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Comparison of Grouping Methods for Beta-Hemolytic Streptococci

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COMPARISON OF GROUPING METHODS FOR BETA-HEMOLYTIC STREPTOCOCCI

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Renee V. Smith

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COMPARISON OF GROUPING METHODS FOR
BETA-HEMOLYTIC STREPTOCOCCI

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BETA-HEMOLYTIC STREPTOCOCCI

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A total of 198 strains of beta-hemolytic streptococci were Lancefield grouped by the conventional nonserological method and the Phadebact Co-agglutination method. These two methods were then compared to the Lancefield precipitin test. The Phadebact Co-agglutination method and the non-serological method were evaluated as to their sensitivity, accuracy, and suitability as methods for serogrouping beta-hemolytic streptococci in clinical laboratories. Due to the difference in their grouping ability only Lancefield Groups A and B could be directly compared. There was a 100 percent agreement between both of these methods for the Lancefield groups A and B, and there were three specimens which were nongroupable by both methods. Identical results for Groups A, B, C, and G were obtained for the Phadebact Co-agglutination method and the Lancefield precipitin test. Only 1.5 percent of the beta-hemolyitc streptococci were nongroupable by Phadebact and the Lancefield precipitin test as compared to 41 percent which were nongroupable by the nonserological method.

A cost-benefit analysis comparison between Phadebact and the nonserological method showed that the Phadebact method was $0.79 less expensive per test than the non-serological method. The Phadebact Co-agglutination method also required only 1 min to group the beta-hemolytic streptococci; however, the nonserological method required an additional 18-24 h.

INTRODUCTION

The streptococci represent a large group of fastidious gram positive bacteria. Most streptococci are facultative anaerobes and non-motile. They tend to grow in chains of variable length and are catalase negative (Clewell 1981).

Streptococci can be divided into serological groups by the Lancefield system on the basis of the presence of a group specific carbohydrate in the cell wall. This permits the arrangement of the streptococci into a number of antigenic groups termed group A, B, C, D, etc.

The last two decades have witnessed important changes in the understanding and in the pattern of interaction of streptococci with the human host. From the mid-1940's until the mid-1960's, there was only a scattering of reports of human infections by streptococci other than Streptococcus pyogenes and the enterococci (Feingold et al. 1966). In 1966, Feingold et al. demonstrated the importance of various serological groups of streptococci as causative agents of extrarespiratory infections (Cunniff and Bump 1976). A similar study by Reinarz and Sanford (1965) revealed that isolation of Lancefield groups other than Group A were found to be pathogens in the human host.

The role of streptococci in suppurative diseases, such as pharyngitis (strep throat), scarlet fever, erysipelas, cellulitis of the skin, and subcutaneous tissue, is well known. Related non-suppurative diseases include acute glomerulonephritis and acute rheumatic fever. Most of these illnesses are associated with infections by Streptococcus pyogenes (Lancefield Group A) which constitutes 48.4 percent of all beta-hemolytic streptococci (Pollock and Dahlgreen 1974). However, non-group A beta-hemolytic strains are being reported as pathogenic agents with increasing frequency (Clewell 1981).

Group B strains of streptococci (Streptococcus agalactiae) have been implicated in a wide variety of human infections (Kirkegaard and Field 1977). The Group B streptococci comprise 19.9 percent of all beta-hemolytic streptococci (Pollock and Dahlgreen 1974). The high mortality and morbidity associated with Group B streptococcal infections in newborns such as neonatal meningitis and septicemia have made the rapid and accurate identification of this organism a high-priority item in most clinical laboratories (Fenton and Harper 1979).

Group C streptococci comprise approximately 11 percent of all beta-hemolytic streptococcal isolates. Group C streptococci frequently cause infections similar to those of Group A (Feingold et al. 1966). Although they are considered to be a low pathogenicity, Group C streptococci

may cause serious and fatal diseases such as acute endocarditis (Finnegan et al. 1974).

Approximately 7 percent of all beta-hemolytic streptococci are Group G. This group is known to inhabit the normal pharynx and female genital tract. Most infections caused by this group are puerperal (Baker 1974). Duma et al. (1969) found that patients infected with Group G streptococci generally had some underlying diseases usually some type of sarcoma (Cunniff and Bump 1976).

Other groups, notably D and F, constitute only 2-3 percent of all beta-hemolytic streptococcal isolates (Facklam et al. 1979). Group F streptococci have been associated with severe diseases in man; however, they are also commonly isolated from the head or respiratory tract and thus the presence of the organism does not imply a disease process (Cunniff and Bump 1976). Group D streptococci, a normal inhabitant of the gut, have been found to be involved in endocarditis and urinary tract infections. The Group D streptococci are also frequently implicated in root canal infections (Clewell 1981).

Differentiation of beta-hemolytic streptococci into Lancefield groups is important for the treatment as well as the prognosis of the patient (Moody and Wannamaker 1972). Streptococci belonging to Groups A, B, C, and G are most often associated with human streptococcal infections; therefore, the ability to identify these groups is highly desirable (Wadstrom 1974). The Lancefield Precipitin Test has been used since 1933 and still represents the standard

methodology (Feingold et al. 1966). This method extracts the carbohydrate antigen from the cell wall of the streptococci and reacts the antigen with group-specific antisera. This method, however, is time consuming, and the cost of the antisera makes the method unacceptable for many laboratories (Burdash et al. 1981). Probably the most applicable means for presumptive grouping of beta-hemolytic streptococci is by a battery of five nonserological tests (Facklam et al. 1974). Each test was selected because it was sensitive and highly specific for a particular species or group of organisms. The nonserological methods included determination of hemolysis for all strains, bacitracin susceptibility for Group A streptpcocci, hippurate hydrolysis for Group B, and bile esculin reaction for Group D. Enterococcal Group D streptococci were differentiated from non-enterococcal Group D streptococci by 6.5 percent sodium chloride tolerance. If the beta-hemolytic streptococci are negative for all of the above tests, the organism is said to be beta-hemolytic streptococci not Group A, B, or D. These tests should be used as a battery and not as single entities (Facklam et al, 1974). In the spring of 1980, Dr. L.P. Elliott (personal communication) conducted a survey of 11 clinical laboratories in Kentucky and one laboratory in Indiana. The results of the survey indicated that these laboratories used the five presumptive tests for grouping beta-hemolytic streptococci. The only significant difference was that in grouping the

Group B streptococci, the CAMP test (Darling 1975) was used rather than the hippurate hydrolysis.

Other methods which allow for grouping pathogenic streptococci have been tested. The immunofluorescence test is rapid and reliable but requires special training and equipment (Burdash et al. 1981). The agglutination methods, however, have now enabled laboratories to definitely group the clinically important beta-hemolytic streptococci with the aid of a simple slide test (Slifkin and Pouchet-Melvin 1980). This can have important consequences in economically underpriviledged areas of the world where the need for accurate and rapid detection of streptococcal infections is particularly acute. In addition, it is expected that better and more homogenous epidemologic information will be gathered concerning the world-wide distribution of the less common groups of beta-hemolytic streptococci (Clewell 1981).

Recently, kits for serogrouping beta-hemolytic streptococci by latex-agglutination and co-agglutination have become commercially available. The three most recognized serogrouping kits are the Phadebact, Serostat, and Streptex. A comparison of these kits based on suitability, sensitivity, and accuracy showed that the Phadebact Streptococcus Test (Pharmacia Diagnostics) was the most proficient and versatile method for serogrouping beta-hemolytic streptococci (Slifkin and Pouchet-Melvin 1980).

A similar study (Burdash et al. 1981) also showed that Phadebact was the best kit for simple, rapid and reliable grouping of streptococci. The Phadebact test is based on the co-agglutination principle. This technique is sensitive due to the coating of Cowan I non-viable staphylococci (carrier staphylococci) with rabbit gamma globulin, i.e., specific antibodies produced that are specific for Streptococcal groups A, B, C and G. When these specific antibodies, which have their Fc portion absorbed to the staphylococci protein A and its antigen-combining Fab portion directed outward, contact the corresponding antigen from a patient isolate the reaction results in a co-agglutination which is visible to the naked eye (Slifkin and Pouchet-Melvin 1980).

Since most hospitals in Western Kentucky are using the five tests for presumptive identification of streptococci, the purpose of this investigation was to (1) compare the Phadebact method against the presently used presumptive biochemical tests and the Lancefield Precipitin test and (2) conduct a cost-benefit analysis for the method now employed in most Western Kentucky hospitals and the Phadebact method.

MATERIALS AND METHODS

STRAINS

All clinical strains of beta-hemolytic streptococci were obtained from the microbiology laboratory of Greenview Hospital in Bowling Green, Kentucky. There were 200 strains studied over a 14-month period during 1981-1982. Source of the specimen, sex of the patient, and the date of the culture were recorded for each of the strains. Routine subculturing was carried out every 6-7 days on human blood agar plates (Difco Dehydrated Media), incubated aerobically at 35 C for 18-24 h and stored at room temperature. One strain of Staphylococcus aureus, obtained from the microbiology laboratory at Greenview Hospital, Bowling Green, Kentucky, was shown to produce beta-hemolysin and was used as the indicator organism.

ISOLATION OF STREPTOCOCCI

All strains being tested were streaked on tryptic soy agar plates with 5 percent sheep blood (BAP) (Gibco Laboratories, Madison, Wisconsin) to obtain isolated colonies. The plates were incubated at 35 C under aerobic conditions for 18-24 h. The plates were observed for the presence of beta-hemolytic streptococci, and the colonial

morphology was studied using a Quebec Colony Counter. Gram stains and catalase activity were performed from these initial plates for all 200 isolates. Isolated colonies of beta-hemolytic streptococci from the BAP were grouped by two different methods: (1) nonserological (physiological) and (2) serological.

GROUPING OF STREPTOCOCCI

Physiological Testing

The beta-hemolytic colonies suspected of being streptococci were tested for bacitracin sensitivity to differentiate group A from the other Lancefield groups (Stoner 1978). Commercial bacitracin disks (Taxo A 0.04 U disks) were purchased from Gibco Laboratories. Tryptic soy agar (Gibco Laboratories) plates with 5 percent sheep blood were marked into six regions, and each region was streaked with a loop (3 mm) containing 4-5 colonies in two different directions across the specific region of the plate. The bacitracin disk was placed in the center of each inoculum. The plates were inverted and incubated aerobically at 35 C for 18-24 h (Stoner 1978). The isolates were considered positive when there was no growth up to the bacitracin disk (Facklam et al. 1974).

For the presumptive identification of Group B streptococci the CAMP test was employed. A stock culture of beta-toxin-producing Staphylococcus aureus was used as the "indicator" organism. The indicator strain was streaked

in a straight line across the center of the BAP. Strains of streptococci to be tested were streaked in a straight line 2-3 cm in length and at right angles of the indicator strain with care taken not to touch the indicator organism. The plates were inverted and incubated aerobically in a candle jar at 35 C for 18-24 h. The production of a distinct "arrowhead" of hemolysis constituted a positive CAMP reaction (Darling 1975).

Commercial Bile Esculin agar (Gibco Laboratories) was inoculated with 2-3 colonies of beta-hemolytic streptococci. The plates were inverted and incubated at 35 C for 24-48 h. The production of a brownish-black color was indicative of Group D streptococci (Facklam et al. 1974). Enterococcal group D streptococci were differentiated from non-enterococcal group D streptococci by 6.5 percent sodium chloride tolerance.

Serological Tests

The Phadebact Streptococcus Colony Test Method was performed according to the manufacturers instructions (Pharmacia Diagnostic, Pitcataway, NJ). The Phadebact Test is based on the principle of co-agglutination antibodies being produced in rabbits and specific against groups A, B, C, and G streptococci which bind to Protein A on the surface of the non-viable cells of Staphylococci. From an 18-24 h BAP culture, 5-6 colonies were transferred by a wooden applicator stick to a drop of buffer solution on a serological

slide. A drop of the group antisera was added, and the mixture was rocked for 1 min by tilting the slide to an angle of 45^{o} every 2 sec. Co-agglutination was determined by using an indirect light against a dark background.

The Lancefield Precipitin Test was used as the reference method. The carbohydrate antigen was extracted by the autoclave method (Rantz and Randall 1955). An 18-24 h culture from a BAP (BBL Cockeysville, MD) was inocualted in 30 ml of Todd Hewitt Broth. After incubation of the broth at 35 C for 18-24 h, the cells were packed by centrifugation at 10,000 rpm for 10 min (IEC International Centrifuge, rotor radius 12 cm). After autoclaving the tubes for 15 minutes at 121 C, the tubes were centrifuged at 1300 rpm for 2 min (IEC International). Lancefield grouping was performed using the capillary precipitin test (Slifkin and Pouchet-Melvin 1980). Commercial antisera (Burroughs Wellcome Laboratories, Greenville, NC) for Groups A, B, C, D, and G were employed in the performance of the precipitin test. The antisera were centrifuged to make sure that no turbidity was present. This was done by constructing a small-pointed tube from a Pasteur pipette. The tip of the pipettes was removed and also cut on the other end to make the tube 8.5 cm long. The tapered end was heated and sealed. This tube was inserted into a small screw-capped test tube (10 cm Pyrex) and autoclaved. The antisera were

pipetted into the small inner tube and centrifuged at 1300 rpm for 1 min and then were centrifuged again at 850 rpm for 2 min (IEC Clinical Centrifuge). The tubes were then stored in a refrigerator until use.

A capillary tube (1.2-1.5 mm outside diameter, Kimble borosilicate glass with both ends open and lightly fire-polished) was dropped into the serum (in screwcap vials) until a column about 1 cm long had been drawn up by capillary action. The tube was next dipped into the extract, and an amount equal to the serum column was collected. The tube was inserted into Plastine until a small plug filled the opening. The tube was inverted and gently inserted into a wooden rack. After 3-5 min, the tubes were examined with a bright light against a dark background. A white cloud or ring at the center of the column represented a positive result.

COST-BENEFIT ANALYSIS

The total cost for both the presumptive identification test and the Phadebact Streptococcus Test was determined by taking the average time for one test (six strains), the cost of the materials employed, and the cost of the labor ($7.00/h).

RESULTS

A total of 200 isolates was received from Greenview Hospital. The frequency of occurence of certain groups of streptococci in clinical specimens is presented in Table 1. The Lancefield grouping in Table 1 is based on the physiological tests done by a microbiologist at Greenview Hospital. After retesting the isolates from the hospital to confirm the presence of beta-hemolytic streptococci, two of the 200 isolates were identified as Staphylococci. The highest percentage (42%) of the total number of beta-hemolytic streptococci grouped by Greenview Hospital was not group A, B, or D.

The comparison between the physiological grouping and the Phadebact co-agglutination grouping method is shown in Table 2. A total of 198 strains of beta-hemolytic streptococci was compared. Agglutination was easily detected in the Phadebact Method, and the agglutination occured within 1 min. The physiological tests took an additional 18-24 h before the tests for grouping could be read as either positive or negative. The physiological tests were designed to group only the Lancefield Group A, B, or D, whereas the Phadebact reagents could group the Lancefield types A, B, C, and G. Since no

Table 1. Frequency of occurence of serological groups of beta-hemolytic streptococci in clinical specimens from Greenview Hospital.

Group	Number of Isolates
Serogroup A	81
B	35
D	0
Nongroupable as A, B, or D	84
Total	200

Table 2. Comparison of physiological grouping and Phadebact grouping of beta-hemolytic streptococci.

Group	Physiological		Phadebact	
	No. of isolates	% of total isolates	No. of isolates	% of total isolates
A	81	40.9	81	40.9
B	35	17.8	35	17.8
C	NT		63	31.8
D	0	0.0	NT	
G	NT		16	8.0
Non-groupable	82	41.0	3	1.5
Total	198	99.7	198	100.0

NT = Non-testable

Group D beta-hemolytic streptococci were isolated in this study, only Groups A and B were directly comparable. There was 100 percent agreement between both methods for grouping Lancefield Group A and B, and there were three specimens which were non-groupable by either method. The Phadebact Co-agglutination test grouped 79 out of the 84 isolates which were considered non-groupable by the physiological tests.

Table 3 compares the Phadebact Co-agglutination Method and the physiological tests with the Lancefield Precipitin Test. All of the 198 strains were tested and compared. In comparison with the Lancefield test there was a 100 percent agreement for both Phadebact and the physiological tests for Lancefield Group A and B, and there were three strains which were non-groupable by all three methods. For the Lancefield Groups C and G, there was a 100 percent agreement between Phadebact and the Lancefield Precipitin Test; however, the physiological method was non-testable for these two groups. There were no Group D streptococci identified by either the Lancefield Precipitin Test or by physiological testing. There were three strains which were non-groupable by the Phadebact and Lancefield methods compared to 82 strains which were non-groupable by the physiological tests.

Technician time and material cost were considered in the cost-benefit analysis as presented in Table 4. The most time-consuming and expensive component of the

Table 3. Comparison of the Lancefield Precipitin Test, Phadebact Co-agglutination Test and the physiological tests for the grouping of beta-hemolytic streptococci.

Group	Lancefield		Phadebact		Physiological	
	No. of isolates	% of isolates	No. of isolates	% of isolates	No. of isolates	% of isolates
A	81	40.9	81	100	81	100
B	35	17.8	35	100	35	100
C	63	31.8	63	100	NT	
D	0	0.0	NT		0	100
G	16	8.0	16	100	NT	
Non-groupable	3	1.5	3	100	82	41
Totals	198	100	198	100	198	40.3

NT = Nontestable

Table 4. Physiological tests vs. Phadebact test: cost-benefit analysis

Physiological tests:	
Bacitracin -	
Average time for 1 test	4 minutes
Materials cost	$0.40
Labor	$0.44
Total Cost	$0.84
CAMP test -	
Average time for 1 test	3 minutes
Materials cost	$0.22
Labor	$0.33
Total Cost	$0.55
Bile Esculin -	
Average time for 1 test	11 seconds
Materials cost	$0.31
Labor	$0.02
Total Cost	$0.33
Total Cost for Physiological Tests	$1.72
Phadebact:	
Average time for 1 test	1 minute
Material Cost	$0.81
Labor	$0.12
Total Cost	$0.93
Total Cost for Phadebact	$0.93

physiological tests was the bacitracin test. Approximately 4 min were required to perform the test, and the cost per test was $0.84. Since the physiological tests generally are performed as a unit, the total cost for all the tests was $1.72. The 1981 Phadebact kit cost was $65.00, and the kit provided enough reagents for approximately 80 tests. The total cost for one Phadebact Co-agglutination Test was $0.93 per test.

The number of isolates according to source and Lancefield Grouping are listed in Table 5. The majority of isolates (76.3%) came from the upper respiratory tract. As may be expected, the greatest number of isolates (40.9%) were of the Lancefield type Group A.

Table 5. Distribution of isolates according to source and Lancefield Group.

Source	Group A	B	C	D	G	Nongroupable	Total	Percent
Wounds	1	4	5	0	2	0	12	6.0
Upper Respiratory Tract	75	10	52	0	11	3	151	76.3
Ears	4	0	2	0	0	0	6	3.0
Stool	0	6	2	0	3	0	11	5.6
Urine	0	6	1	0	0	0	7	3.5
Vaginal Tract	0	9	1	0	0	0	10	5.0
Blood	1	0	0	0	0	0	1	0.5
Total	81	35	63	0	16	3	198	99.9
Percent	40.9	17.8	31.8	0.0	8.0	1.5		

DISCUSSION

The results from this research confirm those of previous studies indicating that the Phadebact co-agglutination method is simple, rapid and reliable for grouping beta-hemolytic streptococci (Burdash et al. 1981; Slifkin and Pouchet-Melvin 1980). A 100 percent agreement was found with Phadebact co-agglutination method and the Lancefield precipitin method for Groups A, B, C, and G. The nonserological (physiological) method was also in 100 percent agreement with the Lancefield precipitin test and the Phadebact co-agglutination method for Groups A, B, and D. The physiological method could not group 82 (41%) of the 198 strains of beta-hemolytic streptococci as compared to the Phadebact co-agglutination method which did not group 3 (1.5%) of the 198 strains of beta-hemolytic streptococci. The Phadebact co-agglutination method allowed for the grouping of Lancefield types C and G, whereas the physiological method was not capable of grouping these two human pathogenic groups. The Group C (31.8%) and the Group G (8.0%) streptococci made up 39.8 percent of the 198 streptococci. This was a high percentage of pathogenic streptococci not being accurately grouped in clinical laboratories. Thus, data generated by serologically grouping the beta-hemolytic streptococci were more important epidemiologically. There

was no substantial difference in the complexity of the execution of the Phadebact co-agglutination method and the physiological method although there was a definite time difference in the performance of the tests and in the read-out of the results. The performance of the test and the results for the Phadebact method required approximately 1 min. The physiological method required approximately 7 min and 11 sec to set-up the tests and then another 18-24 h before the results could be read. This makes the data generated by the physiological tests less clinically relevant. For example, if the isolates were from blood and the streptococci are Group A, B, C, or G, benzyl penicillin or other penicillin should be administered. If the streptococci are of group D enterococcus then penicillin should not be used and a drug susceptibility test performed so the proper antibiotic can be administered.

Differences in the cost of the test showed that the Phadebact co-agglutination method was $0.79 less than the physiological test. The Phadebact co-agglutination method was approximately $0.93 as compared to $1.72 for the physiological test. In calculating the total cost for the physiological method, the cost for differentiating Group D enterococcus from Group D non-enterococcus by 6.5% NaCl tolerance was not included for this reason: there were no Group D streptococci isolated thus no data were available to calculate the cost. The final results were

calculated based upon Greenview Hospital's work-load, technician salaries, and organizational structure; therefore, the cost analysis will be different for each individual laboratory.

The 200 clinical isolates from Greenview Hospital were examined by the gram reaction and catalase activity to confirm the presence of streptococci. Two of the 200 clinical specimens, which were identified as non-groupable streptococci by the hospital, were found to be catalase positive and were identified as Staphylococci. This points out the necessity for determining Gram stain characteristics and catalase activity. In a busy clinical setting, such errors of this type could be made. Before performing any type of test for the grouping of streptococci the colonial morphology needs to be observed. The streptococci generally have a large zone of beta-hemolysis and the colonies are quite small. The colonies are translucent to slightly opague and have entire edges. There was no distinct odor. The Gram stain showed small gram positive cocci generally occuring in chains or singly. As for the colonial morphology between the different groups it was difficult to clearly differentiate between them. The Group A streptococci were generally much smaller (pinpoint colonies) as compared to Groups B, C, and G. The Group A streptococci also had a much larger zone of beta-hemolysis around the colonies, but this could have been caused by the smaller colonies making the zone of beta-hemolysis appear larger. The other streptococcal groups

showed no noticeable difference in their colonial morphology. The majority of the streptococci isolates (76.3%) was obtained from the upper respiratory tract of patients. This included all throat, sputum, bronchial washings and nose specimens. These data correlate with results published by Pollock and Dahlgreen (1974), who obtained 77.9 percent of their streptococcal isolates from the upper respiratory tract.

The Lancefield Group A was found to be the most predominant group isolated. Since Group A comprised 81 (40.9%) of the 198 streptococci isolated, the microbiologist may find the determination of hemolysis and bacitracin susceptibility to be sufficient procedures for investigating throat and skin specimens as recommended by Facklam et al. (1974). This shows that most illnesses associated with streptococci are Group A and agrees with Clewell (1981); however, the beta-hemolytic streptococci Groups B, C, and G were also found to be associated with a number of streptococcal infections from various clinical sources. Pollock and Dahlgreen (1974) also found Group A streptococci to be the most dominant organism in streptococcal infections. Their results showed Group A comprised 48.4 percent of the clinical isolates. However, Cunniff and Bump (1976), showed that the streptococcal Groups B, C, and G were also important as pathogens for the human host. In their results they obtained 50 percent as Group B, 11.8 percent as Group G and 2.8 percent as Group C. In this research the Group C

comprised a total of 31.8 percent of the isolates while Group B comprised 35 percent and Group G 8 percent.

There was no beta-hemolytic streptococci Group D isolated in this study. Since the Phadebact only identifies Groups A, B, C, and G, alternate tests would have to be applied to Group D, namely, bile esculin agar (Facklam et al. 1974).

CONCLUSION

The Phadebact Co-agglutination Direct-Plate Method and the physiological method evaluated in this investigation permitted the correct serogrouping of 195 beta-hemolytic streptococci by Phadebact and 116 by the physiological tests, out of 198 clinical isolates of the beta-hemolytic streptococci. In contrast to the nonserological method, which is employed by most Western Kentucky hospitals, the Phadebact Co-agglutination method represented a more reliable, inexpensive, and rapid method for the detection and grouping of beta-hemolytic streptococci. With the availability of this simple agglutination method, the data obtained show that both the small and large laboratories would greatly benefit from the use of this agglutination test over the conventional, nonserological method presently being used.

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