Trends in Peptide and Protein Sciences
Volume 5 (2020): e9

DOI: http://dx.doi.org/10.22037/tpps.v5i0.33390

Original Article

A Rapid and Cost-Effective Method for the Purification of *Streptococcus pneumoniae* Antiserum Using Tangential Flow Filtration Method

Mitra Moghiman[a,b], Vahid Kia[c], Zahra Panahi[a,b], Rahman Shokri[b*], Mahdi Paryan[b*]

[a] *Danesh Alborz University, Qazvin, Iran.*
[b] *Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, Tehran, Iran.*
[c] *Department of Medical Biotechnology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.*

Article history:
Received: 20 December 2020
Accepted: 31 December 2020

Keywords:
Agglutination
Antigen
ELISA
Immunoglobin G
Streptococcus pneumoniae

HIGHLIGHTS

- A rapid and cost-effective method for the purification of *Streptococcus pneumoniae* antiserum.
- IgG antibody was prepared and purified by using tangential flow filtration method.
- The purified antibody can be used in ELISA and agglutination diagnostic kits.

ABSTRACT

Antibodies are important agents in the laboratory diagnosis of various microorganisms such as *Streptococcus pneumoniae*. In this study, we prepared and purified IgG antibody against *Streptococcus pneumoniae* using novel methods to be used in ELISA and agglutination diagnostic kits. First, *Streptococcus pneumoniae* was cultured, harvested, and inactivated. Then, bacteria were injected into four mature New Zealand white rabbits, and antisera were developed. Afterward, immunoglobulins were purified using ammonium sulfate precipitation, diafilteration using Tangential Flow Filtration, and ion-exchange chromatography. The purified antibody was then biotinylated and used in ELISA. Gel electrophoresis results showed that the antibody was highly pure. Agglutination test on the primary antigen and clinical samples was four-plus. ELISA results showed that the sensitivity and specificity of the test were 95% and 100%, respectively. Results indicated that our fast method was suitable for anti-*Streptococcus pneumoniae* IgG purification. Repetitive qualitative and quantitative experiments confirmed high purity of the immunoglobulin. Thus, it could be a suitable candidate to be used in laboratory diagnostic kits.

Cite this article as: Moghimana, M., Kia, V., Panahi, Z., Shokri, R. and M. Paryan (2020). A rapid and cost-effective method for the purification of *Streptococcus pneumoniae* antiserum using tangential flow filtration method, *Trends Pept. Protein Sci.*, **5**: e9.

M. Moghiman: https://orcid.org/0000-0002-7788-142X
R. Shokri: https://orcid.org/0000-0002-1996-5799
M. Paryan: https://orcid.org/0000-0002-2592-3528

[*] *Corresponding Authors:*
Email: mparyan@gmail.com, m_paryan@pasteur.ac.ir (M. Paryan)
Email: shokrei@gmail.com (R. Shakori)

Introduction

Streptococcus pneumoniae is an important cause of mortality especially in children under 5 years old and the elderly (Obaro and Adegbola, 2002; Paton and

Trappetti, 2019). In addition, it is the second cause of meningitis in youngsters and adults (Winegarner and Wittkopp, 2020), and the main cause of bacterial otitis media and bacteremia with an incidence rate of 1.5% (Austrian, 1981; Austrian, 1999). In 2000, at least one million children died because of pneumococcal infections (Williams et al., 2002). The early diagnosis of *Streptococcus pneumoniae* is the most important measure to control and treat it (Paton and Trappetti, 2019). In addition, the correct diagnosis can reduce antibiotic usage, drug resistance, and mortality rate (Arbique et al., 2004; CDC, 2015). At present, sensitivity to optochin and solubility in bile are the most common methods of pneumococcus laboratory diagnosis. These methods do not confirm the presence of the bacteria, and other complementary tests and quality control measures are required (Williams et al., 2002). In most countries, Immunoglobin G (IgG) is regularly used in pneumococcus infection diagnostic kits since it is precise, rapid, and cost-benefit (Lalitha et al., 1996; Williams et al., 2002).

Generally, antibodies used in diagnosis, research, and treatment are divided into 2 groups: polyclonal and monoclonal (Lipman et al., 2005). Monoclonal antibody production is limited since it needs specific laboratory and cell culture equipment. It is also time-consuming and expensive. In addition, monoclonal antibodies are extremely sensitive to extreme physical and chemical conditions, and thus, their storage is difficult (Hendriksen and Hau, 2002). Mammal's serum is an important and economical source of IgG, which is widely used for detecting diseases (Ostler et al., 2002). Polyclonal antibodies are obtained from immunized animals such as rabbits, goats, and sheep. These polyclonal antibodies include various antibodies against different antigenic epitopes of immunogens (Hanly et al., 1995).

In this study, we aimed to prepare IgG against pneumococcus to be used in diagnostic agglutination and ELISA kits, as well as, designing an ELISA kit for detecting *Streptococcus pneumoniae*. Diafiltration and Tangential Flow Filtration (TFF) were used to improve the purity of the antibody.

Materials and Methods

Streptococcus pneumoniae culture

Streptococcus pneumoniae strain IBRC-M 10819 was purchased from IBRC and cultured in Brain Heart Infusion medium (Merck, Cat. No. 110493). The cultures were incubated at 37 °C for 24 h. The bacteria were then inactivated by heating at 60 °C for one hour in a shaker water bath. Then, the bacteria were harvested using phosphate buffer pH 7.2. McFarland 3 standard of the bacteria was prepared for injection into rabbits.

Animal treatment and blood sampling

Four New Zealand white rabbits were used for injection. One mL of the antigen including bacterial suspension (9×10^8 CFU/mL) was intravenously injected into the rabbits ear vein, weekly for five weeks. One week after the last injection, 2 mL blood samples were collected from the ear vein, repeated for 6 weeks. After each sampling, the samples were centrifuged at 3000 RPM at 4 °C for 30 min to obtain the sera. The sera were then stored at −70 °C until use.

Purification and quality assessment of the antibodies

Ammonium sulfate (Merck, Cat. No. 101217) was used to precipitate the antibodies. Briefly, equal volumes of the serum and 0.15 M NaCl were mixed and placed on a stirrer at 200 RPM at 4°C. Then, an equal volume of saturated ammonium sulfate was added to the mixture dropwise and stirred for 30 min. The final mixture was centrifuged at 1500 RPM for 30 min at 4 °C. The supernatant was discarded and the precipitate was washed with semi-saturated ammonium sulfate. The centrifugation step was repeated, and the final precipitate was resuspended in one-third of the initial 0.15 M cold NaCl. TFF (Labscale TFF system, Millipore) was used to remove ammonium sulfate and concentrate the sample. A 100 kDa cut-off filter (Millipore, Pellicon® XL50 with Biomax® 100 kDa Membrane, C screen, 50 cm², Cat. No. PXB100C50) and PBS 0.07 M (Gibco, Cat. No.18912014) pH 6.3 were used in TFF.

Nesler test was used to confirm ammonium sulfate removal. Briefly, 150 µL of the purified protein, 20 µL Nesler reagent, and 25 µL potassium hydroxide (Merck, Cat. No.105033) were mixed in a 1.5 mL microtube. The transparency of the mixture indicates ammonium sulfate removal.

Ion-exchange chromatography was then used to remove the remaining impurities. The column was equilibrated using 50 mL PBS pH 7.6. Then, the samples were applied to the column. Protein was eluted from the column using the same buffer. The quality and quantity of the purified antibodies were evaluated using SDS-PAGE and Bradford assay.

Biotinylation of the purified antibody

One milligram biotin (Sigma, Biotin-NHS, Cat. No. 35013-72-0) was resuspended in 180 µL injection water and incubated at room temperature for 50 min. The antibody stock solution (2 mg/mL) was then added to biotin solution (26.6 µL/mL of antibody), and then, gel filtration chromatography (Sephadex® G-25 Medium, Sigma, Cat. No. GE17-0033-01) was used to exchange the buffer.

ELISA test

The primary antibody (1, 2, 5, 10 µg/mL) and bicarbonate coating buffer were added to a 96-well plate, and the plate was incubated overnight at 4 °C on a shaker. Then, the solutions in the wells were discarded, and the wells were washed 3 times with TBST washing buffer (20 mM Tris-HCl, Merck, Cat. No. 648317 and 150 mM NaCl). Next, 250 µL of blocking buffer and 0.01 g/mL bovine serum albumin (BSA, Sigma, Cat. No. A2153) was added to each well, and the plate was incubated at room temperature for 2 h on a shaker. After washing, 100 µL of inactivated bacteria (the antigen, at 3 McFarland standard concentration) was added to each well, and the plate was incubated at 37 °C for 1 h. Four wells were considered for blank and negative control. The wells were then washed 3 times, the biotinylated antibody (5, 10, 20, 40, 80 µg/mL) was added, and the plate was incubated at 37 °C for 30 min. The wells were then washed 4 times. HRP-streptavidin conjugate (Sigma, Cat. No. RABHRP3, 600 UL) was then added to all wells except for the blank, and the plate was incubated at 37 °C for 30 min. After 5 washing steps, 100 µL of the chromogenic substrate was added, and the plate was placed in a dark room for 15 min. 50 µL of stop solution was then added to the wells, and the plate was read at 450 nm using Hiperion MP^{4++} ELISA reader system.

Sensitivity and specificity of the assay

Twenty positive clinical samples of *Streptococcus pneumoniae* were used to determine the sensitivity of the assay. *E. coli, Pseudomonas aeruginosa, Neisseria meningitides*, and *Staphylococcus aureus* were used to determine the specificity of the assay. All samples were from the archive of positive and negative samples of Day Hospital, Tehran, Iran.

Agglutination test

The immunization antigen, positive clinical samples, and *Neisseria meningitides* whole-cell antigen were used as a test, positive control, and negative control, respectively. A single colony of patients' sample was resuspended in one drop of normal saline on a glass slide, and 30 µL of the antiserum was added. After one minute, the agglutination was evaluated.

Results and Discussion

According to the WHO report in 2005, nearly 1.6 million individuals died because of pneumococcal infection, more than 1 million of whom were children under 5 years old (WHO, 2005). One of the measures for controlling the disease is the rapid diagnosis of the patients (CDC, 2018).

Glover and Korschgen evaluated two methods of antiserum preparation (Glover and Korschgen, 1980). These methods differed in the injection method. Wolfe used IV injection (Wolfe, 1935), while Proom used the IM method (Proom, 1943). In Wolfe method, less time was needed, the steps of the process were fewer, and preparation/implementation of the antigen was easier. We used Wolfe method in this study. We also used the protocol developed by Leenaars and Hendriksen (Leenaars and Hendriksen, 2005). The concentration of the antibody they purified was 25.925 mg/mL, which indicates standard principles of injection, serum collection, and purification processes.

Antibody purification and qualification

Non-reducing 10% acrylamide gel electrophoresis was performed to assess the quality of the antibody. Electrophoresis was performed after the purification of the antibody using ion-exchange chromatography and TFF. The presence of a single 150 kDa band indicates the high purity of the antibody (Fig. 1).

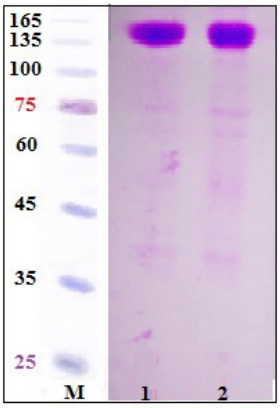

Figure 1. Analysis of the purified antiserum on 12% resolving SDS PAGE. M: pre-stained molecular ladder (25-165kDa). Lane 1, after ion-exchange chromatography. Lane 2, after tangential flow filtration, a single band of 150 kDa was observed.

Parker and his co-workers analyzed the purity of antibodies against pneumococcal anti-capsular polysaccharide (CPS) using SDS-PAGE (Parker et al., 2017). Their results showed that the purity of the anti-capsular IgG was more than 95%. We used TTF and ion-exchange chromatography (Diethylaminoethyl Sepharose Fast Flow) to eliminate impurities. SDS-PAGE analysis showed a single band of 150 kDa under non-reducing conditions. This means a highly pure antibody. Moreover, the color change from orange to clear in the Nessler test indicates the removal of ammonium sulfate (Fig. 2).

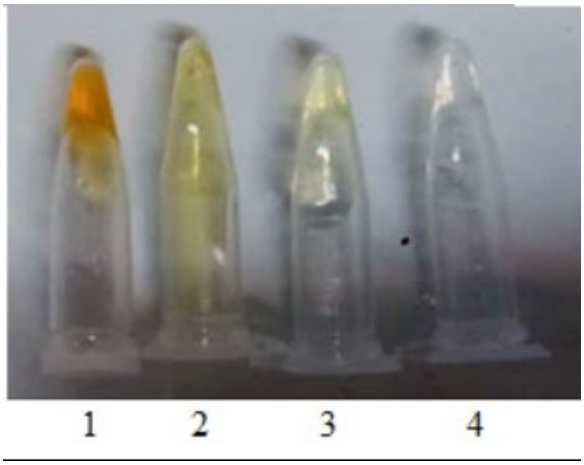

Figure 2. Nessler test. In the ammonia test, Nessler reagent (K_2HgI_4) reacts with the ammonia present in the sample (under strongly alkaline conditions) to produce a yellow species. The color change from orange to colorless after diafiltration indicates the complete removal of ammonium sulfate from the purified antibody. 1: unprocessed sample, 2-4: after passing 100-800 mL of buffer through the filter.

ELISA test

The ELISA method is an integral part of pneumococcus diagnosis. To determine the quality of the anti-pneumococcal immunoglobulins, an ELISA test was designed. The optimum concentration of the primary antibody was first determined, and then the absorbance was measured at 450 nm. The results showed that the absorbance of the blank and negative control was zero, and that of the positive samples was proportional to the different concentrations (Table 1). The highest absorbance was at 1 µg of the primary antibody and 80 µg of the biotinylated antibody. The optimum concentration of the primary and biotinylated antibody was 2.5 and 40 µg, respectively. After the optimization of the antibody concentration, the assay was used to evaluate the clinical samples. Table 2 shows the result of the assay on clinical *Streptococcus pneumoniae* samples and some other bacteria. The latter samples were used to determine the specificity of the assay.

Table 1. Absorbance of primary and biotinylated antibody.

				Biotinylated antibody concentration (µg)									
				5		10		20		40		80	
		B	C⁻	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Primary antibody concentration (µg)	1	0	0	0.318	0.304	0.486	0.447	0.676	0.730	1.144	1.202	1.983	1.818
	2.5	0	0	0.332	0.388	0.439	0.450	0.644	0.691	1.107	1.147	1.827	1.772
	5	0	0	0.312	0.347	0.458	0.512	0.661	0.664	1	1.049	1.873	1.685
	10	0	0	0.298	0.311	0.407	0.452	0.611	0.591	0.983	1.073	1.804	1.619

B: Blank, C⁻: negative control, R1: repeat one, R2: repeat two.

Table 2. The results of ELISA using the purified antibody and clinical samples. Optical density for the blank and the negative control should be less than 0.05 and 0.1, respectively. Cut-off value = optical density of the negative control mean +0.15. Cut-off Index (COI) = optical density of sample/cut-off value (values above 1/1 are positive). All clinical samples (CS) except CS17 sample were positive.

Optical density in 450 nm												
	CS13	CS14	CS15	CS16	CS17	CS18	CS19	CS20	Men	*E. coli*	Seu.ae	Staph
R1	0.651	1.761	1.161	0.925	0.017	0.382	0.341	1.230	0.01	0.001	0.007	0.000
R2	0.576	1.521	1.157	0.844	0.011	0.391	0.410	1.344	0.017	0.000	0.010	0.044

Optical density in 450 nm													
	B	C⁻	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	CS11	CS12
R1	0	0	0.454	0.361	1.211	0.870	1.47	1.244	0.425	0.871	1.112	1.232	0.411	0.766
R2	0	0	0.481	0.370	1.242	0.844	1.399	1.28	0.468	0.854	1.140	1.430	0.417	0.791

R1: Repeat one, R2: repeat two, B: blank, C⁻: negative control, CS1-CS20: clinical samples, Men: *Meningococcus*, Seu.ae: *Pseudomonas aeruginosa*, Staph: *Staphylococcus aureus*

Agglutination test

Slide agglutination tests have always attracted attention considering the low price of specific anti-pneumococcal antiserum and easy handling of agglutination tests. Agglutination test is highly valuable for pneumococcus diagnosis. Porter and colleagues in 2014 studied the precision, time, sample volume, and ease of agglutination test compared to Quellung test (Porter et al., 2014). Fig. 3 shows the positive test result for positive clinical samples and negative results for other bacterial samples in our study.

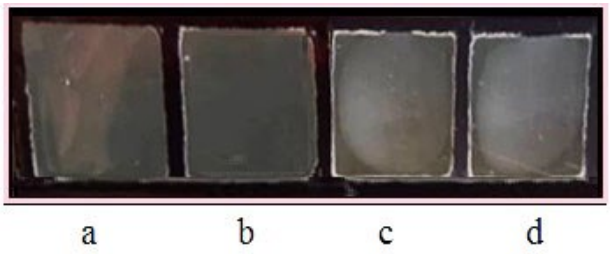

Figure 3. Agglutination test. The agglutination test using a) *E. coli* b) Meningococcal c) clinical Pneumococcal sample and d) Pneumococcal antigen. The test was positive using pneumococcal antigen and pneumococcal clinical samples (c and d), but it was negative using irrelevant samples (a and b).

Specificity and sensitivity

In this study, the specificity of the assay increased using the purified antiserum. Nowadays, several methods are used to eliminate undesirable antibodies against antigenic determinants and increase the sensitivity/ specificity of IgG. These methods include ammonium sulfate precipitation, dialysis, and chromatography. In this study, we performed dialysis using a novel TFF system with 30 and 100 kDa filters, precipitation by saturated ammonium sulfate, and followed by ion-exchange chromatography.

Twenty confirmed positive samples of *Streptococcus pneumoniae* were used to examine the sensitivity of the assay. The assay detected 19 samples, and thus, the sensitivity of the assay was determined to be 95%. To determine the specificity of the assay, non-pneumococcal bacterial samples were used, and no positive result was obtained. Therefore, the specificity was determined to be 100%.

The sensitivity and specificity analyses of ELISA test, which was developed for qualitative evaluation of prepared antibodies, showed that the developed assay could detect pneumococcus and had not any cross-reaction with other bacteria.

Conclusion

According to the qualitative and quantitative tests, the extracted IgG was highly pure. The results showed that the assay using prepared antiserum is sensitive and specific enough. Therefore, it can be a suitable replacement for commercial pneumococcal ELISA kits and can be used in research and clinical laboratories.

Ethical Statement

All the procedures of the study have been approved by the Ethics Committee of the Pasteur Institute of Iran, Tehran, Iran and has been confirmed by the provisions of the Declaration of Helsinki.

Acknowledgements

The authors appreciate the Pasteur Institute of Iran, Tehran, Iran for providing technical support.

Competing Interests

The authors declare no conflict of interest.

Funding Support

This work was funded by Pasteur Institute of Iran (Grant No: 1442).

Authors' Contribution

M. Moghiman contributed in the writing of the original draft. V. Kia and Z. Panahi performed data curation and R. Shokri was the supervisor of project. M. Paryan contributed in the design of methodology and validation.

References

Arbique, J.C., Poyart, C., Trieu-Cuot, P., Quesne, G., Maria da Glória, S.C., Steigerwalt, A.G., Morey, R.E., Jackson, D., Davidson, R.J. and R.R. Facklam, (2004). "Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov." *Journal of Clinical Microbiology*, **42**(10): 4686-4696.

Austrian, R. (1981). "Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention." *Reviews of Infectious Diseases,* **3**(Supp. 1):S1-7.

Austrian, R. (1999). "The Pneumococcus at the Millennium: Not down, not out." *The Journal of Infectious Diseases,* **179**(Supp. 2): S338-S341.

Centers for Disease Control and Prevention (CDC) (2018). "Global Pneumococcal disease and vaccine." Retrieved from: https://www.cdc.gov/pneumococcal/global.html.

Glover, R.L. and L.J. Korschgen, (1980). "Evaluation of two methods of preparing antisera for precipitin tests." *Wildlife Society Bulletin*, **8**(2): 118–122.

Hanly, W. C., Artwohl, J. E. and B. T. Bennett, (1995). ″Review of polyclonal antibody production procedures in mammals and poultry.″ *ILAR Journal,* **37**(3): 93-118.

Hendriksen, C. and J. Hau, (2002). ″Production of polyclonal and monoclonal antibodies.″, In: Hau, J. and G. L. Van Hoosier (Eds), *Handbook of Laboratory Animal Science, Second Edition: Essential Principles and Practices*. CRC Press, Boca Raton, pp. 391-412.

Lalitha, M.K., Pai, R., John, T.J., Thomas, K., Jesudason, M.V., Brahmadathan, K.N., Sridharan, G. and M.C. Steinhoff, (1996). ″Serotyping of *Streptococcus pneumoniae* by agglutination assays: a cost-effective technique for developing countries.″ *Bulletin of the World Health Organization*, **74**(4): 387–390.

Leenaars, M. and C. F. M. Hendriksen, (2005). ″Critical steps in the production of polyclonal and monoclonal antibodies: Evaluation and recommendations.″ *ILAR Journal,* **46**(3): 269-279.

Lipman, N.S., Jackson, L.R., Trudel, L.J. and F. Weis-Garcia, (2005). ″Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources.″ *ILAR journal*, **46**(3): 258-268.

İşeri, M.N., Dinleyici, M., Kılıç, Ö., Ceyhan, M., Gürler, N. and E.Ç. Dinleyici, (2019). ″Meningitis due to *Streptococcus pneumoniae* serotype 24B in a patient with cochlear implant previously vaccinated with the pneumococcal vaccine.″ *Mikrobiyoloji Bulteni*, **53**(4): 451-456.

Obaro, S. and R. Adegbola, (2002). ″The pneumococcus: carriage, disease and conjugate vaccines.″ *Journal of Medical Microbiology*, **51**(2): 98-104.

Ostler, E.L., Resmini, M., Brocklehurst, K. and G. Gallacher, (2002). ″Polyclonal catalytic antibodies.″ *Journal of Immunological Methods*, **269**(1-2): 111-124.

Parker, A.R., Lock, E., Iftikhar, A., Barber, R., Stubbs, P.D., Harding, S. and G.L. Wallis, (2017). ″Purification and characterization of anti-pneumococcal capsular polysaccharide IgG immunoglobulins.″ *Clinical Biochemistry*, **50**(1-2): 80-83.

Paton, J.C. and C. Trappetti, (2019). ″*Streptococcus pneumoniae* capsular polysaccharide.″ In: Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A., Braunstein, M. and J.I. Rood (Eds), *Gram-Positive Pathogens*, ASM Press, Canad, pp. 1-15.

Porter, B. D., Ortika, B. D. and C. Satzke, (2014). ″Capsular serotyping of *Streptococcus pneumoniae* by latex agglutination.″ *Journal of Visualized Experiments*. **25**(91):e51747.

Proom, H. (1943). ″The preparation of precipitating sera for the identification of animal species.″ *The Journal of Pathology and Bacteriology*, **55**(4): 419-426.

Williams, B.G., Gouws, E., Boschi-Pinto, C., Bryce, J. and C. Dye, (2002). ″Estimates of world-wide distribution of child deaths from acute respiratory infections.″ *The Lancet Infectious Diseases*, **2**(1): 25-32.

Winegarner, J.H. and J. Wittkopp, (2020). ″*Streptococcus pneumoniae* meningitis associated with over-the-counter sinus irrigation.″ *Cureus*, **12**(5): e8258.

Wolfe, H.R. (1935). ″The effect of injection methods on the species specificity of serum precipitins.″ *The Journal of Immunology*, **29**(1): 1-11.

World Health Organization (WHO) (2005). ″ International travel and health: Pneumococcal disease.″ Retrieved from: https://www.who.int/ith/diseases/pneumococcal/en/