# Development of Coagglutination Reagents for Serological Grouping of Streptococci

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**Abstract**

Coagglutination reagents for the rapid serological grouping of groups A, B, C, F and G *Streptococcus* have been developed. Antisera to groups A, B, C, F and G *Streptococcus* were raised in rabbits. After absorption with cross-reacting antigens, the specific antibodies were coated on *Staphylococcus* protein-A and used as group-specific coagglutination reagents. The sensitivity of the reagents for groups A, C and G *Streptococcus* was 100 per cent and the specificity was 100, 100, and 98.77 per cent, respectively. The sensitivity and specificity of these reagents were consistent up to 12 months, although specificity declined with longer storage. The in-house coagglutination reagents for groups A, C and G streptococcus were also tested in comparison with the commercially available *Streptococcus* Phadebact® test and yielded almost identical results. Sensitivity of the in-house of group B *Streptococcus* reagent was low, while the group F reagent gave a high incidence of false positive reaction.

**Key word :** Coagglutination, Streptococci

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Streptococcal infection is one of the most frequent bacterial diseases found in tropical areas. The organisms produce various clinical patterns of disease, especially, upper respiratory tract infection and particularly important, serious sequelae like rheumatic fever and acute glomerulonephritis following group A streptococcal infection. For that reason, serological grouping must be performed when β-hemolytic streptococcal colonies are obtained from the culture. Several methods have

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been described for identifying groups of streptococci, these include the bacitracin test[1] for detecting group A streptococci, precipitation techniques with group-specific antiserum and extracts of streptococcal group antigens[2-6], immunofluorescent method[7] and agglutination techniques[6,8,9].

Coagglutination for rapid diagnosis of streptococci, first described by Christensen and associates[8], has become the method of choice in streptococcus grouping. The technique, based on the immunological reaction between the C carbohydrate antigens of streptococci and their group-specific antibodies adsorbed to formaldehyde-and heat-treated Cowan I staphylococci has been claimed to be accurate, rapid and simple to perform. Many modifications have been described in order to simplify the technique and decrease the time consumed for identification of the microbes in culture[10-12]. The coagglutination reagents for various groups of streptococci are commercially available and widely used in many microbiology laboratories. However, the reagents are costly for developing countries such as Thailand.

To minimize the cost of testing, we have developed group-specific coagglutination reagents for group A, B, C, F and G streptococci by using hyperimmune rabbit sera produced in our laboratory. The reagents were standardized and tested for their precision as well as their shelf-life with various reference culture strains. We also compared our reagents with the commercially available streptococcus test for the serological grouping of streptococci freshly isolated from clinical specimens.

## MATERIAL AND METHOD

**Streptococcal strains** Reference isolates of group A, B, C, F and G streptococci were obtained from the National Streptococcus Reference Center of Thailand and the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. These included reference strains for antisera preparation originally provided by Dr. Kinjiro Takizawa, Kanagawa Public Health Laboratory, Japan, and the strains used for serological testing, i.e., 403 isolates from stock cultures and 90 isolates from clinical specimens. All strains were grown overnight on blood agar plates or in Todd Hewitt broth at 37°C and grouped by using the commercial Phadebact® Streptococcus test (Boule Diagnostics AB, Huddinge, Sweden).

**Antigen preparation** Group A, C and G streptococcal antigens for immunization were prepared as previously described[13], with some modifications. Briefly, after growing the bacteria in 250 ml Todd Hewitt broth at 37°C for 18 hours, the cultures were harvested by centrifugation and the bacterial cell pellets were washed once in normal saline solution. The cells were resuspended in 25 ml phosphate-buffer pH 7.8 containing 25 mg trypsin and kept at room temperature overnight. The treated bacteria were washed once, resuspended in phosphate buffer pH 7.8 and autoclaved. After further washing, the cells were finally resuspended in 25 ml phosphate buffer pH 7.8 containing 0.2 per cent formalin, aliquoted and stored at 4°C until used.

Group B and F antigens were prepared by a similar procedure, with the exception of trypsin treatment.

**Immunization** The streptococcal antisera were produced in rabbits according to the immunization scheme described by Rotta and Facklam[13] with modifications. Briefly, rabbits weighing 2.5-3 kg were injected intradermally with 1 ml of antigen-incomplete Freund's adjuvant suspension in the first week. From the second to the fifth week, one ml of antigen was given intravenously on three successive days of the week. The animals were bled 5-7 days after the last injection and the antisera were tested for the presence of specific antibodies by precipitation and agglutination methods. If the titer was unsatisfactory, the immunization was continued for 2-3 weeks until a clear and strong reaction was obtained.

The antisera to each group of *Streptococcus* were tested for any cross-reactivity with heterologous strains by agglutination. If agglutination occured, the antisera were absorbed with the cross-reacting strains. Briefly, 1.5 ml of cross-reacting bacterial sediment was added to 5 ml of antisera. After incubation at 37°C for 2 hours, the mixture was placed at 4°C overnight. The serum was separated by centrifugation, steriled by filtration and re-tested for cross-reactivity. The antisera that were used for coagglutination reagent preparation showed no cross-reactivity.

**Preparation of coagglutination reagents** Coagglutination reagents were prepared as described previously[8]. Briefly, 1 ml of 10 per cent staphylococcus protein-A suspension (Sigma Chemical, USA Lot No.32H6818) was added with 0.1 ml of the group specific rabbit antistreptococcal

antisera at appropriate titer. The mixture was mixed thoroughly, incubated at room temperature for five minutes and washed twice in 0.03 M phosphate buffered saline pH 7.3. The sediment was resuspended in 10 ml phosphate buffered saline containing 0.1 per cent sodium azide. The reagents were aliquoted and kept at 4°C until used.

**Coagglutination test** The coagglutination test was carried out on locally available plastic card slides. One bacterial colony from a pure culture growing overnight on blood agar was picked with a sterile toothpick and smeared on the plastic card slide. Ten microlitres of group-specific coagglutination reagent were added, mixed and the slide was rotated by hand for 2 minutes. The results were read with the naked eye. The test was interpreted as positive if agglutination occurred within 2 minutes. The reaction was graded from $1^+$ to $4^+$ depending on the degree of clumping and clarity of the mixture. Weak reactions were considered as to be a negative test result.

**Sensitivity and specificity testing** The sensitivity and specificity of the group specific coagglutination reagent was determined by using homologous and heterologous streptococcus as reference strains. Each group-specific coagglutination reagent was tested every month for a period of 12 months in order to determine their shelf-life.

**Comparison of the coagglutination with the Phadebact® streptococcus test.** The in-house group-specific streptococcus coagglutination reagents were used for identifying the clinical isolates of β-hemolytic streptococcal colonies on blood agar. The results were compared with those obtained by using the commercially available streptococcus coagglutination Phadebact® test.

## RESULTS

**Coagglutination test** The in-house streptococcal coagglutination reagents were tested with homologous and heterologous streptococcus reference strains. As shown in Table 1, the coagglutination reagents of group A, C, F and G streptococci gave 100 per cent positive result as tested against all homologous strains, no cross-reactivity with heterologous strains was seen using group A and C reagents, whereas, the reagent of group B, F and G showed some cross-reactivity. The sensitivity of the group B reagent was considered low, it reacted with only 52 out of 100 homologous isolates. The reagent of group F streptococci, eventhough it displayed agglutination with all 10 homologous strains tested, it broadly reacted with various groups A, B, C and G strains. However, considering the strength of the reaction, coagglutination with homologous strains usually produced a greater or more rapid degree of reaction than cross-reactions (data not shown).

Sensitivity and specificity, including positive and negative predictive value of each reagent, are shown in Table 2.

**Table 1. Coagglutination test of reference streptococcal strains of group A, B, C, F and G.**

| Coagglutination reagent of *Streptococcus* group | Per cent positive with reference streptococcal strains (number tested) | | | | |
|---|---|---|---|---|---|
| | group A | B | C | F | G |
| A | 100 (100) | 0 (50) | 0 (50) | 0 (15) | 0 (50) |
| B | 0 (45) | 52 (100) | 0 (50) | 10 (10) | 0 (47) |
| C | 0 (48) | 0 (50) | 100 (95) | 0 (9) | 0 (47) |
| F | 32 (50) | 10 (50) | 18 (50) | 100 (10) | 10 (50) |
| G | 4 (50) | 0 (50) | 0 (50) | 0 (12) | 100 (98) |

**Table 2. Sensitivity, specificity, and positive and negative predictive values of the in-house coagglutination reagents.**

| Coagglutination reagent of streptococcus group | Sensitivity % | Specificity % | Positive predictive value % | Negative predictive value % |
|---|---|---|---|---|
| A | 100 | 100 | 100 | 100 |
| B | 52 | 99.35 | 98.11 | 76 |
| C | 100 | 100 | 100 | 100 |
| F | 100 | 82.5 | 22.22 | 100 |
| G | 100 | 98.77 | 98 | 100 |

**Table 3. Comparison on the grouping of *Streptococcus* from clinical isolates by the in-house and Phadebact® streptococcal coagglutination reagents.**

| Streptococcus group | No. of isolates tested by Phadebact® | No. (%) of positive reaction with in-house coagglutination reagents |
|---|---|---|
| A | 55 | 52 (94.5) |
| C | 5 | 5 (100) |
| G | 30 | 30 (100) |

Streptococcal coagglutination reagents were also used for identifying the clinical isolates of β-hemolytic streptococcal colonies on blood agar compared to the commercially available streptococcus Phadebact® test. There was significant correlation between the results obtained with the two methods for group C and G reagents. With group A reagent, 52 (94.5%) out of 55 strains were positive with our reagent, whereas, 3 escaped detection. The results are shown in Table 3.

**Stability of the coagglutination reagents**

All reagents were tested each month for up to 12 months in order to check their shelf-life. The reagents of group A, C and G were perfectly reactive (100%) with all homologous strains throughout the study period. Meanwhile, slight cross-reactivity was observed when group C and group G reagents reacted with strains of group A (2-4%) and when reagents of group A reacted with strains of group F (10-20%). The reagents of group B showed a decrease in sensitivity from 52 per cent in the first month to 37.5 per cent in the twelfth month, whereas, reagents of group F maintained one hundred per cent reactivity with homologous strains. Cross-reactivity gradually increased with all heterologous strains after 3 months (data not shown).

## DISCUSSION

The coagglutination technique first described by Kronvall[14] has been used for serodiagnosis of many bacterial infections with relatively high sensitivity and specificity[15]. The technique was considered rapid, simple and reliable. With streptococcal infections, application of the coagglutination technique is increasing[16]. The reagents are commercially available and the test has been carried out in many clinical microbiology laboratories. Although the use of commercially available kits is convenient, it incurs relatively high expense. For routine serological identification with the Phadebact® *Streptococcus* test, the cost for sero-grouping of streptococcus A, B, C, G and F amounts to about 160 baht (~4 US$) per isolate in our laboratory.

The group-specific antisera used in the present study were raised in rabbits. Those showing cross-reactivity were absorbed before being coated on staphylococci and tested against a panel of streptococcus strains of group A, B, C, F and G taken directly from the blood agar plates. We found the sensitivity of reagents for groups A, C, G and F to be as high as 100 per cent (Table 2). These findings are in agreement with the results obtained from many previous reports using the same coagglutina-

tion technique[8,17-19]. Moreover, neither subculture in broth nor enzyme treatment were necessary in our experiments thus resulting in rapid performance of the assay after growing the bacteria in primary culture. The coagglutination results of group A, C and G reagents also corresponded with that of the commercially available Phadebact$^{R}$ reagent when testing with the colonies of clinical isolates β-hemolytic streptococci. Interestingly, group F which did not have any agglutination with other groups before coating on staphylococcus protein A showed a cross reactive result with other groups in this study. The result remained the same even when we used the serum absorbed with the highest cross-reacting group A streptococci. Therefore, reagents of group F were not tested with clinical specimens. It is possible that group F might contain some common antigens that cannot be completely absorbed. With group B streptococcal reagents, although producing high specificity, the sensitivity was considered too low (52%) for diagnostic purposes. This may be due to the antisera used that may not cover all serotypes of group B streptococci. To increase the sensitivity of the group B reagent, polyvalent antisera obtained after immunizing the rabbits with more than one subtype of the bacteria, or antisera representing polytype reagents may be required as has been described by other investigators[7,19].

Coagglutination reagents of group A, C and G *Streptococcus* maintained their activity up to 12 months when stored at 4°C. Although a slight cross-reactivity was observed, there were no difficulties in interpreting the results (even with the group F reagent), since the reaction intensity was always stronger and quicker with specific strains when compared to the cross-reactive ones (i.e. $3^{+}$ -$4^{+}$ *vs* 1-$2^{+}$). However, the investigator should pay special attention when comparing the strength and time taken for the reaction with each reagent.

The coagglutination reagents used in this study, especially for group A, C and G streptococci, offer a sensitive and specific test for the identification of β-hemolytic streptococci. The test is easy to perform, rapid and inexpensive. Only a small quantity of reagents, including antisera and *Staphylococcus aureus*-protein A, are required per test. The cost of the reagents prepared in-house has been roughly calculated as less than 1 US$ per isolate, which is cheaper than the commercially available kit.

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# การพัฒนาน้ำยาโคแอกกลูติเนชั่น สำหรับการตรวจซีโรกรุ๊ปของเชื้อ*สเตร็ปโตคอคคัส*

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งานวิจัยนี้ได้พัฒนาน้ำยาตรวจวินิจฉัยเชื้อ*สเตร็ปโตคอคคัส*กลุ่ม A, B, C, F, G ด้วยวิธี coagglutination ซึ่งเป็นวิธีที่สามารถแยกเชื้อ*สเตร็ปโตคอคคัส*กลุ่มต่าง ๆ ได้อย่างรวดเร็ว โดยได้เตรียมแอนติซีรั่มต่อเชื้อ*สเตร็ปโตคอคคัส* กลุ่ม A, B, C, F, G ขึ้นเองจากการ immunized ในกระต่าย นำแอนติซีรั่มที่ได้ ดูดซับเอาแอนติบอดีที่ทำปฏิกิริยาข้ามกลุ่มออกแล้วเคลือบบน *Staphylococcus* protein A เพื่อเป็นน้ำยาสำหรับ coagglutination test จากการทดสอบกับเชื้ออ้างอิง (reference strain) ต่าง ๆ พบว่า coagglutination reagent ของเชื้อ*สเตร็ปโตคอคคัส* กลุ่ม A, C และ G มีความไว 100% ทั้ง 3 กลุ่ม, ความจำเพาะ 100%, 100% และ 98.77% ตามลำดับ และจากการเปรียบเทียบผลการทดสอบระหว่างน้ำยาที่ผลิตขึ้น กับน้ำยาสำเร็จรูป Phadebact® เมื่อทดสอบกับเชื้อที่ได้จากสิ่งส่งตรวจของผู้ป่วย พบว่าให้ผลการทดลองสอดคล้องกันเกือบทุกราย นอกจากนี้ได้ทดสอบอายุการใช้งาน (shelf life) ของน้ำยาทั้ง 3 ชนิด เป็นเวลา 12 เดือน พบว่าน้ำยาทุกตัวยังคงให้ผลความไว และความจำเพาะใกล้เคียงกับระยะแรก โดยพบว่ามีปฏิกิริยาข้ามกลุ่มกับ*สเตร็ปโตคอคคัส*กลุ่มอื่นเพิ่มขึ้นเล็กน้อยในระยะเดือนท้าย ๆ และปฏิกิริยามักจะเป็นผลบวกอ่อน (weakly positive) ส่วน coagglutination reagent ของ*สเตร็ปโตคอคคัส*กลุ่ม B มีความจำเพาะสูงแต่ความไวค่อนข้างต่ำ ในขณะที่กลุ่ม F นั้น ยังมีปัญหาผลบวกเทียมอยู่

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