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Preparation of antisera for serological detection of streptococcal pneumoniae antigens.*

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Streptococcus pneumoniae is an important etiologic agent of lower respiratory tract infection. Serological detection of Streptococcus pneumoniae antigens from clinical specimens is a useful diagnostic method. In this study, we have prepared antisera against the antigens of Streptococcus pneumoniae type 1, 19, 5, 6, and 23, which are the common serotypes found in Thailand. The reference strains of Streptococcus pneumoniae were kindly provided from Streptococcal Department, Statens Seruminstitut, Denmark. The antisera were prepared by using 5 rabbits for each strain with at least 5 weeks period of immunization.

The geometric mean titers of antisera against Streptococcus pneumoniae type 1, 19, 5, 6, and 23 were 194, 2435.5, 1024, 55, and 194 respectively. There were cross-reactions between type 1 antiserum and type 6 antigen, type 6 antiserum and type 1 antigen, type 6 antiserum and Hemophilus influenzae antigen, antisera of type 1, 6, 23 and Escherichia coli. Respective immune immunoglobulins were purified by affinity chromatography on a Protein A-sepharose CL-4B column and coupled to latex particles for testing against various heterologous bacteria from clinical specimen. The preliminary testing of this latex agglutination kid showed good result without cross-reaction.

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กำจร ตติยกวี, ไหม รัตนวรารักษ์, ผ่องพรรณ นันทาภิสุทธิ์, ฉุทัย สกุลแรมรุ่ง, นิภา รุจิธรรมกุล. การเตรียมแอนตี้เซรา ต่อแอนติเจนของ สเตร็ปโตคอคคาล นิวโมเนีย. จุฬาลงกรณ์เวชสาร 2533 กรกฎาคม; 33 (7): 501-508

Streptococcus pneumoniae เป็นจุลชีพสำคัญที่ทำให้เกิดโรคติดเชื้อของระบบหายใจส่วนล่าง การตรวจหาแอนติเจนของ Streptococcus pneumoniae จากสิ่งส่งตรวจต่าง ๆ โดยวิธีทาง serology เป็นอีกวิธีหนึ่งซึ่งช่วยการวินิจฉัยโรคติดเชื้อที่เกิดจากจุลชีพดังกล่าว และเป็นวิธีที่มีประโยชน์อย่างยิ่งในกรณีที่การเพาะเชื้อไม่ได้ผล งานวิจัยนี้เป็นการเตรียมแอนติเซรุ่มเพื่อให้ในการตรวจหาแอนติเจนของ Streptococcus pneumoniae โดยเลือกสายพันธุ์ที่ทำให้เกิดโรคติดเชื้อได้บ่อยจากรายงานในประเทศไทยเพียง 5 สายพันธุ์คือ 1, 19, 5, 6 และ 23 ทำการเตรียมวัคซีนสำหรับฉีดกระต่ายด้วยเชื้อมาตรฐานที่ใช้อ้างอิงซึ่งได้รับมาจาก Streptococcal Department, Statens Seruminstitut, Denmark ฉีดวัคซีนโดยใช้กระต่าย 5 ตัว สำหรับการเตรียมแอนติเซรุ่มแต่ละสายพันธุ์ การฉีดวัคซีนให้กระต่ายแต่ละชุดใช้เวลาประมาณ 5 สัปดาห์จึงเจาะเลือดแยกแอนติเซรุ่ม

ผลการศึกษาพบว่าการตอบสนองสร้างแอนติบอดีของกระต่ายต่อเชื้อแต่ละสายพันธุ์มีมัชฌิมเรขาคณิตของ Titer ของแอนติบอดีต่อสายพันธุ์ 1, 19, 5, 6 และ 23 เป็น 194, 2435.5, 1024, 55 และ 194 ตามลำดับ เมื่อนำแอนติเซรุ่มที่เตรียมได้ไปทดสอบหาปฏิกิริยาข้ามกลุ่มระหว่างสายพันธุ์ พบปฏิกิริยาข้ามกลุ่มกันชัดเจน ระหว่างแอนติเซรุ่มต่อสายพันธุ์ 1 และ 6 การทดสอบปฏิกิริยาข้ามกลุ่มกับเชื้ออื่น ๆ พบว่าแอนติเซรุ่มต่อสายพันธุ์ 6 มีปฏิกิริยาข้ามกลุ่มต่อ Hemophilus influenzae และ แอนติเซรุ่มต่อสายพันธุ์ 1,6,23 มีปฏิกิริยาข้ามกลุ่มต่อ Escherichia coli นำแอนติเซรุ่มไปแยกสกัดเฉพาะ Immunoglobulins โดยวิธี affinity chromatography ด้วย Protein A sepharose CL-4B cloumn นำไปประยุกต์ประดิษฐ์ชุดทดสอบ Latex agglutination ซึ่งจากการทดสอบเบื้องต้นพบว่าชุด Latex agglutination ที่เตรียมขึ้นใช้เองนี้มีประสิทธิภาพดี

Communicable diseases remain the main health problem in Thailand. Streptococcus pneumoniae is one of the most common causes of lower respiratory tract infection especially in children under 2 years[1,2]. The potentially serious nature of the disease requires the early diagnosis of infection to ensure the institution of appropriate antimicrobial treatment. Different kinds of soluble pneumococcal capsular polysaccharide are used to produce more than 80 type-specific commercial diagnostic antisera[3]. However, some epidemiological studies in Thailand indicated that only a few types of Streptococcus pnemoniae, including type 1, 6, 19, 5 and 23, frequently caused infections in hospitalized patients[4].

At present Counter-Immunoelectrophoresis (CIE)[5-7] and latex agglutination (LA) are two of the most acceptable serological methods for detection of Streptococcus pneumoniae from clinical specimens.[8-10] However, the performing of CIE requires electrophoretic equipments and uses more standard antisera than the LA method. In addition, the LA method is easy to interpret and suitable for bed-side diagnosis[11,12].

In this paper, we describe a simple method in the preparation of specific antisera against Streptococcus pneumoniae serotypes 1, 6, 19, 5 and 23. The aim of the present investigation is to evaluate the prepared antisera compared with the commercial antisera. Furthermore it is to use the prepared antisera with the appropriate serological method, latex aglutination, for Streptococcus pneumoniae screening.

Materials and Methods

1. Lyophilized standard strains of Streptococcus pneumoniae serotypes 1, 5, 6, 19 and 23 (kindly provided from Streptococcal Department, Staten Seruminstitut, Copenhagen, Denmark) were used for vaccine preparation.

2. Standard commercial antisera for serotypes 1, 5, 6, 19 and 23 were obtained from Staten Seruminstitut, Copenhagen, Denmark.

3. White New Zealand rabbits, weighing 2-2.5 kilogram, were used for immunizations.

Vaccine preparation

Vaccine preparations followed Lund's method[13]. Each selected strain of lyophilized Streptococcus pneumoniae was dissolved in brain heart infusion broth and then added to sheep blood agar. The culture was incubated at 37°C in 5% CO_2 for 24 hours; this was a control of their sterility. Then, a single isolated colony from the pure culture was inoculated into 10 ml of 5% horse serum broth and incubated at 37°C for 18 hours. In the morning the serum-broth culture was expanded into a flask of 1500 ml of 5% horse serum broth. The flask was shaken and incubated again at 37°C, and the growth was sampled by culture in sheep blood agar every hour.

Gram stain was performed to exclude impurity. The density of the growth in each sample was estimated, and the capsule formation is tested by Quellung's reaction,[14] by its specific antiserum. The optimal density was reached after about 6 hours; then the growth was stopped by the addition of 2% formalin. The flask remained in the incubator at 37°C until the following morning. The culture was then centrifuged 3000 g for 30 min. The sediment was suspended in 0.5% formalin in Sorensen buffer solution (20 ml of M/15 KH_2PO_4 + 80 ml of M/15 $Na_2HPO_4 . 2H_2O$ + 300 ml of 0.9% NaCl solution). The density of the finished vaccine was estimated by means of a standard containing 1.2×10^9 organisms per ml. The vaccine was divided into several small tubes and stored at =.−30°C.

Immunizations

Each strain of Streptococcus pneumoniae needed 5 white rabbits for immunization. At first, blood samples of all rabbits were taken, and then their sera were poured and freezed. The vaccine was warmed up to 37°C prior to the injections, which was given slowly and intravenously into their ears. The rabbits were injected with increasing dosages of vaccine every other day (three times a week) during a period of 5 weeks. The schedule of immunizations was arranged as in the following table (Table 1).

Production of antisera

After 5 weeks of immunization, blood samples were taken by venepuncture to estimate the potency of the antisera. The various rabbits did not produce the same amounts of antibody. Further immunization was given by repeating the 5th week's dosage scheme of the schedule (Table 1) in the rabbits that produced low titre of antibody. When the sera were found to possess sufficiently high titre, the rabbits were bled by venepuncture or heart puncture to take at least 30 ml of blood from each rabbit.

The blood samples were left standing in cold-room for 24 hours. After centrifugation, 1% thimerosal was added to the obtained sera and stored at −30°C.

All sera were examined for agglutination titre with microagglutination method, and tested for their specificities with Quellung's reaction.

Microagglutination method[15]

The prepared antisera were serially two fold diluted with 0.85% saline in wells of microtitre plates. The type-specific Streptococcus pneumoniae vaccines used

as antigens were added to the correspondent wells (Table 2). One to two drops of 10% methylene blue were added to enhance interpretations. The positive antigen-antibody reactions showed agglutinations at the bottom of the wells.

Table 1. The schedule of immunization within 5 weeks.

	day 1	day 3	day 5
week 1	0.25 ml	0.5 ml	0.5 ml
week 2	1.0 ml	1.0 ml	1.0 ml
week 3	1.5 ml	1.5 ml	1.5 ml
week 4	2.0 ml	2.0 ml	2.0 ml
week 5	2.0 ml	2.0 ml	2.0 ml

Table 2. Titration by microagglutination procedure in microtiter plate.

	well 1	well 2	well 3	well 4	well 5	well 6	well 7	well 8	well 9
0.85% saline	<----------50 μl---------->								50μl
antibody	100μl								
	50μl	50	50	50	50	50	50	50	discard
Ab* titer	1	2	4	8	16	32	64	128	-ve
antigen	<----------**50 μl**---------->								**50μl**

Ab* : antibody

Immunoglobulin G extraction for latex agglutination

Immunoglobulin G was extracted from the prepared antisera by affinity chromatography method. Protein A coupling to sepharose CL-4B was used to bind IgG molecules from the applied sera[16]. Protein A is a protein isolated from the cell wall of some strains of staphylococcus aureus that specifically reacts with IgG molecules of subclass IgG1, IgG2 and IgG4. The column of Protein-A sepharose CL-4B saturated in Tris-NaCl at pH 8.6, adsorbed IgG from the sera. Then the unbound substances was washed out of the column; and the purified IgG was recovered by changing the pH by acetate-NaCl pH 4.3 buffer. The concentrations of the eluted protein were determined by optical density at 280 nm (OD_{280}). The purity of IgG was tested with anti-rabbit IgG and anti-rabbit serum by immunodiffusion technique.

The protein in extracted solutions were concentrated by Amicon Ultrafiltration of membrane size YM 100. The final volumes were the same as the beginning volumes of antisera before extractions. The concentrations of IgG were determined by Lowry method[17].

Results

1. Titres of the prepared antisera before extractions of IgG were determined by microagglutination method. Geometric mean of each antisera was calculated with the following formula :

Geometric mean = n^{th} root of ($titer_1 \times titer_2 \times titer_3 \ldots \times titer_n$) ($titer_{1,2,3\ldots n}$ is the agglutination titers of a type-specific antisera from the first, second, third immunized rabbits and so on.) The geometric means of type 1, 5, 6,19, and 23 were 194, 2435.5, 55, 1024, and 194 respectively (Table 3).

Table 3. Titer of the prepared Streptococcus pneumoniae antisera.

Antisera	range	Geometric mean
Pn* 1	64-512	194
Pn 5	512-8192	2435.5
Pn 6	8-512	55
Pn 19	256-16384	1024
Pn 23	128-256	194

Pn* = Streptococcus pneumoniae

2. Cross-reactions to the different serotypes and some of the different organisms, particularly Hemophilus influenzae type b, Escherichia coli, and Klebsiella species, were done by microagglutination method. The most cross-reactions were caused by the antiserum of type 6. Other cross-reactions are showed in table 4.

3. The same type antisera were pooled together. The pooled antisera were found to react specifically to their original vaccines by Quellung's reaction.

4. IgG extractions by affinity chromatography were done after absorption of the antisera with the cross-reacted E.coli. The titers of the antisera type 1, 5, 6, 19, and 23 were decreased to 64, 128, 256, 512 and 64 respectively (Table 5).

Table 4. Cross-reactions against other Streptococcus pneumoniae and other organisms.

	Antisera				
Antigen	**Pn1**	**Pn5**	**Pn6**	**Pn19**	**Pn23**
Other Pn*					
Pn 1	+	−	+	−	−
Pn 5	−	+	−	−	−
Pn 6	+	−	+	+	−
Pn 19	−	−	+	+	−
Pn 23	−	−	−	±@	+
Other bacteria					
H. influenzae b	−	−	+	−	−
E.coli	+	±@	+	−	+
Klebsiellae sp.	−	−	−	−	−

Pn* = Streptococcus pneumoniae
±@ = 2 from 5 antisera caused cross-reactions

Table 5. Comparison of antisera titer before and after IgG extractions.

Antisera	before extraction	after extraction
Pn 1	256	64
Pn 5	1024	128
Pn 6	512	256
Pn 19	2048	512
Pn 23	256	64

Pn* = Streptococcus pneumoniae

5. The extracted antisera were filtrated by Amicon Ultrafiltration. The final volume of each antisera was equal to its original volume before extraction, and its protein concentration was determined by Lowry method. The most concentrated antiserum was antiserum of type 19, 5.80 mg/ml, while the least was type 23, 0.68 mg/ml (Table 6).

6. Latex agglutination kid was prepared by these IgGs and tested for cross-reactions. The antisera type 5 and 19 cross-reacted to some different types of Streptococcus pneumoniae, but did not cross-reacted to Hemophilus influenzae (Table 7).

Table 6. Protein determinations for IgG by Lowry's method.

IgG of	concentration (mg/ml)
Pn* 1	1.88
Pn 5	1.26
Pn 6	3.20
Pn 19	5.80
Pn 23	0.68

Pn* = Streptococcus pneumoniae

Table 7. Tests for cross-reactions of Latex agglutination kit.

	Latex agglutination against				
Antigen	Pn1	Pn5	Pn6	Pn19	Pn23
Pn* 1	+	+	−	−	−
Pn 5	−	+	−	+	−
Pn 6	−	−	+	+	−
Pn 19	−	+	−	+	−
Pn 23	−	+	−	−	+
Hib #	−	−	−	−	−

Pn* = Streptococcus pneumoniae
Hib # = Hemophilus influenzae

The Latex agglutination kid was tested with various clinical specimens. The preliminary result was comparable to standard culture procedure (data not shown).

Summary and discussion

Streptococcus pneumoniae antisera specific to serotype 1, 5, 6, 19, and 23 were prepared. White rabbits were immunized with vaccines prepared from the reference strains provided by Staten Seruminstitut, Denmark. The antibody productions from the immunized rabbits were varied. The rabbits immunized with Streptococcus pneumoniae type 5 vaccine showed the highest antibody response, while immunization with Streptococcus pneumoniae type 6 vaccine showed poor response. However, the overall antibody responses were satisfactory. The titers' geometric means of type 1, 5, 6, 19, and 23 were 194, 2435.5, 55, 1024, and 194 respectively.

Antiserum of Streptococcus pneumoniae type 6 was found to have cross-reaction with Hemophilus influenzae type b. In addition, the antiserum also cross-reacted with E.coli. This is believed to be due to their antigens contain the same sugar, ribitol, in their structures (18,19) (fig. 1).

H.influenzae type b ; -->3)-B-d-Rib f-(1-->1)- D-ribitol-5 phosphate (1,4)
S.pneumoniae type 6 ; -->2)-L-D-Gal p-(1-->3)-L-Rhap-(1-->3)- D-ribitol-5 phosphate
E.coli K100 ; Ribosyl (1-2) ribitol linkage

Figure 1. Antigenic structures of Streptococcus pneumoniae type 6, Hemophilus influenzae type b and Escherichia coli K 100.

Affinity chromatography, using Protein-A sepharose CL-4B, was performed to extract IgG from the prepared antisera. The antibody titers were decreased during the extraction processes. However, the extracted IgGs were pure and suitable for coating with latex particles. Our prepared Latex agglutination kit was effective in the detection of the specific types of Streptococcus pneumoniae from the cultures. The preliminary testing of the kit with clinical specimens showed good result. This study is beneficial for preparations of Streptococcus pneumoniae antisera. Development of home made serological diagnostic kits will decrease the expenditure of using commercial antisera or kits, as well as valuable for bed side diagnosis of certain infectious diseases.

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