group A infections occurred in wounds and the upper respiratory tract. One-third of all group A infections occurred in children under 10 years of age, the majority a result of skin infections. Spanning all age groups, 60% of S.pyogenes infections were associated with skin or wound infections. Duma et al. found in their study of streptococcal bacteremia that almost half of the patients with group A streptococcal bacteremia had an associated skin or wound infection, and frequently the skin was considered the source and/or portal of entry (26). The second most frequent associated infection was the respiratory tract.

Unlike the majority of previous reports though, S.pyogenes was not the major cause of infection overall. Of the hemolytic streptococci, group B predominated being responsible for 45% of hemolytic streptococcal infections, and 55% of all non-respiratory infections. It is also significant that S.agalactiae was the only streptococcal species recovered from every type of clinical specimen, from males and females similarly. This is in keeping with a recent study by

Cliniff and Bump in which group B was responsible for 49.6% of all extrarespiratory infections (19).

The association of group B streptococci with neonatal and urinary tract infections has become increasingly well known. The association of group B to pulmonary infections is not as common a topic of inquiry and they may be more important than expected in view of the fact that the upper respiratory passages harbor these organisms. Sixty per cent of the group B respiratory isolants during this work were recovered from the upper respiratory tract. In a much larger study including 919 group B strains, Pollock and Dahlgren reported a 17% recovery rate of group B streptococci from the upper respiratory passages and a 41% occurrence of this species in both the lower and upper respiratory tract (79). In view of the apparent anatomical ubiquity of this species, no source should be considered out of its range.

In humans, group C streptococci may be found normally in the vagina, pharynx, or on the skin. They have most commonly been associated with skin and pureperal infections (15, 26). The group C isolants of this study were equally distributed between wounds and the respiratory tract. S.equisimilis is often viewed as seldom causing serious disease in man (106) but serious infections have been reported (34, 81). Although none were found in this study, the not infrequent reports of incidences of bacitracin sensitive group C strains (34, 63, 79) could easily lead to misidentification and possible oversight of associated complications (26).

Andrews and Horder called group F streptococci S.anginosus because these organisms were first isolated from the pharynges of

patients with sore throats or "angina" (1). Man appears to be the only animal with which this species is associated. S. anginosus can be isolated from the throat, vagina, skin, or feces (16). Group F streptococci have been associated with infections in the sinuses, wounds, and the meninges (26). Rantz (83) and Duma et al. (26) both reported cases of bacteremia following tooth extraction. Cliniff and Bump reported a high incidence of group F infections in wounds (19). This study produced only one group F infection in a wound with the remaining three isolations of dubious significance from the upper respiratory tract.

Due to poor definition in the past, Lancefield group G is a streptococcus without a name. The designation S. canis has been suggested due to its frequent association with infections in dogs (26). Normally, it can be cultured from the skin and genito-urinary tract, and less commonly from the pharynx and the intestinal tract (26). The most frequent infections with which it has been associated have been puerperal, skin, wound, CSF, and occasionally septicemia (19, 26, 34, 51). In this study, 2/3 group G isolants were recovered from wound infections with the remaining isolant being obtained from the respiratory tract. Probable portals of entry for this organism have been reported to vary widely including the genital tract, biliary tract, pharynx, and skin (2, 26). Armstrong et al. found skin, gastrointestinal tract, and respiratory tract infections due to group G second in frequency only to group A (2).

Table 10 outlines a recommended identification scheme for the hemolytic streptococci. Bacitracin sensitivity is probably the most

widely used test in the identification of the streptococci. In this work, this test was greater than 90% accurate with beta hemolytic colonies. Because of the susceptibility of certain alpha streptococci to bacitracin, the test should not be performed with these isolants, nor would there be any justification to do so. There has been some suggestion that a 10mm zone of inhibition is necessary to establish a presumptive group A identification (19, 31). If such criteria were used in this work, 17% of group A isolants would have been categorized as non-group A. Obviously, the accuracy of the test will in large part depend on the number of beta hemolytic non-group A streptococci which occur within a given patient population. In a study of more than 4000 streptococci, Pollock and Dahlgren found that groups C and G gave the highest rate of false-positive readings (79). Armstrong et al. found no uniform consistency whatsoever in distinguishing between group A and group G with 2 unit bacitracin discs (2). Ederer et al. found group B organisms had a greater per cent of sensitive strains (27). Therefore, as misidentification could occur among beta hemolytic non-group A isolants, this test must be regarded as a presumptive test only and not the final word on identification.

The identification of group B streptococci has come to be of primary importance in a clinical laboratory. The ability to hydrolyze sodium hippurate to glycine and benzoic acid has been recognized as being unique to S. agalactiae, the majority of other hemolytic streptococci being unable to do so. The two methods used to detect the hydrolysis of hippurate differ not only in the tested final end product but, perhaps more importantly from a practical standpoint, in the

length of time needed to complete the test. Facklam et al. described a 48 hour test in which ferric chloride was used to detect benzoic acid (33). Hwang and Ederer described a 2 hour test in which ninhydrin reagent was used to detect glycine (52). The obvious advantage of the latter no doubt is in the ability to obtain results within a very short time. In this study both methods were tried. Facklam's method proved reliable but impractical for quick results. Hwang's method proved reliable and useful but modifications were needed that extended the incubation time to a minimum of four hours. Incubating the tests for periods longer than four hours also heightened the reaction with six hours proving to be the optimal. The strength of the reaction was not always consistent with weak positives not being unusual. At this point, the positive and negative controls became of utmost importance. Even so, in some instances the controls and unknowns had to be tested over to insure that the results were correct. The size of the inoculum was important and no doubt contributed in some cases to varying reaction strengths.

The CAMP test proved to be the method of choice for the identification of group B streptococci. All group B isolants gave unequivocal results when tested in this manner after an overnight incubation. Darling reported that candle jar incubated plates could be read within 5-6 hours (24) and under these circumstances the CAMP test proved no more lengthy than the rapid hippurate method of Hwang and Ederer. Furthermore, the size of the inoculum did not appear to influence results in the CAMP test. This could be of significance in a laboratory situation in which the number of colonies on a primary isolation

plate is not large.

At this point in time, there have been no presumptive test procedures reported that readily and reliably differentiate the hemolytic streptococci of groups C, F, and G. Facklam has reported that S. anginosus (group F) can be distinguished on the basis of its colonial morphology: pinpoint colonies with wide zones of beta hemolysis (31). This is subjective at best and, as reported, prone to error. In this study it was found that non-group B bacitracin negative hemolytic streptococci which did not produce acid from glycerol were presumptively identified correctly as S. anginosus. The non-group B bacitracin negative hemolytic streptococci which were glycerol positive were presumptively identified as group G if it hydrolyzed esculin, and group C if it were esculin negative. The hemolytic strains which were bacitracin positive and glycerol positive were also distinguished as group G on the basis of a positive esculin test. As previously pointed out, conclusions are difficult to establish when only a small number of isolants are obtained. These results deserve further consideration after studying a greater number of organisms.

Serologic grouping is of the most consequence in the final identification on the hemolytic streptococci. Two antigen extraction methods were tried: the autoclave method of Rantz and Randall (88) and the use of combined lysozyme and S. albus filtrate by Watson, Moellering, and Kunz (101). Without doubt the non-autoclave method of Watson was much preferred. Not only did this procedure prove simpler and faster, but it also produced stronger precipitin reactions which were much easier to read. Also, the latter method allowed for

the grouping of those group D strains (notably S. avium) which had failed to give a reaction under the Rantz and Randall method. The simplicity of the Watson method makes it a very feasible procedure that even the smallest laboratory could adopt.

The interesting facet concerning the antibiotic sensitivity patterns of the hemolytic streptococci is the close similarity among groups A,C,F, and G. Group B in almost every case differs from other hemolytic species displaying greater resistance for all antibiotics except kanamycin, carbenicillin, and gentamicin. As previously pointed out, this deviation is due in part to the number of group B isolants obtained from urinary tract infections. Also, group A showed greater resistance than group B to tetracycline but this is not surprising as a large percentage of group A have been reported to be highly resistant to this drug (26). Although Duma et al. found 83% of their group B isolants sensitive to tetracycline (26), Patterson and El Batool Hafeez recently reported that there is an increasing shift nationwide toward tetracycline resistance among group B streptococci (77). This is reflected in this study which found only 59% of group B strains sensitive to tetracycline. Jones et al. found that among strains of hemolytic streptococci from groups A,B,C,F, and G, those of groups B,C, and G were least sensitive to penicillin and erythromycin while those of group A were the most sensitive. Group B strains displayed the greatest resistance to penicillin (56).

Division II: The Alpha Streptococci

The various members of this division are normal inhabitants of the

mouth, throat, and gastro-intestinal tract and thus are commonly cultured from the upper respiratory tract and feces. The role of S. pneumoniae in respiratory illness has been well reported. S. mutans is a leading candidate for the most important cause of dental caries. S. sanguis, S. salivarius, and S. mitis are frequently related to endocarditis (15, 109). The role of these organisms in other infections has not been well studied. It may be that their role is greater than presently known but has been overlooked because the presence of these streptococci is often dismissed as innocuous. This work has shown that all members of this division have been found in various infectious situations in substantial numbers. It could be argued that these organisms were in fact bystanders or contaminants but such is not likely to be the case for so many based on the manner in which they were chosen for inclusion in this study.

Over the years the alpha streptococci have not been subjected to as much scrutiny as the hemolytic or group D streptococci. The speciation of them has heretofore often been overlooked and regarded as unnecessary. Part of the reason could be due to the simple fact that this group of organisms was just too difficult to place into neat categories. They have been collectively, albeit erroneously, known as S. viridans and thought to be just an undefinable cluster of organisms. Serogrouping does not define the individual species as it does for the hemolytic streptococci. Nor does it define the group as a whole as it does for the group D streptococci. Colman and Williams, in their review on the taxonomy of some human alpha streptococci, emphasized the fact that the alpha streptococci may react with a

variety of Lancefield antisera (21). Therefore reliance on physiological tests is of greater importance for definition of this division of organisms than for either of the others.

With the alpha streptococci, the tests for differentiation which proved to be of most merit were optochin sensitivity, arginine hydrolysis, the production of acid from mannitol, sorbitol, and inulin, and esculin hydrolysis (Table 11).

As is commonly known, optochin sensitivity is the identification hallmark of S.pneumoniae. The ability to ferment inulin has in the past been designated as a unique characteristic of this organism also (109). However, the results of this work do not support this as 8/9(89%) S.pneumoniae isolants were unulin negative. Positive results with inulin and esculin characterize S.sanguis and S.salivarius. These two species can then be differentiated by arginine hydrolysis: S.sanguis being arginine positive and S.salivarius being arginine negative. Sorbitol and mannitol differentiate S.mutans from the other alpha streptococci, all of which are negative for these two carbohydrates. S.mitis is distinguished by its failure to react in the ascribed tests. Thus, with these biochemicals an identification could be accomplished with a considerable degree of confidence.

Only in their uniform sensitivity to ampicillin and their relative resistance to streptomycin did the alpha streptococci agree in their reactions to the antibiotics tested. Except for ampicillin, no greater than 67% of S.mutans isolants were sensitive to any of the drugs tested. Four of six S.mutans strains were obtained from urinary tract infections. These isolants displayed an increased resistance to

every antibiotic tested. This is striking in comparison to the remaining S.mutans strains, isolated from the blood and respiratory tract, which with only one exception were sensitive to every antibiotic tested. (The respiratory tract isolant was resistant to streptomycin.) This increased pattern of resistance for these S.mutans urinary tract isolants is similar to that seen with the group B strains isolated from this same source.

Alpha streptococci isolated from the blood and respiratory tract displayed relatively similar antibiotic resistance patterns. This could lead to the implication of the respiratory tract as a likely portal of entry for these organisms into the blood stream. There appeared to be no consistent relationship between sensitivity patterns of respiratory-urine or respiratory-wound isolants.

Division III: The Group D Streptococci

As group A streptococci received literary attention in the past, so the group D streptococci have within the recent past been the subject of an increasing number of publications. The members of group D have been associated with infections in tissues, the blood stream, the genito-urinary tract, the meninges, and can produce endocarditis (16,26,29,89).

S.faecalis is by far the most frequent isolant of this group. It is very commonly linked to urinary tract infection. In this study 69/111(62.2%) S.faecalis strains were cultured from this source. This accounted for 61% of all urine isolants. Approximately 65% of these occurred in patients over the age of 50. Of these, almost 60%

were females in contrast to previous findings in which group D urinary tract infections occurred almost four times as often in elderly males (26). Group D urinary tract sepsis has also been found to be the most frequent antecedent to group D streptococcal bacteremia (26).

S.faecalis and S.faecium, the "enterococci", play no small role in bacteremia and endocarditis. Although this study can report that only 3% of its enterococcal strains were recovered from blood cultures, the percentages from other studies range from 15%-83% (26,29,51). The low incidence in this study is no doubt due in part to the inability to obtain every streptococcal blood isolant that came into the laboratory.

Although S.faecalis and S.faecium can be cultured from similar sources (16), the incidence of S.faecium is much less. Out of 117 enterococcal strains encountered during the course of this work only 5% were S.faecium. This is similar to the finding of Gross et al. that S.faecium constituted 7% out of 2503 group D clinical isolants (42). Likewise, Toala et al. reported that 5% of 382 enterococcal clinical isolants were S.faecium (97). Although no enterococci were recovered from CSF or the respiratory tract, isolations from these sources have been reported (29).

The streptococcal species included in the designation "nonenterococci" are S.bovis, S.equinus, and S.avium. S.equinus is the predominant streptococcus of horse feces (94). No S.equinus strains were obtained from any clinical source during this current study. This was not surprising as other studies reported failure to encounter any human isolations of this species also (29,42).

In 1970, based on their investigations, Facklam and Moody concluded that S. avium should rarely be of consequence to a clinical laboratory concerned with the diagnosis of human infection (32). In 1972, in support of this, a study by Facklam of 262 human group D isolants resulted in no S. avium strains being encountered. In 1975, Gross et al. reported that S. avium constituted 2% of all group D species isolated from a variety of clinical sources (42). This study has found S. avium accounting for 4% of all group D isolations. This progression would appear at first glance to reflect a trend of increasing occurrence of S. avium infections, but one must be aware that such a conclusion would be based on a small amount of available data. Nevertheless, it is an assessment that bears watching in light of future studies even though it may appear speculative at the moment.

The S. avium isolations in this study were obtained from infections in the lower respiratory tract, the urinary tract, and wounds. No isolations were obtained from blood or CSF. Gross et al. recovered S. avium most frequently from postsurgical infections of the abdominal or rectal area, or abscesses of the extremities. They found few in urine or genito-urinary areas and none in blood or CSF (42).

S. bovis has attained importance of late as a significant agent involved in producing endocarditis (29,42,62,89). One half of the S. bovis strains in this study were recovered from blood cultures. Facklam recovered 42/44(98%) S. bovis isolants from blood cultures of which 30/43(70%) were from patients with endocarditis (29). Gross et al. reported that out of 169 S. bovis isolations (6% of all group D isolants) 18(11%) were from endocarditis patients. (They did not

specify the total number of S.bovis isolations from blood.) The remaining 151 strains were recovered most frequently from adult female genito-urinary areas, urine, or newborn infants, either colonized or infected (42). Facklam's remaining S.bovis isolation was obtained from urine (29). In this study, urine and a wound served as sources. This information is in keeping with the observation that genito-urinary and gastro-intestinal antecedents are as common in S.bovis endocarditis as in enterococcal endocarditis (102).

When Sherman first established his criteria for the differentiation of the streptococci in 1937 (93), the terms enterococcus and group D were synonymous. The widespread acceptance of serogrouping changed this definition concluding in the addition of S.bovis, S.equinus, and S.avium to the group D streptococci. The inclusion on these nonenterococci to this group indicates that presumptive procedures, as well as serogrouping, are not sufficient in order to accurately obtain species identification. In this study, 7% of the group D isolants were nonenterococci. This finding is similar to that by Gross et al. who found that out of 2503 group D isolants, 8% were nonenterococci (42). As the nonenterococci have antibiotic sensitivity patterns which differ from the enterococci, differentiation of the group D species deserves attention.

The group D streptococci was the division in which the most definitive speciation was obtained. A select spectrum of tests including arabinose, bile esculin medium, arginine hydrolysis, and glycerol from Stage I plus lactose, raffinose, and sorbose from Stage II allowed for successful differentiation of this division of streptococci

(Table 12).

A laboratory must first identify an isolant as belonging to group D and then it can further identify it as to species. The successful use of the bile esculin reaction (BEM) in identifying group D strains corresponds to the finding of others (32,33) that this is the best nonserological method available for the identification of group D streptococci. A medium much in use for this same purpose is Pfizer's Selective Enterococcus Medium (PSE) which is a modified bile esculin medium (54). PSE has less bile than BEM and publications have attested to its lesser selectivity in differentiating streptococci (29,42,68) with the misidentification of alpha streptococci as group D streptococci the most common error.

It may be readily apparent that the widely accepted use of 6.5% NaCl in differentiating enterococci from nonenterococci is missing from the recommended group of tests. This is because of the ability of S. avium to grow in this medium and thus having the potential of being misidentified as an enterococci. It was found that arginine hydrolysis served the purpose much more accurately. Besides being a simple test to perform and interpret, it reliably differentiates between enterococci and all nonenterococci. The usefulness of this test has been advocated by Gross et al. (42) although its significance has often been overlooked by others (29,31).

The remaining tests in the scheme were chosen to provide sufficient information to confidently differentiate among the species. The main purpose of arabinose was to single out S. faecium. Glycerol distinguished S. faecalis as well as S. avium. Although S. faecium can also

be glycerol positive, its reaction time was much longer than that of S. faecalis. (A definitive separation could be accomplished by performing the test anaerobically as S. faecalis is the only group D species that is glycerol positive under those conditions.) Sorbose proved unique to S. avium as had been similarly reported by Gross et al. (42). The use of raffinose and/or lactose to distinguish between S. bovis and S. equinus appears to be of merit. Since no S. equinus strains were encountered, it is difficult to form any definite conclusions. No raffinose negative S. bovis variants were encountered in this study as well as others (42) but several raffinose positive strains have been reported (29). Based on this, lactose should be used as the more reliable index.

The terms "enterococci" and "nonenterococci" have their greatest clinical significance in relation to therapy. Of particular concern is the identification of the group D species causing endocarditis because of the important therapeutic implications. The differentiation of the members of group D attains special significance when a main goal is the prevention of overtreatment with potentially toxic drugs.

It has been reported that since 1949 group D streptococci have become increasingly resistant to antibiotics, both in the level of resistance to particular drugs and in the multiplicity of this resistance (97). Observing that this increase has paralleled that seen in the Enterobacteriaceae and Staphylococcus, in which it is known that resistance to many antibiotics is plasmid borne, Jacob and Hobbs proceeded to demonstrate the presence of plasmid born transferrable resistance in a strain of S. faecalis var zymogenes (97). This discovery may

afford an explanation for the great increase in group D antibiotic resistance, notably that of the enterococci. Enterococci are difficult to kill with single antibiotics in vitro and in vivo (97) necessitating combined drug therapy (89). Even combined drug therapy is not always effective as some enterococcal strains can also develop resistance to antibiotic synergism (110).

Reports on antibiotic susceptibility of the nonenterococci generally are related to S.bovis because of the recent recognition of S.bovis as significant in endocarditis. (Very little can be found regarding S.avium and S.equinus.) The clinical picture and drug sensitivity patterns found in S.bovis endocarditis are more closely related to the alpha streptococci than to the enterococci (100). S.bovis strains have been reported to be much more sensitive to antibiotics in vitro and in vivo (89).

In this study, S.bovis demonstrated the least resistance to the antibiotics used in this in vitro test system. Belonging as it does to the nonenterococci, it was expected that S.avium would demonstrate drug susceptibility patterns similar to S.bovis. Such was not the case, for S.avium exhibited significantly increased resistance to 8 of the 12 antibiotics. It is interesting that this nonenterococcal species displayed greater overall resistance to the antibiotics tested than did the enterococcal species S.faecium. Finally, as could be anticipated, S.faecalis displayed the greatest antibiotic resistance of all the group D species.

Summary:

1. The type of animal blood used in preparing blood agar plates did not significantly affect the demonstration of hemolysis by any streptococcal species except for S. faecalis. S. faecalis more consistently displayed an alpha reaction on sheep blood agar while expressing a beta reaction on horse and rabbit blood agars.
2. A recommended scheme for identifying streptococci, adaptable to most any clinical laboratory, was developed and is outlined in Table 13.
3. S. agalactiae (group B) was the most frequent beta hemolytic streptococcal isolant. Significantly, it was the only species overall to be recovered from every clinical source. The CAMP test proved to be the best method for the presumptive identification of the group B streptococci.
4. The alpha streptococci were isolated in substantial numbers from a variety of infectious situations suggesting that their role in infection may be greater than previously believed.
5. S. avium accounted for a greater percentage of group D isolations than previously reported in other studies of a similar nature.
6. The bile-esculin test was supported as the best nonserological method for distinguishing the group D streptococci. Arginine hydrolysis was shown to be the best method for distinguishing between the enterococci and the nonenterococci. The test commonly used for this purpose, growth in 6.5% NaCl, resulted in the misidentification of S. avium as an enterococcal species.
7. Urinary tract isolants from every division (notably S. agalactiae,

S.mutans, and S.faecalis) exhibited patterns of overall greater antibiotic resistance than those strains isolated from other sources.

8. Among the group D species, S.faecalis displayed the greatest antibiotic resistance followed by S.avium, S.faecium, and S.bovis. S.avium, a nonenterococcus, showed greater drug resistance than S.faecium, an enterococcus. S.bovis was the least resistant with susceptibility patterns more closely related to the alpha streptococci.

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VII. APPENDIX

Division I: Physiological Reactions of Hemolytic Streptococci
(Not Group D) to a Spectrum of Tests

	<u>S. Pyogenes</u>	<u>S. agalactiae</u>	<u>S. equisimilis</u>	<u>S. anginosus</u>	Group G
Stage I					
Hemolysis Production	β	β/α/-	β	β	β
Bacitracin Sensitivity	+	- ^v	-	-	-
Optochin Sensitivity	-	-	-	-	-
Arabinose (acid)	-	-	-	-	*
Bile-esculin Medium	-	-	-	-	*
Growth in 6.5% NaCl	- ^v	- ^v	-	-	*
Arginine Hydrolysis	+	+	+	+	+
Glycerol (acid)	-	+	+	-	+
Hippurate Hydrolysis	-	+	-	-	-
Columbia Agar	-	+	-	-	-
CAMP Test	-	+	-	-	-
Stage II^a					
Growth on: 10% bile	-	+	+	*	*
40% bile	-	+	-	- ^v	-
Carbohydrates (acid):					
Lactose	+	- ^v	+	+ ^v	+
Mannitol	-	-	-	-	-
Salicin	+	+	v	+	v
Sorbitol	-	-	-	-	-
Trehalose	+	+	+	+ ^v	+
Inulin	-	-	-	-	- ^v
Esculin Hydrolysis	-	-	- ^v	-	+
Nitrofurazone Sensitivity	*	*	*	*	*
Serologic Grouping	A	B	C	F & G	G

Key: + = 85-100% strains positive

+^v = 16-84% strains positive (negative)

- = 0-15% strains positive

v = variable reactions by various strains

* = not known

Table 2

Division II: Physiological Reactions of Alpha Streptococci
(Not Group D) to a Spectrum of Tests

	<u>S. sanguis</u>	<u>S. pneumoniae</u>	<u>S. salivarius</u>	<u>S. mitis</u>	<u>S. mutans</u>
Stage I					
Hemolysis Production	α	α	α/-	α	α/-
Bacitracin Sensitivity	-	-	-	-	-
Optochin Sensitivity	-	+	-	-	-
Arabinose (acid)	-	-	-	-	-
Bile-esculin Medium	-	-	-	-	v
Growth in 6.5% NaCl	-	-	-	-	-
Arginine Hydrolysis	+	-	-	-	-
Glycerol (acid)	-	-	-	-	-
Hippurate Hydrolysis	-	-	-	-	-
Columbia Agar	-	-	-	-	-
CAMP Test	-	-	-	-	-
Stage II ^a					
Growth on: 10% bile	+ ^v	-	+	- ^v	+
40% bile	- ^v	-	- ^v	-	v
Carbohydrates (acid):					
Lactose	+	+	+ ^v	+ ^v	+
Mannitol	-	-	-	-	+
Salicin	+	-	+	+ ^v	+
Sorbitol	-	-	-	-	+
Trehalose	+	- ^v	+ ^v	- ^v	+
Inulin	+ ^v	+ ^v	+	-	-
Esculin Hydrolysis	+ ^v	-	+	-	v
Nitrofurazone Sensitivity	S	*	R	S	R
Serologic Grouping	H	-/0	K	OKM	*

Key: + = 85-100% positive

+^v(-^v) = 16-84% positive (negative)

- = 0-15% positive

v = variable reactions by various strains

* = not known

S,R = sensitive, resistant

Division III: Reactions of Group D Streptococci
to a Spectrum of Tests

	---Enterococci---			-Nonenterococci-		
	<u>S. faecalis</u>	<u>S. faecium</u>	<u>S. faecium</u> <u>var durans</u>	<u>S. bovis</u>	<u>S. equinus</u>	<u>S. avium</u>
Stage I						
Hemolysis Production	-/β	-/α	β	α/-	α	α
Bacitracin Sensitivity	-	-	-	-	-	-
Optochin Sensitivity	-	-	-	-	-	-
Arabinose (acid)	-	+	-	-	-	+
Bile Esculin	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	-	-	+
Arginine Hydrolysis	+	+	+	-	-	-
Glycerol (acid)	+	-	-	-	-	+
Stage II ^b						
Citrate	+	-	-	-	-	-
Carbohydrates (acid):						
Lactose	+	+	+	+	-	+
Mannitol	+	+	-	v	-	+
Raffinose	-	-	-	+	-	-
Salicin	+	+	v	+	+	•
Sorbitol	+	-	-	-	-	+
Sucrose	+ ^v	•	-	+	+	+
Trehalose	+	+	v	v	-	+
Sorbose	-	-	-	-	-	+
Serologic Grouping	D	D	D	D	D	DQ

Key: + = positive reaction

(+) = delayed positive

- = negative reaction

• = not known

Table 4

Division I: Hemolytic Streptococci from Human Sources Showing Reactions to a Spectrum of Tests

	<u>S. pyogenes</u>	<u>S. agalactiae</u>	<u>S. equisimilis</u>	<u>S. anginosus</u>	<u>Group G</u>	Total
No. of Isolants	30	34	4	4	3	75
% of Total	40.0	45.3	5.3	5.3	4	
Stage I						
Hemolysis:						
Sheep Blood Agar	30B	28B, 6 α	4B	4B	3B	
Horse Blood Agar	30B	27B, 6 α , 1-	4B	4B	3B	
Rabbit Blood Agar	30B	27B, 6 α , 1-	4B	4B	3B	
Bacitracin Sensitivity	30+ ¹	1+, 33-	4-	4-	1-, 2+	
Optochin Sensitivity	30-	34-	4-	4-	3-	
Arabinose (acid)	30-	33-, 1(+)	4-	4-	3-	
Bile Esculin Medium	30-	34-	4-	4-	3-	
Growth in 6.5% NaCl	27-, 1+, 2(+)	27-, 3+, 4(+)	3-, 1+	4-	3-	
Arginine Hydrolysis	30+	33+, 1-	4+	4+	3+	
Glycerol (acid)	28-, 2(+)	30+, 3(+), 1-	2+, 2(+)	4-	3(+)	
Hippurate Hydrolysis	30-	34+	4-	4-	3-	
Columbia Agar		24+, 10-				
CAMP Test		34+				
Stage II ^a						
Growth on: 10% Bile	26-, 4(+) ²	34+	3+, 1-	3-, 1+	3(+)	
40% Bile	29-, 1(+)	34+	3-, 1+	4-	2-, 1(+)	
Carbohydrates (acid):						
Lactose	30+	19-, 9+, 6(+)	4+	3+, 1-	3+	
Mannitol	30-	34-	4-	4-	3-	
Salicin	28+, 2-	30+, 2(+), 2-	4+	4+	2+, 1-	
Sorbitol	30-	34-	4-	4-	3-	
Trehalose	29+, 1-	28+, 4(+), 2-	4+	4+	2+, 1-	
Inulin	29-, 1+	32-, 2+	4-	4-	3-	
Esculin Hydrolysis	30-	34-	4-	4-	3+	
Serologic Grouping	30A	34B	4C	4F	3G	

¹Zone of inhibition varied from 9-16 mm.²Bile agar (+) = 72 hours

Key: + = positive reaction
 (+) = delayed positive
 - = negative reaction

Table 5

Division II: Alpha Streptococci from Human Sources Showing Reactions to a Spectrum of Tests

	<u>S. sanguis</u>	<u>S. pneumoniae</u>	<u>S. salivarius</u>	<u>S. mitis</u>	<u>S. mutans</u>	<u>Total</u>
No. of Isolants	14	9	15	98	6	142
% of Total	9.9	6.4	10.6	69.0	4.2	
Stage I						
Hemolysis:						
Sheep Blood Agar	18,13 α	9 α	11-,4 α	18,97 α	4 α	
Horse Blood Agar	18,13 α	9 α	11-,4 α	18,97 α	4 α	
Rabbit Blood Agar	18,13 α	9 α	11-,4 α	18,92 α ,5-	4 α	
Bacitracin Sensitivity	14-	9-	14-,1+	93-,5+	6-	
Optochin Sensitivity	14-	9+	15-	98-	6-	
Arabinose (acid)	13-,1+	9-	15-	98-	4-,2(+)	
Bile Esculin Medium	14-	9-	14-,1+	96-,2+	6-	
Growth in 6.5% NaCl	13-,1+	9-	10-,5+	83-,15+	4+,2-	
Arginine Hydrolysis	14+	9-	15-	93-,5+	6-	
Glycerol (acid)	14-	9-	15-	98-	5-,1(+)	
Stage II ^a						
Growth on: 10% Bile	7+,7-	9-	12+,3-	76-,22+	6+	
40% Bile	7+,7-	9-	11+,4-	87-,11+	1+,4(+),1-	
Carbohydrates (acid):						
Lactose	14+	9+	14+,1-	88+,2(+),7-	6+	
Mannitol	14-	9-	15-	98-	6+	
Salicin	10+,4-	9-	14+,1-	72-,26+	5+,1-	
Sorbitol	14-	9-	15-	98-	6+	
Trehalose	14+	6+,3-	14+,1-	65-,33+	6+	
Inulin	12+,2-	8-,1+	13+,1(+),1-	96-,2+	4-,2+	
Esculin Hydrolysis	11+,3-	8-,1+	13+,2-	88-,10+	4+,2-	
Nitrofurazone	7S,7R		8R,7S	62R,36S	3R,3S	
Sensitivity						
Serologic Grouping	11+,2NG	3NG	3NG	10NG,40	2NG	

Key: + = positive reaction
 (+) = delayed positive
 - = negative reaction
 NG = nongroupable
 S,R = sensitive, resistant

Table 6

Division III: Group D Streptococci from Human Sources Showing Reactions to a Spectrum of Tests

	-----Enterococci-----			-----Nonenterococci-----			<u>Total</u>
	<u>S. faecalis</u>	<u>S. faecium</u>	<u>S. faecium</u> <u>var durans</u>	<u>S. bovis</u>	<u>S. equinus</u>	<u>S. avium</u>	
No of Isolants	111	6	0	4	0	5	126
% of Total	88.1	4.8		3.2		4.0	
Stage I							
Hemolysis							
Sheep Blood Agar	58,106 α	6 α		4 α		18,4 α	
Horse Blood Agar	338,77 α ,1-	6 α		4 α		18,4 α	
Rabbit Blood Agar	338,73 α ,5-	18,4 α ,1-		4 α		18,4 α	
Bacitralin Sensitivity	110-,1+	6-		4-		5-	
Optochin Sensitivity	111-	6-		4-		5-	
Arabinose (acid)	109-,2+	6+		4-		4+,1-	
Bile Esculin Medium	111+	6+		4+		5+	
Growth in 6.5% NaCl	111+	6+		4-		5+	
Arginine Hydrolysis	110+,1-	6+		4-		5-	
Glycerol (acid)	108+, 3(+)	1-,5(+)		4-		4+,1(+)	
Stage II ^b							
Citrate	10+,97(+),4-	6-		4-		5-	
Carbohydrates (acid):							
Lactose	103+,7(+),1-	6+		4+		5+	
Mannitol	111+	5+,1-		3-,1+		5+	
Kaffinose	108-,2+,1(+)	4-,1(+),1+		3+,1-		5-	
Salicin	111+	6+		4+		4+,1(+)	
Sorbitol	109+,1(+),1-	6-		4-		5+	
Sucrose	105+,3(+),3-	6+		4+		5+	
Trehalose	111+	6+		4+		5+	
Sorbose	111-	6-		4-		5+	
Serologic Grouping	15D	6D		4D		5D	

Key: + = positive reaction
 (+) = delayed positive
 - = negative reaction

Table 7

In Vitro Sensitivity of Streptococcal Isolants to Twelve Antibiotics

	No. tested	% of Isolants Sensitive to:											
		P 10µg	E 15µg	Me 5µg	Am 10µg	CR 30µg	S 10µg	Te 30µg	C 30µg	K 30µg	Cl 2µg	Cb 50µg	G 10µg
Division I:													
Hemolytic Streptococci													
<i>S. pyogenes</i>	30	100	97	97	100	100	7	43	97	0	97	23	17
<i>S. agalactiae</i>	34	82	82	68	94	88	56	59	88	53	82	71	73
<i>S. equisimilis</i>	4	100	100	100	100	100	0	75	100	0	100	50	50
<i>S. anginosus</i>	4	100	100	100	100	100	0	75	100	0	100	0	0
Group G	3	100	100	100	100	100	33	67	100	0	100	0	0
Total	75	92	91	84	97	95	29	55	93	24	92	44	43
Division II:													
Alpha Streptococci													
<i>S. sanguis</i>	14	100	93	86	100	100	43	71	100	64	79	71	71
<i>S. pneumoniae</i>	9	100	89	67	100	67	33	67	89	56	89	67	67
<i>S. salivarius</i>	15	100	73	60	100	100	7	60	73	43	56	93	93
<i>S. mitis</i>	98	82	83	75	95	97	28	76	94	54	80	59	58
<i>S. mutans</i>	6	67	50	33	100	67	17	50	33	67	33	50	50
Total	142	86	82	72	97	95	27	72	92	55	75	64	63
Division III:													
Group D. Streptococci													
<i>S. faecalis</i>	111	46	32	3	46	73	3	28	92	39	1	16	11
<i>S. faecium</i>	6	67	83	0	100	67	17	100	83	17	17	33	33
<i>S. bovis</i>	4	100	75	75	100	100	25	25	100	75	25	100	75
<i>S. avium</i>	5	100	40	0	60	100	40	0	60	40	60	0	0
Total	126	49	37	5	51	75	6	30	91	39	5	19	14
Total all isolates	343	74	67	50	80	87	20	53	92	42	53	44	41

Symbols: P = penicillin, E = erythromycin, Me = methicillin, Am = ampicillin, CR = cephalothin, S = streptomycin, Te = tetracycline, C = chloromycetin, K = kanamycin, Cl = clindamycin, Cb = carbenicillin, G = gentamycin

Table 8

Distribution of Streptococci in Clinical Specimens

Streptococcal Species	Respiratory		Miscellaneous*		Urine		CSF		Blood		Total	% per Division
	No.	%	No.	%	No.	%	No.	%	No.	%		
Division I												
<u>S. pyogenes</u>	12	12.8	18	15.1	0	0	0	0	0	0	30	40.0
<u>S. agalactiae</u>	5	5.3	16	13.5	11	9.7	1	100	1	6.7	34	45.3
<u>S. equisimilis</u>	2	2.1	2	1.7	0	0	0	0	0	0	4	5.3
<u>S. anginosus</u>	3	3.2	1	0.8	0	0	0	0	0	0	4	5.3
Group G	1	1.1	2	1.7	0	0	0	0	0	0	3	4.0
Total	23	24.5	39	32.8	11	9.7	1	100	1	6.7	75	
Division II												
<u>S. sanguis</u>	8	8.5	6	5.0	0	0	0	0	0	0	14	9.9
<u>S. pneumoniae</u>	6	6.4	3	2.5	0	0	0	0	0	0	9	6.3
<u>S. salivarius</u>	7	7.5	3	2.5	3	2.6	0	0	2	13.3	15	10.6
<u>S. mitis</u>	48	51.1	21	17.7	23	20.2	0	0	6	40.0	98	69.0
<u>S. mutans</u>	1	1.1	0	0	4	3.5	0	0	1	6.7	6	4.2
Total	70	74.5	33		30	26.3	0	0	9	60.0	142	
Division III												
<u>S. faecalis</u>	0	0	40	33.6	69	60.5	0	0	2	13.3	111	88.1
<u>S. faecium</u>	0	0	4	3.4	1	0.9	0	0	1	6.7	6	4.8
<u>S. faecium</u> var <u>durans</u>	0	0	0	0	0	0	0	0	0	0	0	0
<u>S. bovis</u>	0	0	1	0.8	1	0.9	0	0	2	13.3	4	3.2
<u>S. equinus</u>	0	0	0	0	0	0	0	0	0	0	0	0
<u>S. avium</u>	1	1.1	2	1.7	2	1.8	0	0	0	0	5	4.0
Total	1	1.1	47	39.5	73		0	0	5	33.3	126	
Total per Clinical Source	94		119		114		1		15		343	

*Abscesses and wounds from various anatomical origins; genito-urinary tract; umbilicus; placenta; postmortem specimens.

Table 9

Relationship of Streptococcal Species to Patient Sex and Age for 253 Isolants

	0-10 years			11-20 years			21-30 years			31-40 years			41-50 years			51-60 years			61-70 years			71-80 years			81-90 years		
	No.	M	F	No.	M	F	No.	M	F	No.	M	F	No.	M	F	No.	M	F	No.	M	F	No.	M	F	No.	M	F
Division I:																											
Hemolytic Streptococci																											
<i>S. pyogenes</i>	10	7	3	2	1	1	3	0	3	3	2	1	2	1	1	5	1	4	1	1	0	2	0	2	1	1	0
<i>S. agalactiae</i>	4	0	4	1	1	0	3	0	3	5	1	4	6	3	3	2	0	2	0		0	1	0	1	0		0
<i>S. equisimilis</i>	1	1	0	0			0			1	0	1	0			0			0		0			0			0
<i>S. anginosus</i>	1	0	1	0			0			1	0	1	0			0			0		0			0			0
Group G	0			0			0			1	0	1	0			0			0		0			0			0
Total	16	8	8	3	2	1	6	0	6	11	3	8	8	4	4	7	1	6	2	2	0	3	0	3	1	1	0
Division II:																											
Alpha Streptococci																											
<i>S. sanguis</i>	1	1	0	1	0	1	0			1	0	1	0			2	1	1	1	1	0	1	0	1	0	1	0
<i>S. pneumoniae</i>	3	1	2	1	1	0	0			0	0	1	0			0			2	2	0	0			0		0
<i>S. salivarius</i>	1	0	1	1	0	1	0			3	0	3	0			0			1	1	0	0			0		0
<i>S. mitis</i>	11	6	5	11	3	8	3	2	1	4	3	1	9	7	2	7	3	4	13	11	2	6	1	5	1	0	1
<i>S. mutans</i>	0			0			0			0			0			0			1	0	1	2	2	0	0		0
Total	16	8	8	14	4	10	3	2	1	8	3	5	9	7	2	9	4	5	18	15	3	9	3	6	1	0	1
Division III:																											
Group D Streptococci																											
<i>S. faecalis</i>	18	7	11	3	2	1	10	3	7	9	7	2	7	4	3	13	5	8	23	11	12	15	7	8	2	0	2
<i>S. faecium</i>	0			1	1	0	1	1	0	0			0			1	0	1	0			0			0		0
<i>S. bovis</i>	1	0	1	0			0			0			0			1	1	0	0			0			0		0
<i>S. avium</i>	1	1	0	0			0			0			0			1	1	0	2	2	0	0			0		0
Total	20	8	12	4	3	1	11	4	7	9	7	2	7	4	3	16	7	9	25	13	12	15	7	8	2	0	2
Total all isolants	52	24	28	21	9	12	20	6	14	28	13	15	24	15	9	32	12	20	45	30	15	27	10	17	4	1	3

Table 10

Recommended Identification Scheme for the Hemolytic Streptococci (Division I)

Test	<u>S. pyogenes</u>	<u>S. agalactiae</u>	<u>S. equisimilis</u>	<u>S. anginosus</u>	Group G
Hemolysis	β	β	β	β	β
Bacitracin Sensitivity	+	-	-	-	-/+
CAMP Test	-/+	+	-	-	-
Acid from Glycerol	-	+	+	-	+
Esculin Hydrolysis	-	-	-	-	+
Serologic Grouping	A	B	C	F	G

Key: + = 85-100% strains positive

- = 0-15% strains positive

-/+ = 16-84% strains positive

Table 11

Recommended Identification Scheme for the Alpha Streptococci (Division II)

Test	<u>S. sanguis</u>	<u>S. pneumoniae</u>	<u>S. salivarius</u>	<u>S. mitis</u>	<u>S. mutans</u>
Hemolysis	α	α	α	α/-	α/-
Optochin Sensitivity	-	+	-	-	-
Arginine Hydrolysis	+	-	-	-	-
Acid from: Mannitol	-	-	-	-	+
Sorbitol	-	-	-	-	+
Inulin	+	-	+	-	- ^v
Esculin Hydrolysis	+	-	+	-	+ ^v

Key: + = 85-100% strains positive

- = 0-15% strains positive

+^v(-^v) = 16-84% positive (negative)

Table 12

Recommended Identification Scheme for the Group D Streptococci (Division III)

Test	-----enterococci-----			-----nonenterococci-----		
	<u>S. faecalis</u>	<u>S. faecium</u>	<u>S. faecium var durans</u>	<u>S. bovis</u>	<u>S. equinus</u>	<u>S. avium</u>
Hemolysis	$\alpha/-/\beta$	$\alpha/-$	α	$\alpha/-$	α	α
Bile-esculin	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	-	-	-
Acid from:						
Arabinose	-	+	-	-	-	+
Glycerol	+	-	-	-	-	+
Sorbose	-	-	-	-	-	+
Lactose	+	+	+	+	-	+
Raffinose	-	-	-	+	-	-

Key: + = 85-100% strains positive

- = 0-15% strains positive

