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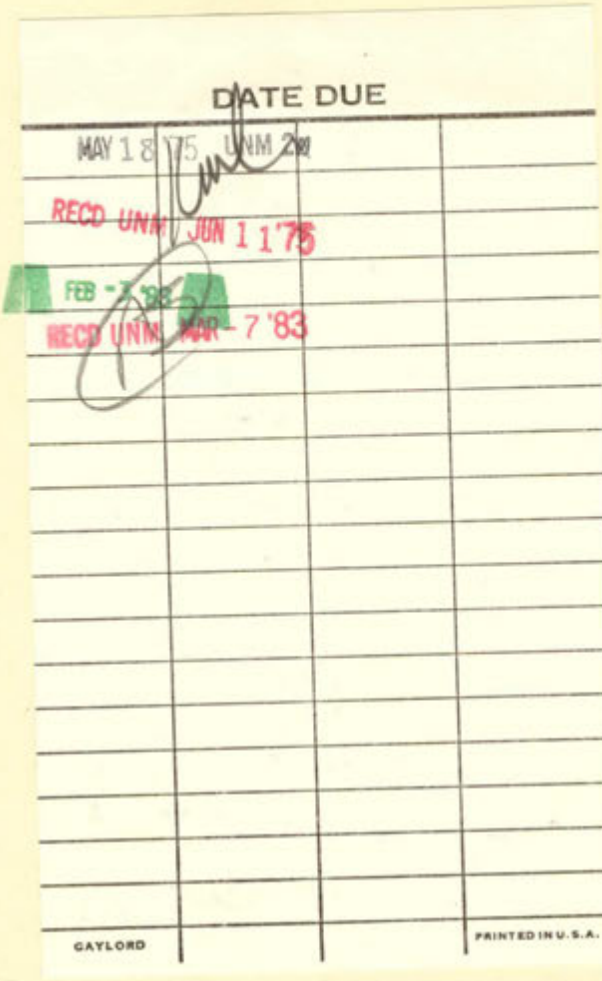
Rapid Identification of Streptococcal Infections Using Fluorescent Antibody Techniques

James H. Meadows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RAPID IDENTIFICATION OF STREPTOCOCCAL INFECTIONS USING FLUORESCENT ANTIBODY TECHNIQUES

By

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CHAPTER I

INTRODUCTION

Apparently, man is the most susceptible of all animals to streptococcal infections and it appears that no tissue or organ of his body is completely immune to these infections. Streptococci can cause epidemic diseases such as scarlet fever, erysipelas and epidemic sore throat. They also can cause puerperal fever, probably cause rheumatic fever and possibly rheumatoid arthritis. The ubiquity of this organism undoubtedly contributes to its causing a greater variety of clinical types of disease than any other organism (67). In addition to the above list of specific diseases for which this organism is responsible, other clinical infections such as carbuncles, impetigo, cellulitis, lymphangitis, septicemia, sinusitis, tonsillitis, pharyngitis, laryngitis, otitis media, mastoiditis, meningitis, brain abscesses, pneumonia, and wound infections should be added. In this latter group of clinical infections, it appears that the hemolytic streptococci are usually secondary invaders superimposed upon other bacterial and particularily viral infections (60).

With all due respect to the suppurative type diseases caused by streptococci, the non-suppurative sequelas, that is, acute glomerulonephritis, rheumatic fever, and rheumatoid arthritis, now

attributed to streptococcal infections, appear far more important both medically and socio-economically. Rheumatic fever is the sequalae noted in the majority of cases.

For many years an "unknown virus" plus infections by the pleuropneumonia-like organisms were thought to be the etiological agents causing rheumatic fever. However, today the laboratory and clinical evidence is almost conclusive that rheumatic fever follows an infection by group A streptococci. It appears that many different types of group A streptococci are involved yet no one metabolite has been definitely incriminated. Some investigative work published recently has demonstrated the possibility of a streptococcal cellular component, a macromolecular complex of group specific C-polysaccharide and a peptide. This complex possesses the capability of producing chronic remittent and intermittent reactions in the dermal connective tissue of rabbits (23, 55).

It has been estimated that rheumatic heart disease causes between 30,000 and 60,000 deaths in the United States each year and accounts for 90 per cent of deaths by heart disease in the four to 20 year age group (47). Further estimates reveal that about two million people living in the United States today have already had or will develop rheumatic fever at sometime in their life. Of these, more than 500,000 probably will die either directly from the rheumatic process or by some complication developing from it (25).

First attacks of rheumatic fever can be prevented through prompt treatment of streptococcal infections which entails the use of an antibiotic, preferably penicillin, for a period of 10 days. Herein lies the present clinician's dilemma because "strep throat" cannot always be distinguished clinically from sore throats caused by other organisms, but every "strep throat" is a potential cause of rheumatic fever (25). The per cent of correct clinical diagnoses of streptococcal throat infections ranges from 43 per cent (62) to 70 per cent (4); therefore, resortment to a laboratory diagnosis is imperative. There are many objections to indiscriminate penicillin therapy, and the clinician usually considers it advisable to delay treatment for two to three days until the true causative agent can be identified by a throat culture. He repeatedly is forced to decide whether to postpone treatment until a definite diagnosis is made, maybe to save the patient money as well as lower the risk of penicillin sensitivity, or to treat the infection with penicillin immediately on the chance it is of streptococcal origin.

To date, prevention of this first attack of rheumatic fever has been almost an impossibility, or a calculated risk, since there was no rapid and certain method available to the clinician to identify pathogenic streptococci.

The pursuit and subsequent identification of these hemolytic streptococci ends in the clinical laboratory. Except in the hands of

an expert, laboratory identification of these streptococci, frequently has failed and this failure unfortunately has not fostered the use of throat culturing by many clinicians (35).

The obstacles to receiving a quick and certain diagnosis of beta hemolytic streptococcal infections conceivably can be removed by application of a fluorescent antibody technique. The fluorescent antibody techniques are essentially immunological in nature therefore possessing the same capabilities and limitations but by comparison are more rapid to perform.

CHAPTER II

LITERATURE SURVEY

The basic fluorescent antibody concepts were conceived by Dr. Albert Coons, in 1939, while on a vacation in Germany (16). The concept stating the Aschoff nodule was a result of a local hypersensitive reaction involving components of the group A streptococci was popular at this time. He proposed testing this hypothesis by demonstrating an antigen or antibody, preferably both, in these lesions. The idea of labeling an antibody molecule with a visible label, it reacting with the antigenic material, then subsequently observing microscopically for the label, was an obvious approach.

In the early 1940's, Coons and coworkers of the Harvard Medical School introduced a method for coupling a fluorescent dye to antibodies (17, 18, 22) making it possible to relate microscopic observations to antigen-antibody reactions in fixed material. According to the methodology of Coons, antibodies are labeled with a fluorescein derivative, fluorescein isocyanate. When fluorescein is illuminated with ultraviolet or near-ultraviolet light, it has a yellow-green fluorescence which is visible in 1:1,000,000 dilution. It appears to be the most sensitive dye known at the present time.

In recent years antibodies have been labeled with other fluorochromes but the reactions of fluorescein-labeled antibodies

are brighter appearing microscopically (2). An isocyanate derivative of fluorescein was employed earlier for chemical coupling (19) but fluorescein isothiocyanate now has been proven to be quite successful (43, 53) and is preferred. Fluorescein isothiocyanate reacts readily with the crude globulin fractions of serum to form the fluorescein labeled antibody. The specific globulins involved, and loci of the chemical bonding has been of significance (24, 29, 52) mainly to explain non-specific and specific antigen-antibody reactions employing the fluorescent antibody methods.

The continued use of the fluorescent antibody methods as a histochemical tool (11, 12, 20, 21, 32, 37, 38) gave this new concept validity and popularity enough, whereas, its application was expanded to other systems of antigen-antibody reactions. In 1953, Goldman (27, 28) employed the method for differentiation of Endamoeba histolytica from Endamoeba coli and Sheldon demonstrated leptospira in human muscle (56). Somewhat later its application to viruses was investigated (69), the results of which were very encouraging for they demonstrated the rapidity and specificity sorely needed for rapid diagnosis of viral type infections (5, 30, 40-42). With this impetus the method was considered as a means for diagnosing clinical bacterial infections (26, 33, 44, 45, 58, 59, 63-65, 72).

The use of fluorescent antibody methods (10, 13-15) for diagnosis of streptococcal infections is an encouraging approach to the

problems in the epidemiology and prophylaxis of streptococcal infections and possible resulting sequelae (6, 34, 57). The efficacy of its use in identification of streptococci found in clinical material has been well established (31, 44), and its application in identification of streptococcal infections by reference laboratories is becoming rather widespread (9, 35, 44, 48, 51, 74).

With the tenets, rapidity and accuracy of the fluorescent antibody methods substantiated, the indication is that if general application were practiced some seven million cases of streptococcal infection could be treated selectively each year in the United States (34). This proposal of general application presents the smaller clinical laboratories with the problem of establishing the fluorescent antibody technique as a routine diagnostic procedure (68). Within these laboratories lies the paramount value of a rapid, accurate diagnosis because the clinician is available immediately with the patient at hand.

Basically this technique is simple and apparent. An antibody against a known antigen is produced by immunization of a laboratory animal, and this antibody then is labeled with a fluorochrome. When suspected material containing the homologous antigen is fixed upon a slide and allowed to come into contact with the fluorescent antibody an immunochemical reaction may take place. Granted this basic assumption, the excess or unreacted material will wash away leaving only the conjugated fluorescent antibody to homologous antigen. When a

fluorescence microscope equipped with a high intensity illumination is available the actual manipulations necessary to demonstrate antigenic material microscopically are very simple. Within the last year the fluorescein labeled antibodies against beta hemolytic streptococci have become available commercially thereby becoming accessible to the small clinical laboratory. The only other costs are in permanent equipment outlay for procurement of a high intensity ultraviolet light source equipped with proper exciter and barrier filters, plus, if not on hand already, a reflecting dark-field condenser.

This study was undertaken to test the application of the fluorescent antibody technique to clinic patients suspected of harboring beta hemolytic streptococci. An evaluation of two possible methods of diagnosis: a direct smear method which obviously would be advantageous for the immediate detection of a group A streptococcal infection; and a 3-4 hour broth culture method which, though not so rapid, would be considered such, in comparison with conventional methods requiring from 24-72 hours for identification. The conventional techniques of culture and identification (39, 50, 54, 70, 71) are used for comparison purposes in the final analysis of recovery rates and specificity of the two fluorescent antibody techniques employed.

CHAPTER III

MATERIALS AND METHODS

Collection of Throat Smears

Two hundred thirty-four throat scrapings were obtained from clinically sore throats sent to the Laboratory Service by the clinicians in the Pediatric Clinic, Adult Dependents Clinic, Hospital Wards, and Emergency Room. These clinicians were asked not to express their opinion on the request forms as to whether or not streptococcal infections were suspected but to send to the laboratory all those patients complaining of a "sore throat." They also were requested not to send any patient to this study who was receiving or had received any antibiotic or chemotherapeutic agent within the previous 14 days.

One throat scraping was obtained by scraping in the peritonsillar area with a modified plastic Ayre "cell biopsy" scraper (Clay-Adam Inc.) rather than a cotton or dacron swab. The selection of this scraper over cotton or dacron swabs, was made on the premise that it would produce a more representative and better diagnostic specimen. The scraper possesses edges that are rigid and rather sharp allowing collection of sub-surface material. Furthermore, the scraper disallows any possible loss of material that conceivably could be trapped within a swab, and when used to inoculate the broth media it will not absorb out any portion of the broth.

Preparation and Conjugation of Antigenic Material

A representative portion of the material from the scraper was smeared-out within a 1-1.5 centimeter diameter circle drawn with a "Lab Tek Plastic Marker" on a 1 x 3 inch microscopic slide, heat fixed, and properly coded. The remaining material on the scraper then was washed by agitation into 0.5 milliliters of trypticase soy broth (TSB-Difco) to which one drop of sterile whole sheep blood had been added (7).

After the TSB-sheep blood culture had incubated in a water bath for 3-4 hours at 37° C another smear was made in the same manner as the direct smear by using two bacteriological loopfuls of the well-shaken culture. Also at this time sheep blood agar plates with gentian violet added (final conc. 1:1,000,000) were inoculated with the remainder of the broth culture. These culture plates were used for beta hemolytic streptococci isolation and subsequent identification by conventional serologic testing.

Conjugation of Fluorescent Antibody to Antigen

The two smears thus obtained were overlayed with commercially obtained fluorescein labeled group A streptococci immune globulin (Sylvana Chemical Company, Orange, New Jersey, Lot #210611 and #210612) for 20 minutes in a moist chamber. They then were washed in two five minute washes of Sorensen's buffered, pH 7.0 (66), isotonic saline, with clearing in a distilled water rinse for five minutes, and

then air dried. After air drying, the slides were mounted under a cover slip using a glycerol-saline solution (nine parts glycerol and one part buffered saline) for mounting media.

Examination of Slides for Fluorescing Antigenic Material

Examination was performed with a binocular Zeiss microscope using a Zeiss power supply for constant line voltage to an HBO-200 Osram lamp mounted in a Zeiss housing, which was used for a high intensity source of ultraviolet light. A Zeiss BG12 with transmission range of 320-510 millimicrons, was used for exitation, and the Zeiss carrier mounted 500 millimicron and 440 millimicron filters were used for barrier filtering (8, 15, 17). Searching for the fluorescent streptococci was performed with high, dry Zeiss Neofluor objective lens, eight power oculars and a reflecting dark-field condenser.

Slides possessing the homologous antigen will show the organisms fluorescing with an intense yellow-green fluorescence with maximum fluorescence at the periphery of the organisms (45). Plate I demonstrates this fluorescence obtained using group A streptococci and homologous labeled antisera.

Specificity of the Labeled Immune Sera

It is quite apparent the fluorescent antibody method is only as good as its specificity. The specificity of each lot of antisera to group A beta hemolytic streptococci must be determined as well as any cross

reactivity to other streptococci possessing similar antigenic structures.

To ascertain this specificity, known groups of lyophilized streptococci, groups A, B, C, D, E, F, G, H, K, and L were obtained from the Biologicals Division, Walter Reed Army Institute of Research, Walter Reed General Hospital, Washington, 25, D. C. These organisms were recovered in thioglycollate broth (Difco), subcultured onto two consecutive sheep blood agar plates then used for study from trypticase soy broth stock cultures. Organisms from these broth cultures were subjected to the fluorescent antibody technique described above and observed for fluorescence.

Absorption Technique for Obtaining Group-Specific Labeled Antisera

It was anticipated that cross-reactions would occur using the labeled anti-A sera against groups C and G (44, 51). A method prescribed by Moody et al. (44) was employed to rid the labeled anti-A sera of cross-reactivity to these groups. Group C and G organisms were grown for 24 hours at 37° C in broth, killed by adding formalin (final concentration 0.2 per cent) and refrigerated overnight. The organisms were collected by centrifugation and washed three times in buffered saline. The labeled antisera was absorbed for 2 hours at 37° C using an equal volume of packed cells to antisera. The antisera was recovered by centrifugation and well shaken fractions checked microscopically for any retained organisms.

Experimental Controls

The controls consisted of heat-fixed preparations of known streptococcal groups A, C and G from trypticase soy broth cultures. The use of group A is to demonstrate maximum fluorescence expected and use of groups C and G is to observe that amount of cross-reactivity expected with organisms so antigenically similar (71).

Identification by Conventional Methods

The remaining portion of the trypticase soy culture as mentioned above was inoculated to sheep blood agar, incubated at 37° C for 24 hours and observed for beta hemolysis. Typical colonies were picked and inoculated onto a sheep blood agar and into tryptose phosphate broth (Difco). The plate contained an implanted bacitracin disk (Taxos A. Difco) (36) for routine laboratory reporting purposes and the tryptose phosphate broth was used in the cell recovery required for subsequent serologic grouping (39, 50). After twenty-four hours growth of these streptococci in 40 milliliters of tryptose phosphate broth they were centrifugalized, the broth decanted, and 0.5 milliliter of normal saline added to the sedimented cells. The organisms were resuspended by gentle shaking and then autoclaved for 15 minutes at 20 pounds pressure. The debris was sedimented by centrifugation and the clear supernatant used for the precipitin test in capillary tubes. Anti-streptococcus sera for groups A, B, C, D, F and G (Cappel Laboratories Division, West Chester, Pa.) was drawn up into capillary

tubes followed by the recovered extracts. Reactions were read within 30-45 minutes at room temperature.

Fluorescent Antibody Identification of Conventionally Isolated Organisms

A smear of the typical beta hemolytic isolates was subjected to the labeled antibody and observed for fluorescence. If maximum expected fluorescence as denoted by the controls used, was observed, they were considered to be group A.

CHAPTER IV

EXPERIMENTAL RESULTS

The Specificity of the Immune Sera

As was anticipated, the labeled anti-A immune sera demonstrated cross reactivity with other groups of the beta hemolytic streptococci. The groups cross reacting and the intensity of these cross reactions are shown in Table 1.

The use of only three terms to designate intensity of fluorescence is contrary to some authors using a one to four plus system. Their designations: 4+ equals cells sharply outlined, bright green fluorescence; 3 + equals cells sharply outlined, dull green fluorescence; 2 + equals cells not sharply outlined, slight green fluorescence; + equals cells not outlined, only faint green discernability in denser areas of the smear (44, 45, 51) offers too much interpretative latitude that could easily give conflicting results by an individual or between individuals.

The cross reactions noted in Table 1, i.e., Lot. 210611 with group C and Lot. No. 210612 with group G necessarily would make their differentiation impossible from group A by these techniques. By use of the absorption techniques employing group specific cellular material, the cross reaction fractions were lost to a degree sufficient for use in differentiation by the schema: plus (+) denotes maximum expected yellow-green fluorescence by the group A control (Plate I); plus-minus (±) denotes yellow-green fluorescence of a lower intensity

(Plate III) but discernible from plus (+); minus (-) denotes no yellow-green fluorescence present (Plate IV). The reactions of the absorbed labeled anti-A with other groups of beta hemolytic streptococci are shown in Table 2. Note in Table 2 that in Lot. No. 210611 the cross reaction with group C was reduced to plus-minus (±) and in Lot. No. 210612 the plus (+) cross reaction with G was reduced also to plus-minus (±). An interesting by-product of the absorption of Lot. No. 210612 with group G organisms reduced the group L cross reaction to no fluorescence.

This procedure of determining the specificity of anti-A labeled sera and absorbing out the cross reactivity if necessary, obviously is required in performing these fluorescence antibody techniques. Consequently, the various groups of beta hemolytic streptococci must be kept in stock culture for labeling as well as for obtaining large amounts of group specific material for absorption.

Results of These Techniques Applied to 234 Random Clinical Specimens

Using the absorbed labeled anti-A sera, 234 patients were submitted to the three techniques as described above. Table 3 denotes the results obtained.

Reduction of Accumulated Data

The data shown in Table 3 is accumulated and reduced in Table 4. This reduction is to the origin of specimens and results of the

direct, broth, and conventional methods employed.

Grouping of the Beta Hemolytic Streptococcal Isolates

The 48 isolates from conventional media were grouped by the fluorescent antibody method and by the extraction method. The extraction method demonstrated four isolates not belonging to group A; namely, one group B, one group G and two only designated as not group A. The fluorescent antibody technique applied to the isolates demonstrated the same 44 group A isolates as the extract technique and was not positive when applied to any of the four isolates not in group A. Three, one group G and the two ungrouped beta hemolytic isolates, occurred in the pediatric group and the one group B occurred in the adult group.

Per Cent of Streptococcal Infections as to Origin

The random specimens had their origin in four areas which may be shown as three distinct groups. The Pediatric Clinic only appoints patients to age 13, the Emergency Room sees service enlisted men and officers, the Department of Hospital Clinics appoints adult females (other than obstetric and gynecology patients), and the ward patients in this study were all adult females. The per cent of specimens these groups represent and the per cent of group A streptococci isolated by conventional means on their material is illustrated in Table 5.

Evaluation of the Fluorescent Methods Employed

The efficacy of the fluorescent antibody methods for use in

diagnosing group A beta hemolytic infections depends on their correlation with heretofore established, or conventionally accepted methods. A direct comparison of the three methods employed in this study is shown in Table 6. The number and per cent positives demonstrated by the three methods appear somewhat comparable but this interpretation should be reserved until the correlations between the methods are examined. In Table 7 the positive examinations using the direct smear with labeled antisera are correlated with the other two methods. Table 8 correlates the broth method positives with the direct and conventional methods and Table 9 the conventional isolates with the fluorescent methods.

To further assist in adjudging these techniques Table 10 reveals the above correlations using only total figures of the positive results.

CHAPTER V

DISCUSSION

The direct method demonstrated 40 total positives, but only 25 were confirmed by either or both of the other techniques. The 15 specimens not confirmed could be explained on the basis of their viability, for the direct method will demonstrate both viable and non-viable organisms, whereas the other two methods can conceivably demonstrate only viable ones. Contrary to this, the broth method demonstrated 24 positives and the conventional 23 positives not demonstrated by the direct method. This failure by the direct method may be attributed to population densities obtained on the smear, chance of that portion of inoculum not having the organism, or frank oversight while scanning the slides. This latter problem certainly must be considered because the artifacts are numerous and masking of the organisms by other material is highly probable. The direct method is an extremely laborious procedure if proper interpretation of observations is made. To properly appraise a slide, 15-20 minutes are required before a negative can be reported with assurance. In this study, all negative direct smears which subsequently were positive by one of the other methods, were reviewed to eliminate possible technical error. No more positives were observed than had previously been reported.

The broth method had the highest number of positives with 49 specimens demonstrating the presence of group A streptococci. It must

be assumed these were viable organisms although the possibility exists that the inoculum may have at times been heavy enough with non-viable organisms to be demonstrated in such a dilution. An encouraging aspect noted of this method is its possessing an apparent high degree of specificity when compared to conventional methodology. This is illustrated by its being positive in 43 of the 44 specimens shown to have group A organisms by the conventional method. Furthermore, it was negative in all four of the specimens possessing non-group A beta hemolytic isolates. The failure of this broth method to demonstrate the one other group A isolate shown by the conventional method could be attributed to technique.

Microscopic interpretation of the broth method is simple and easy. The streptococci assume their characteristic chain formations in the broth and population densities apparently are high enough after 3-4 hours that it is not uncommon when observing positive slides, to detect the fluorescing organisms within 3-5 fields at high power magnification.

If the broth method truely possesses the specificity it demonstrates above, then explanation should be made as to why six specimens, positive by the broth method, failed to be confirmed by the conventional method. First, cross-reactivity with the labeled antisera may have occurred with streptococci not considered in the specificity studies. Second, the original inoculum may have contained non-viable organisms in numbers

capable of demonstrating fluorescence. Third, the conventional methods used may have failed due to not using an enrichment broth and strict anaerobic conditions. An enrichment broth was considered for this study but reference to their use prescribed incubation periods up to 24 hours (34), this time period obviously defeats the basic approach of this study. The highly nutritive media selected for this study may be beneficially replaced by an enrichment media. The use of enrichment media for fluorescent antibody work has been established by a recent publication by Peeples, Spielman and Moody (48), using 0.5 milliliters of Todd-Hewitt broth in a field study comparing fluorescent antibody methods very similar to those described herein.

The purpose of this study is to evaluate two adaptations of the basic fluorescent antibody concept for use in establishing a rapid and accurate clinical diagnosis of streptococcal infections. The fluorescent antibody concept is basically a serological technique and is subject to the same limitations and capabilities (31). When subjecting a labeled antibody to homologous and heterologous antigens cross reactions may and do occur as was pointed out earlier in this study. However, by using absorption techniques and proper controls a labeled antibody with a high degree of specificity can be obtained.

Having established that this specificity exists when applied to groups of stock cultures the next attempt would be to establish it on clinical material. The better results denoting this specificity appear in the broth method. Positive results by this method correlated with

the conventional technique on 43 of 44 isolates and was negative in all 4 of the non-group A beta hemolytic isolates. Although this method failed to show positive with one group A conventional isolate, it was positive in 6 specimens not demonstrated by the conventional method. If the specificity of the fluorescent method is conceded, one may consider the broth method to have greater sensitivity than the conventional method.

Concluding that this degree of specificity exists in the labeled antisera, mention must be made of the 15 positive direct smears not confirmed by either of the other methods. If these are to be considered positives and the broth method positives are added to these, the incidence of group A organisms in the clinical material studied is 61 positives in 234 total specimens examined, or 26 per cent. To have approximately one in every four patients presenting themselves with a sore throat positive for a group A beta hemolytic streptococci infection points to a real need for establishing a rapid and accurate method of diagnosis. Moreover, if the specificity toward clinical material exists, the conclusion must be drawn that conventional methods missed six positives if compared to the broth method, and 17 positives if compared to the direct method.

A direct smear of material from a patient with symptoms is indeed the most valuable method if found to be positive. If consideration is given to implementing the direct smear method as a routine

procedure certain inherent problems associated with it should be considered. First, a negative cannot be considered negative. Second, the scanning time required for a valid interpretation of a direct smear can take 20 minutes or more, making it a time consuming procedure. Third, the presence of artifacts and extraneous cellular material may establish doubt in interpretation or mask any organisms present. The later problem could make any interpretation frankly impossible. These problems may possibly be circumvented, at least to some degree, by use of a contrasting fluorescent counterstain proposed by Smith, Marshall and Eveland (61). The counterstain proposed apparently has an affinity for animal cells yet does not interfer with serological systems similar to these used. Conceivably, counterstain employment would make scanning of suspect material simpler and interpretation of any yellow-green fluorescence easier.

The real value of the direct smear method may lie within its ability to measure non-viable organisms. The presence of any group A streptococci in the throat, whether viable or non-viable, may be of aid to the clinician.

The broth method, as adapted to the fluorescent antibody concept, appears to have all the attributes necessary for establishment as a diagnostic tool. Relative to conventional methods, it is a rapid method; moreover it possesses the attributes of being a sensitive, specific and practical laboratory procedure which lends itself well to clinical medicine.

The techniques in performing the test are relatively simple, indicating ease in the training of laboratory technicians. It is also economically less expensive both in materials and time (48, 74). The present, and most obvious, obstacle to its more general use would be the initial cost of the high intensity ultraviolet light source.

The justification of this expenditure could easily be made on the basis of practical applications offered by the fluorescent antibody techniques. The results shown in this study propound their use for rapid diagnosis of beta hemolytic streptococci; furthermore, rapid diagnosis of pathogenic Escherichia coli (46) is already at the clinical level and numerous other applications are to come (2).

Consideration should be made to teach the fluorescent antibody methods in undergraduate studies. To teach and exploit these methods in the diagnosis of streptococci alone, within the State of New Mexico, appears justified. The 1961 epidemiological reports indicate this State has over seven times the number of streptococcal infections than the United States as a whole (1, 49). In addition Winter and Moody (72, 73) have developed a labeled antisera for use in the rapid diagnosis of Pasteurella pestis, another disease peculiar to New Mexico, easily cured if diagnosed rapidly.

The application of fluorescent antibody techniques to the detection or identification of bacteria in clinical specimens, or mainly cultures from them, has developed rapidly in the past six years. A physician

has often been handicapped in choosing a therapeutic agent for treatment by not knowing what organism was inciting the disease. Conventional procedures for the identification may take one to several days for completion. The fluorescent antibody methods potentially provide rapid detection methods conceivably without maintaining viability. The sensitivity is extremely high , for theoretically, a diagnosis could be made by the observation of one fluorescing cell.

It is quite evident that labeled antibodies are potentially applicable to identification of all bacteria. Each antigen-antibody system employing their use must naturally be thoroughly investigated for rapidity, sensitivity and specificity under a severe control system (9).

Clinical studies, such as this one, demonstrate that fluorescent antibody methods for detection and identification of group A streptococcal infections are equal to or may exceed conventional methods. Of paramount importance is the fact that group determinations may be made in 3-4 hours instead of 3-4 days. The need for an early and rapid diagnosis is further borne out by the data shown in Table 5. Note that 75 per cent of the group A streptococci isolates occurred in the 65 per cent of specimens which were of pediatric origin. This is the age group which is apparently more highly infected and more susceptable to the consequences of the infections when not treated adequately. The rapidity, sensitivity and specificity of the methods

proposed in this study implicates their importance in preventing rheumatic fever and other sequelae attributed to streptococcal infections.

CHAPTER VI

SUMMARY

The use of fluorescent microscopy coupled with immuniological techniques offer new methods for the demonstration of antigen-antibody reactions. The adaptation of these techniques to grouping of beta hemolytic streptococci makes feasible their further adaptation toward identification of group A streptococci in clinical material. Commercially available labeled antibodies against group A beta hemolytic streptococci were obtained for use in the study. A method for the determination of labeled antisera specificity is proposed and a technique for absorbing out any heterologous reacting antibody is described. Using this commercial labeled antisera, subsequent to specificity studies plus absorption if necessary, adaptations of the basic fluorescent antibody techniques are suggested for the rapid diagnosis of clinical group A beta hemolytic streptococci infections.

Two adaptations, a direct smear method and a 3-4 hour culture method, are evaluated using conventional methods for comparison. Throat scrapings were obtained from 234 clinic patients with the main complaint of "sore throat." Direct smears were made of the material and broth cultures were inoculated. After 3-4 hours of incubating the broth culture, a smear was made and conventional media inoculated with the broth culture material. The bacteria in two smears were subjected to conjugation with labeled anti-group A streptococci sera and observed for fluorescence.

The results obtained from the fluorescent antibody methods indicate their possessing comparable specificity to conventional methods and conceivably higher sensitivity. The ease of performance and obvious practical application of such rapid techniques for the diagnosis of group A streptococcal infections are rather appealing. The methods appear to be specific and practical, and can be performed in the clinical laboratory as a routine procedure.

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TABLE 1. Cross Reactions With Other Groups of Streptococci

Lot Numbers of Labeled Anti-A Sera	Groups of Beta Hemolytic Streptococci									
	A	B	C	D	E	F	G	H	K	L
	a	c				b				
210611	+	-	+	-	-	±	-	±	-	±
210612	+	-	±	-	-	-	+	±	-	±

a Plus sign denotes maximum expected yellow-green fluorescence

b Plus-minus sign denotes yellow-green fluorescence of organisms present but easily discernible from + .

c Minus sign denotes no yellow-green fluoerescence of organisms noted.

TABLE 2. Cross Reactions With Other Groups of Streptococci Subsequent to Absorption

Lot Numbers of Labeled Anti-A Sera	Groups Beta Hemolytic Streptococci									
	A	B	C	D	E	F	G	H	K	L
	a	c	b							
210611	+	-	±	-	-	±	-	±	-	±
210612	+	-	±	-	-	-	±	±	-	-

a Plus sign denotes maximum expected yellow-green fluorescence of organisms present

b Plus-minus sign denotes yellow-green fluorescence of organisms present but easily discernible from + .

c Minus sign denotes no yellow-green fluorescence of organisms noted.

Lot Numbers of Labeled Anti-A Sera	Group of Beta Hemolytic Streptococci									
	A	B	C	D	E	F	G	H	K	L
210611	+[a]	-	+[c]	-	-	±[b]	-	±	-	±
210612	+	-	±	-	-	-	+	±	-	±

a Plus sign denotes maximum expected yellow-green fluorescence

b Plus-minus sign denotes yellow-green fluorescence of organisms present but easily discernible from +

c Minus sign denotes no yellow-green fluorescence of organisms noted.

TABLE 2. Cross Reactions With Other Groups of Streptococci Subsequent to Absorption

Lot Numbers of Labeled Anti-A Sera	Group of Beta Hemolytic Streptococci									
	A	B	C	D	E	F	G	H	K	L
210611	+[a]	-	±[b]	-	-	±[c]	-	±	-	±
210612	+	-	±	-	-	±	-	±	-	-

a Plus sign denotes maximum expected yellow-green fluorescence of organisms present

b Plus-minus sign denotes yellow-green fluorescence of organisms present but easily discernible from +

c Minus sign denotes no yellow-green fluorescence of organisms noted.

TABLE 3. Results from 234 Clinical Specimens

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
1	PC[b]	Neg	Neg		Neg	
2	PC	Pos	Pos	A	Beta Hemo	A
3	PC	Neg	Neg		Neg	
4	PC	Neg	Neg		Neg	
5	PC	Neg	Neg		Neg	
6	ER[d]	Neg	Pos	A	Beta Hemo	A
7	ER	Neg	Neg		Neg	
8	ER	Pos	Pos	A	Beta Hemo	A
9	ER	Neg	Neg		Neg	
10	PC	Neg	Neg		Neg	
11	PC	Neg	Neg		Neg	
12	ER	Neg	Neg		Neg	
13	PC	Neg	Neg		Neg	
14	ER	Neg	Neg		Neg	
15	PC	Neg	Neg		Neg	
16	PC	Neg	Neg		Neg	
17	PC	Neg	Neg		Neg	
18	PC	Neg	Neg		Neg	
19	DHC[c]	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
20	ER	Neg	Neg		Neg	
21	DHC	Neg	Neg		Neg	
22	DHC	Neg	Neg		Neg	
23	DHC	Neg	Neg		Neg	
24	PC	Pos	Pos	A	Beta Hemo	A
25	PC	Neg	Neg		Neg	
26	DHC	Neg	Neg		Neg	
27	PC	Pos	Pos	A	Beta Hemo	A
28	DHC	Neg	Neg		Neg	
29	DHC	Neg	Neg		Neg	
30	PC	Neg	Neg		Neg	
31	DHC	Neg	Neg		Neg	
32	DHC	Pos	Neg		Neg	
33	PC	Neg	Neg		Neg	
34	ER	Neg	Pos	A	Beta Hemo	A
35	PC	Neg	Neg		Neg	
36	PC	Neg	Pos	A	Beta Hemo	A
37	PC	Neg	Neg		Neg	
38	PC	Neg	Neg		Neg	
39	PC	Neg	Neg		Neg	
40	DHC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
41	PC	Neg	Neg		Neg	
42	ER	Neg	Neg		Neg	
43	PC	Neg	Neg		Neg	
44	PC	Neg	Neg		Neg	
45	DHC	Neg	Neg		Neg	
46	ER	Neg	Neg		Neg	
47	PC	Neg	Neg		Neg	
48	ER	Neg	Neg		Neg	
49	ER	Neg	Neg		Neg	
50	PC	Neg	Neg		Neg	
51	PC	Neg	Neg		Neg	
52	DHC	Neg	Neg		Neg	
53	PC	Neg	Neg		Neg	
54	PC	Neg	Neg		Neg	
55	PC	Neg	Neg		Neg	
56	PC	Neg	Neg		Neg	
57	ER	Pos	Neg	Not A	Pos	B
58	DHC	Neg	Neg		Neg	
59	PC	Neg	Pos	A	Beta Hemo	A
60	PC	Pos	Pos		Neg	
61	PC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
62	PC	Neg	Neg		Neg	
63	DHC	Neg	Neg		Neg	
64	ER	Neg	Pos	A	Beta Hemo	A
65	ER	Neg	Neg		Neg	
66	PC	Neg	Pos	A	Beta Hemo	A
67	PC	Pos	Pos		Neg	
68	PC	Neg	Neg		Neg	
69	PC	Neg	Pos	A	Beta Hemo	A
70	PC	Neg	Pos	A	Beta Hemo	A
71	PC	Pos	Neg		Neg	
72	PC	Pos	Pos	A	Beta Hemo	A
73	PC	Neg	Neg		Neg	
74	Wd 5[e]	Neg	Neg		Neg	
75	PC	Neg	Neg		Neg	
76	PC	Pos	Pos	A	Beta Hemo	A
77	PC	Neg	Neg		Neg	
78	PC	Neg	Neg		Neg	
79	ER	Pos	Neg		Neg	
80	ER	Neg	Neg		Neg	
81	PC	Neg	Pos	A	Beta Hemo	A
82	PC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
83	ER	Neg	Neg		Neg	
84	PC	Neg	Neg		Neg	
85	DHC	Neg	Neg		Neg	
86	PC	Pos	Neg		Neg	
87	PC	Pos	Pos	A	Beta Hemo	A
88	DHC	Neg	Neg		Neg	
89	DHC	Neg	Neg		Neg	
90	PC	Neg	Neg		Neg	
91	ER	Neg	Neg		Neg	
92	DHC	Neg	Neg		Neg	
93	PC	Pos	Pos	A	Beta Hemo	A
94	PC	Pos	Pos	A	Beta Hemo	A
95	PC	Neg	Neg		Neg	
96	PC	Neg	Neg		Neg	
97	PC	Neg	Neg		Neg	
98	DHC	Neg	Neg		Neg	
99	DHC	Pos	Neg		Neg	
100	PC	Neg	Neg		Neg	
101	PC	Neg	Neg		Neg	
102	PC	Neg	Neg		Neg	
103	DHC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
104	PC	Neg	Neg		Neg	
105	PC	Neg	Neg		Neg	
106	Wd 4	Neg	Pos	A	Beta Hemo	A
107	PC	Neg	Pos	A	Beta Hemo	A
108	PC	Neg	Neg		Neg	
109	PC	Neg	Neg		Neg	
110	PC	Neg	Neg		Neg	
111	PC	Neg	Neg		Neg	
112	Wd 4	Neg	Neg		Neg	
113	DHC	Neg	Neg		Neg	
114	PC	Neg	Neg		Neg	
115	PC	Neg	Neg		Neg	
116	DHC	Neg	Neg		Neg	
117	PC	Neg	Neg		Neg	
118	PC	Pos	Neg		Neg	
119	PC	Pos	Neg		Neg	
120	PC	Neg	Neg		Neg	
121	PC	Pos	Neg		Neg	
122	PC	Pos	Pos	A	Beta Hemo	A
123	PC	Pos	Pos	A	Beta Hemo	A
124	PC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
125	PC	Neg	Neg		Neg	
126	PC	Pos	Pos	A	Beta Hemo	A
127	PC	Neg	Neg		Neg	
128	PC	Neg	Neg		Neg	
129	PC	Neg	Pos	A	Beta Hemo	A
130	PC	Neg	Neg		Neg	
131	PC	Neg	Neg		Neg	
132	DHC	Neg	Neg		Neg	
133	PC	Pos	Pos	A	Beta Hemo	A
134	ER	Neg	Pos	A	Beta Hemo	A
135	ER	Pos	Pos	A	Beta Hemo	A
136	DHC	Neg	Neg		Neg	
137	PC	Neg	Neg		Neg	
138	PC	Neg	Neg		Neg	
139	ER	Neg	Neg	Not A	Beta Hemo	Non group-able
140	PC	Neg	Neg		Neg	
141	DHC	Pos	Pos	A	Beta Hemo	A
142	PC	Neg	Neg		Neg	
143	ER	Neg	Neg		Neg	
144	ER	Pos	Neg		Neg	
145	ER	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number [a]	Origin	Fluorescent Technique			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
146	DHC	Neg	Neg		Neg	
147	PC	Pos	Neg		Neg	
148	PC	Neg	Neg		Neg	
149	PC	Neg	Neg	Not A	Beta Hemo	G
150	DHC	Neg	Neg		Neg	
151	PC	Neg	Neg		Neg	
152	PC	Neg	Neg		Neg	
153	ER	Neg	Neg		Neg	
154	PC	Neg	Neg		Neg	
155	PC	Neg	Neg		Neg	
156	PC	Neg	Neg		Neg	
157	PC	Neg	Neg		Neg	
158	DHC	Neg	Neg		Neg	
159	PC	Neg	Neg	A	Beta Hemo	A
160	PC	Neg	Neg		Neg	
161	ER	Pos	Neg		Neg	
162	ER	Pos	Neg		Neg	
163	DHC	Neg	Neg		Neg	
164	PC	Neg	Neg		Neg	
165	PC	Neg	Neg		Neg	
166	PC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
167	PC	Neg	Neg		Neg	
168	PC	Neg	Neg		Neg	
169	PC	Neg	Neg		Neg	
170	ER	Neg	Neg		Neg	
171	ER	Pos	Neg		Neg	
172	PC	Neg	Neg		Neg	
173	PC	Neg	Neg		Neg	
174	PC	Neg	Neg		Neg	
175	PC	Neg	Neg		Neg	
176	PC	Neg	Neg		Neg	
177	ER	Neg	Neg		Neg	
178	PC	Neg	Pos	A	Beta Hemo	A
179	PC	Neg	Neg		Neg	
180	ER	Neg	Neg		Neg	
181	PC	Neg	Neg		Neg	
182	PC	Neg	Neg		Neg	
183	PC	Neg	Neg		Neg	
184	PC	Neg	Neg		Neg	
185	PC	Pos	Pos	A	Beta Hemo	A
186	PC	Neg	Pos	A	Beta Hemo	A

TABLE 3. Results from 234 Clinical Specimens - Contd

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
187	ER	Neg	Neg		Neg	
188	PC	Pos	Pos	A	Beta Hemo	A
189	PC	Neg	Neg		Neg	
190	ER	Neg	Neg		Neg	
191	ER	Neg	Neg		Neg	
192	PC	Neg	Neg		Neg	
193	ER	Neg	Neg		Neg	
194	ER	Neg	Neg		Neg	
195	ER	Neg	Pos	A	Beta Hemo	A
196	DHC	Pos	Pos		Neg	
197	DHC	Neg	Neg		Neg	
198	PC	Neg	Neg		Neg	
199	PC	Neg	Neg	Not a	Beta Hemo	Non group-able
200	PC	Neg	Neg		Neg	
201	PC	Pos	Pos	A	Beta Hemo	A
202	ER	Pos	Pos	A	Beta Hemo	A
203	ER	Pos	Pos	A	Beta Hemo	A
204	ER	Neg	Pos	A	Beta Hemo	A
205	ER	Neg	Pos		Neg	
206	ER	Pos	Pos		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Technique			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
207	PC	Neg	Neg		Neg	
208	PC	Neg	Neg		Neg	
209	DHC	Neg	Neg		Neg	
210	ER	Neg	Neg		Neg	
211	PC	Neg	Pos		Neg	
212	PC	Neg	Pos	A	Beta Hemo	A
213	PC	Neg	Pos	A	Beta Hemo	A
214	PC	Neg	Pos	A	Beta Hemo	A
215	PC	Neg	Neg		Neg	
216	PC	Neg	Neg		Neg	
217	ER	Neg	Neg		Neg	
218	PC	Neg	Neg		Neg	
219	PC	Neg	Neg		Neg	
220	PC	Neg	Neg		Neg	
221	DHC	Neg	Neg		Neg	
222	PC	Neg	Neg		Neg	
223	PC	Neg	Neg		Neg	
224	PC	Neg	Neg		Neg	
225	ER	Neg	Neg		Neg	
226	PC	Neg	Neg		Neg	

TABLE 3. Results from 234 Clinical Specimens - Contd.

Patient Number[a]	Origin	Fluorescent Techniques			Conventional Techniques	
		Direct	Broth	Isolate Groups	Culture	Extract Groups
227	PC	Neg	Neg		Neg	
228	PC	Neg	Neg		Neg	
229	PC	Pos	Neg		Neg	
230	PC	Neg	Neg		Neg	
231	PC	Neg	Pos	A	Beta Hemo	A
232	PC	Neg	Pos	A	Beta Hemo	A
233	ER	Pos	Pos	A	Beta Hemo	A
234	PC	Neg	Neg		Neg	

a All patient names held on confidential file in Laboratory Service, United States Army Hospital, Sandia Base, New Mexico.
b Pediatric Clinic
c Department of Hospital Clinics - This is an adult female clinic.
d Emergency Room - This is the military dispensary used primarily by enlisted and officer personnel.
e Ward (number) refers to inpatients and their location within the hospital.

TABLE 4. Reduction of Accumulated Data

Origin	Number of Specimens	Positive for Group A by Fluorescent Method		Beta Hemolytic Isolates on Conventional Media
		Direct	Broth	
PC[c]	150 (64.1%)	24 (16.0%)	33 (22.0%)	36[a] (24.0%)
ER[d]	47 (20.1%)	13 (27.7%)	13 (27.7%)	10[b] (21.3%)
DHC[e]	34 (14.5%)	3 (8.8%)	2 (5.9%)	1 (2.9%)
Wards[f]	3 (1.3%)	-	1 (33.3%)	1 (33.3%)
TOTAL	234 (100%)	40 (17.1%)	49 (20.9%)	48 (20.5%)

a Three were not group A isolates
b One was not group A isolate
c Pediatric Clinic
d Emergency Room
e Department of Hospital Clinics
f Wards

TABLE 5. Per cent of Total Specimens and Group A Isolates of Three Groups Derived from Random Sampling

	Under 13 Years	Adult Male	Adult Female
% of 234 Specimens	64.1%	20.1%	15.8%
% of Group A Isolates	75.0%	20.5%	4.5%

TABLE 6. Direct Comparison on Methods Employed.

Methods Employed on 234 Clinical Specimens							
Fluorescent Methods				Conventional Methods			
Direct		Broth					
Positive for Group A	%	Positive for Group A	%	Positive Beta Hemolytic	%	Positive for Group A	%
40	17.1	49	20.9	48	20.5	44	18.8

TABLE 7. Correlation of the Direct Smear Method

Positive Direct Smears With:	PC[a]	ER[b]	DHC[c]	Wd[d]	Total
No Correlating Method	8	5	2	0	15
Broth Method Only	2	1	1	0	4
Conventional Method Only	0	0	0	0	0
Broth and Conventional	16	2	3	0	21
			Grand Total		40

a Pediatric Clinic
b Emergency Room
c Department of Hospital Clinics
d Wards

TABLE 8. Correlations of the Broth Method

Positive Broth Smears With:	PC[a]	ER[b]	DHC[c]	Wd[d]	Total
No Correlating Method	1	1	0	0	2
Direct Method Only	2	1	1	0	4
Conventional Method Only	16	5	0	1	22
Direct and Conventional	16	2	3	0	21
		Grand Total			49

a Pediatric Clinic
b Emergency Room
c Department of Hospital Clinics
d Wards

TABLE 9. Correlations of the Conventional Method

Positive Conventional Method With:	PC[a]	ER[b]	DHC[c]	Wd[d]	Total
No Correlating Method	1	0	0	0	1
Direct Method Only	0	0	0	0	0
Broth Method Only	16	5	0	1	22
Direct and Broth Method	16	3	2	0	21
		Grand Total			44

a Pediatric Clinic
b Emergency Room
c Department of Hospital Clinics
d Wards

TABLE 10. Correlation of the Three Methods Employed

	Methods Employed		
	Direct	Broth	Conventional
Total Positive for Group A of 234 Specimens	40	49	44
Correlated with Direct		25	21
Correlated with Broth	25		43
Correlated With Conventional	21	43	

TABLE 10. Correlation of the Three Methods Employed

	Methods Employed		
	Direct	Broth	Conventional
Total Positive for Group A of 235 Specimens	40	49	44
Correlated with Direct		25	41
Correlated with Broth	25		43
Correlated W.U. Conventional	21	40	

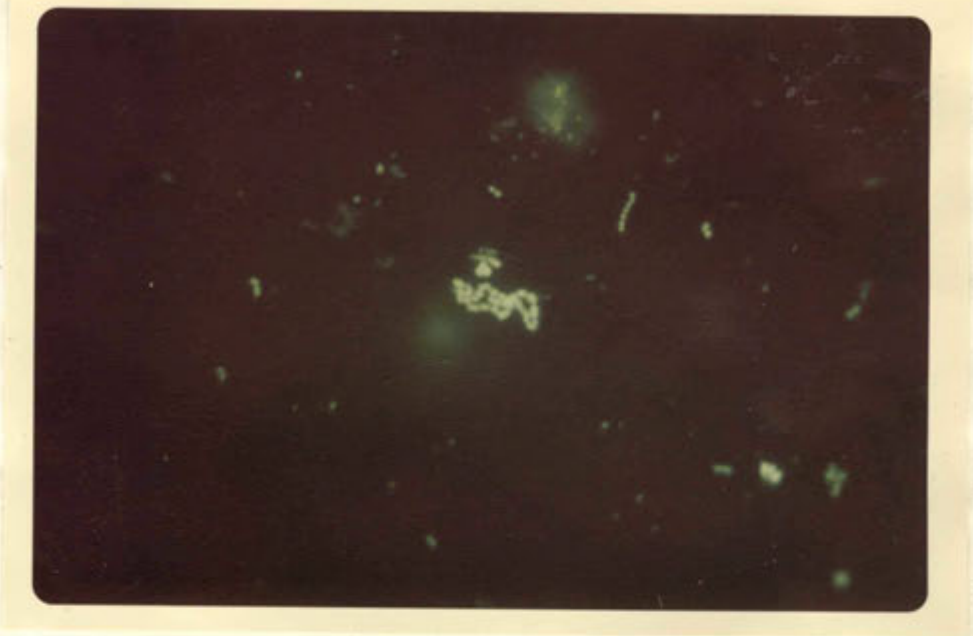

Plate I. Group A beta hemolytic streptococci conjugated with labeled anti-group A sera. x 500, High Speed Ektachrome, 8 min.

Plate II. Group A beta hemolytic streptococci conjugated with labeled anti-group A sera. Prepared from broth culture of Patient #203. x 500, High Speed Ektachrome, 8 min.

Plate I. Group A beta hemolytic streptococci conjugated with labeled anti-group A sera. x 500, High Speed Electrons, 5 min.

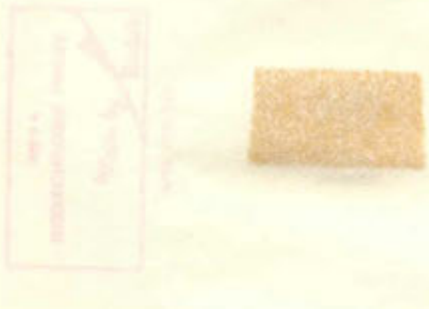

Plate II. Group A beta hemolytic streptococci conjugated with labeled anti-group A sera. Prepared from broth culture or patient #203. x 500, High Speed Electrons, 5 min.

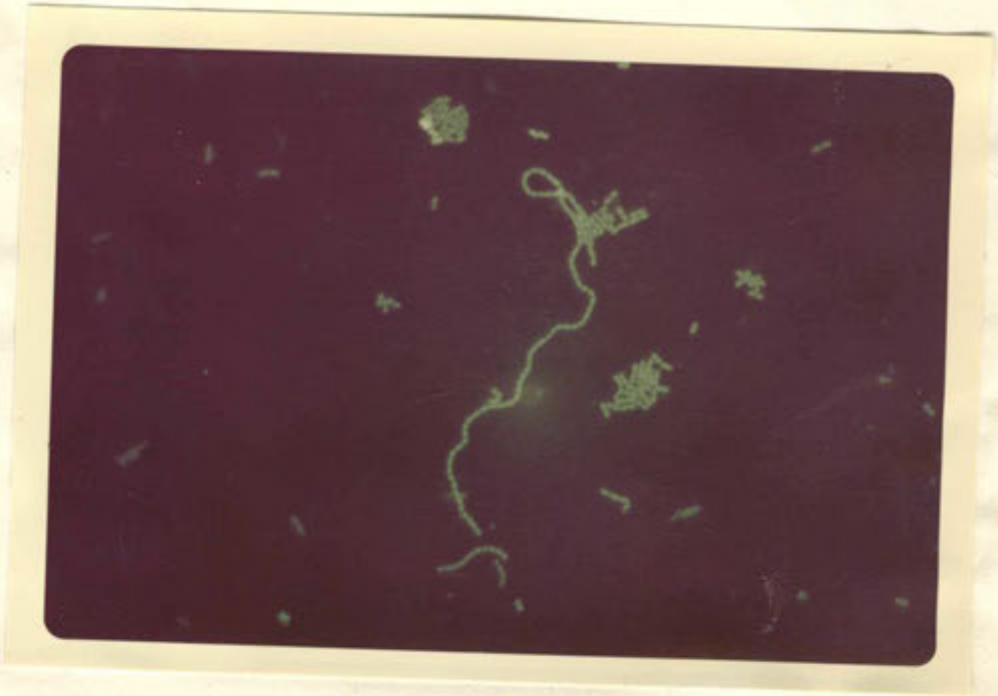

Plate III. Group G beta hemolytic streptococci conjugated with labeled anti-group A sera. x 500, High Speed Ektachrome, 8 min.

Plate IV. Group B beta hemolytic streptococci conjugated with labeled anti-group A sera. x 500, High Speed Ektachrome, 8 min.

IMPORTANT!

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