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# Purification of Capsular Polysaccharide Produced by *Streptococcus pneumoniae* Serotype 19A

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***Streptococcus pneumoniae* is a major cause of invasive infection in young infants and older adults. There are currently 90 capsular serotypes identified and 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F) are responsible for about 90% of invasive disease. Among the more than 90 different *S. pneumoniae* serotypes, serotype 19A is globally very prevalent. A simplified purification procedure including adjustment of cell lysate pH to 4.5, fractionation with 50–80% ethanol, and dialysis rendered capsular polysaccharide (CPS) in a yield of 31.32 ± 3.11 mg from 1 l culture (75% recovery after lyses). The product contained only 69.6 µg of protein (99.78% purity) and 0.8 mg (sum of the precipitants from 50~60%, 60~70%, and 70~80%) of nucleic acid (97.45% purity). The purified CPS was conjugated with bovine serum albumin; the product size ranged from 100 to 180 kDa.**

**Keywords:** Capsular polysaccharide, conjugation, *Streptococcus pneumoniae* type 19A, purification

*Streptococcus pneumoniae* is a Gram-positive, lancet-shaped, coccus-shaped bacterium that usually exists in pairs (diplococci). It is the second most frequent cause (after *Haemophilus influenzae* type b) of bacterial meningitis and otitis media in children [8, 14]. Three major surface layers can be distinguished: plasma membrane, cell wall, and capsule. The latter is the thickest layer, and completely conceals the inner structures in exponentially growing pneumococci [4]. This capsule is a determinant of virulence in humans, because it interferes with phagocytosis by preventing antibodies from attaching to the bacterial cell [3].

At present, two pneumococcal pneumonia vaccines are available. One is a 23-valent pneumococcal polysaccharide vaccine (PPV-23), which is effective in 50−70% of cases in adults but not for children. The other is pneumococcal conjugate vaccine, which is about 80% protective against invasive pneumococcal disease in adults and children [14]. Recently, many new strains, including drug-resistant strains, have been increasingly identified in patients. Among the multidrug-resistant strains, serotype 19A is predominant, especially in pneumococcal disease in Korean infants [16, 20]. The capsular polysaccharide (CPS) of type 19A CPS can be purified in a 16-step, 3-day procedure with a contamination by protein (0.5%) and nucleic acid (0.02%), providing a CPS yield of 70% [2, 20]. The present study describes an alternative simplified and efficient purification process for type 19A CPS and the subsequent conjugation of the CPS with bovine serum albumin (BSA) to investigate simple and optimum conditions of CPS conjugation with other carrier proteins.

*S. pneumoniae* type 19A strain 10357, which expresses a standard type 19A CPS, and the CPS monoclonal antibody (mAb) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Tryptic soy agar with 5% defibrinated sheep blood (TSA) and brain heart infusion (BHI) broth were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The dialysis tubing required for dialysis of purified CPS was purchased from Spectrum Labs (Rancho Dominguez, CA, USA). For the Western blot analysis, hyper-enhanced chemiluminescence (ECL) film, nitrocellulose membranes, and ECL Western blotting system were purchased from GE Health Care (Uppsala, Sweden), and the film developer and fixer reagent were purchased from Sanda (Gwangju, Korea). Sodium deoxycholate

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monohydrate, acetic acid (97%) was purchased from Sigma-Aldrich Korea (Seoul, Korea). Acetic acid (first-grade) was purchased from Shinyo Pure Chemicals (Osaka, Japan). Sodium hydroxide and 95% ethanol (GR grade) were purchased from Duksan Pure Chemicals (Kyonggido, Korea). The protein size marker was purchased from Bio-Rad (Hercules, CA, USA).

*S. pneumoniae* 19A was grown on TSA at 37°C for 24 h. The seed culture was prepared by inoculating a colony into a 250 ml Erlenmeyer flask containing 50 ml of BHI broth and incubating at 37°C for 24 h. Then, 1% (v/v) of the seed culture was added to a 10 l fermentor containing 6 l of BHI broth and fermentation conducted during 24 h at 37°C. Protein concentration was quantified by the Bradford assay using BSA as the standard [5]. The amount of nucleic acid was measured by the absorbance at ultraviolet light at 260 nm, with nucleic acid content (μg/ml) being calculated as the optical density (OD) value × 50.

The antigen effect of purified type 19A CPS was analyzed by dot blotting. One μl of purified type 19A CPS was spotted onto a nitrocellulose membrane. After drying at room temperature (RT), the membrane was blocked for 1.5 h at 24°C using blocking reagent [5% (w/v); Sanda Co., Gwangju, Korea] dissolved in PBST buffer (phosphate-buffered saline, pH 7.6; 0.1% Tween 20), washed 30 min with PBST buffer, and treated with 30 ml of the type 19A CPS monoclonal antibody (mAb) (mAb:PBST ratio of 1:5,000) for 1.5 h at 24°C. After being washed for 30 min using PBST buffer, 30 ml of anti-rabbit immunoglobulin G heavy/light chain Ab (Ab:PBST=1:5,000) was added and the mixture was reacted for 1.5 h at 24°C. After being washed for 30 min, the membrane was treated with 1 ml of ECL reagent for 1 min, and exposed on X-ray film in the dark. The film signal intensity was analyzed by the AlphaEase FC 4.0 program (Alpha Innoteh, San Leandro, USA).

The conjugated mixtures were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane by using a semidry blotting system (Bio-Rad). Membranes were blocked by incubation in PBST (as described in dot blot) containing 5% skim milk for 1 h 30 min. The membrane was washed three times for 10 min with PBST, and a 1:5,000 dilution of 19A CPS antibody in PBST was added to the corresponding membrane and reacted for 1 h 30 min at room temperature. After being washed three times for 10 min in PBST, a 1:5,000 dilution of anti-rabbit immune globulin G (heavy plus light chain) in PBST was added and the mixture was reacted for 1 h 30 min at room temperature. After being washed three times for 10 min in PBST, the membrane was incubated with ECL reagent for 1 min and exposed to X-ray film in the dark room.

The quantity and purity of CPS were determined by high-pressure liquid chromatography with a refractive index detector (RID-10A; Shimadzu, Kyoto, Japan) equipped with a SUGAR KS-805 column (Shodex Co., Kawasaki, Japan) [11]. The CPS standard curve was prepared with a verified concentration of standard 19A CPS (ATCC 252-X; ATCC; Manassas, VA, USA). Distilled water was used as the mobile phase and the flow rate was maintained at 1 ml/min. All samples were prepared as 0.1% (w/v) solutions; 20 μl of solution was injected in each run.

The cell cultures were collected after 20 h ($OD_{600}$ = 0. 85) by centrifugation (12,000 ×*g*, 30 min), and the cell pellet was suspended in distilled water to 0.05% of the initial culture volume. The cells were lysed by the addition of sodium deoxycholate [0.1% (w/v)] for 1 h at 37°C, and boiled at 100°C for 20 min. The supernatants were recovered after centrifugation and the pH was adjusted to 3.5–6 with 5 M acetic acid to assess the effect of lysate pH on the recovery and purity of CPS. After 20 min, the precipitants were removed by centrifugation, and protein and nucleic acid in the supernatants were analyzed as described above. After adjustment of the supernatant pH to 4.5, CPS prepared from the cell lysate was fractionated with different ethanol concentrations. A 95% ethanol solution was added to make a final concentration of 30% and was stored at –20°C for 6 h; the precipitate that developed was recovered by centrifugation (1,000 ×*g*, 30 min). The remaining supernatant received a volume of 95% ethanol to make a final ethanol concentration of 40%, and the precipitant was obtained as previously described. Consecutive ethanol fractionations of 50%, 60%, 70%, and 80% were performed repeatedly, and the corresponding precipitants were collected. Each precipitant was dried at 50°C under vacuum and was dissolved to 2.0% (v/v) with distilled water. The precipitant from each ethanol fractionation was dialyzed against distilled water for 8 h using a dialysis membrane with a cut-off size of 12,000–14,000 at 4°C. The purity of each preparation was determined as described above.

To prepare conjugated CPS with bovine serum albumin, the purified type 19A CPS (6 mg) was oxidized using 10 mM sodium metaperiodate ($NaIO_4$) in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.15 M NaCl, pH 7.2) at 24°C in the dark for 30, 60, or 90 min. At each time period, 2 ml of ethylene glycol was added to stop the $NaIO_4$ reaction and the solution was left at 24°C for an additional 30 min. The resulting oxidized CPS was dialyzed and lyophilized. Each oxidized CPS was mixed with 1 mg of BSA in 1 ml of PBS. Sodium cyanoborohydride was added to each suspension to a final concentration of 50 mM, and the reaction mixture was stirred in sealed vials at 4°C for 3 days.

The effect of pH adjustment on the sodium deoxycholate lysate supernatant was analyzed against CPS recovery and purity (Table 1). CPS was analyzed by dot blot analysis for measuring only CPS without any contaminant. The recovery of CPS after the pH adjustment was 3.77% at pH 3.5, 20.11% at pH 4.0, 81.7% at pH 4.5, 82.96% at pH 5.0,

**Table 1.** Effect of pH adjustment on the sodium deoxycholate lysate supernatant for CPS yield, and concentrations of protein and nucleic acid.

| pH* | Protein[a] (mg/l) | Nucleic acid[b] (mg/l) | CPS yield[c] (%) |
|---|---|---|---|
| Control | 78.29±1.31 | 26.66±2.27 | 100 |
| 3.5 | 0±0.10 | 2.55±1.03 | 3.77±1.26 |
| 4.0 | 0±0.10 | 2.71±0.92 | 20.11±5.03 |
| 4.5 | 0±0.10 | 2.93±0.74 | 81.7±1.26 |
| 5.0 | 0±0.10 | 5.91±0.95 | 82.96±2.51 |
| 5.5 | 2.56±0.15 | 18.14±1.57 | 67.87±5.03 |
| 6.0 | 8.08±0.44 | 24.16±2.28 | 61.59±1.26 |

*After boiling of culture supernatant with sodium deoxycholate, pHs were adjusted with the addition of 5 M acetic acid before ethanol precipitation.
[a]Protein was calculated by the Bradford assay using BSA as the standard.
[b]Nucleic acid was calculated by detection at UV 260 nm (OD value × 33=μg/ml).
[c]Signals on dot blot were analyzed by the AlphaEase FC 4.0 program and CPS yield was calculated by comparison of spots before and after pH adjustment.
Control; pH 6.8 after finishing culture.
100% CPS ranged from 40 to 45 mg/l of culture.

**Table 2.** Analysis of CPS after each ethanol fractionation.

| Cumulative final ethanol* concentration | Protein[a] (mg/l) | Nucleic acid[b] (mg/l) | CPS yield[c] (%) |
|---|---|---|---|
| pH 4.5 | 0** | 2.93±0.64 | - |
| Up to 30% | 0 | 0.06±0.01 | 2.05±1.02 |
| 30–40% | 0 | 1.09±0.03 | 6.15±2.06 |
| 40–50% | 0 | 0.85±0.05 | 9.23±1.03 |
| 50–60% | 0 | 0.46±0.05 | 25.13±2.56 |
| 60–70% | 0 | 0.23±0.05 | 31.28±2.57 |
| 70–80% | 0 | 0.15±0.06 | 32.31±5.64 |

*After pH 4.5 adjustment, the supernatant was precipitated by the particular ethanol concentration. The precipitants were dissolved in distilled water and analyzed.
**As in Table 1.
[a]As in Table 1.
[b]As in Table 1.
[c]As in Table 1.
100% CPS ranged from 32.9 to 37.3 mg/l of culture.

67.87% at pH 5.5, and 61.59% at pH 6.0 (Fig. 1). The protein concentration contained in the CPS preparation from 1 l culture after adjustment of pH to 3.5–5.0 was 0 mg (no detection by Bradford assay), 2.56 mg at pH 5.5, and 8.08 mg at pH 6.0. Compared with the control (without pH adjustment; pH 6.8), which contained 78.29 mg of protein, the reduction of contaminated protein by pH control was significant. The nucleic acid concentration contained in CPS (prepared from 1 l culture) after adjustment of pH was to 24.16 mg at pH 6.0, 18.14 mg at pH 5.5, 5.91 mg at pH 5.0, 2.93 mg at pH 4.5, 2.71 mg at pH 4.0, and 2.55 mg at pH 3.5. A pH of 4.5 for the pH adjustment of lysate was chosen for subsequent CPS recovery.

To improve the CPS purity further, the ethanol concentration for the fractionation step was optimized [2]. The CPS yield after the final 30% ethanol precipitation was 2.05%. When the ethanol concentration in the supernatant was from 30% to 40%, 6.15% of control CPS (with 100% corresponding to the quantity of CPS before ethanol fractionation) was further recovered. The ethanol concentration was gradually increased as 40–50%, 50–60%, 60–70%, and 70–80%. As a result, CPS recovery also gradually increased from 9.23% to 32.31%, relative to control CPS (Fig. 2). Fractionation of CPS with 50–80% ethanol after adjustment of the cell lysate pH to 4.5 produced a CPS yield of 89%

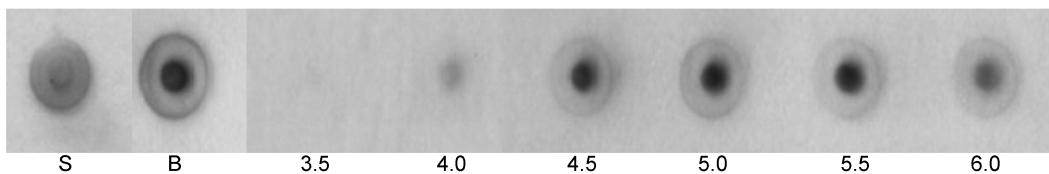


**Fig. 1.** Dot blot for the identification of *S. pneumoniae* serotype 19A capsular polysaccharide recovery ratio at various pHs.
Each supernatant of DOC cell lyzates was adjusted for the pH. After removal of precipitant from each sample, supernatants were analyzed by dot blot. The spots were analyzed by AlphaEase FC program and the CPS recovery ratio was calculated by comparison of the before spot. (S: ATCC 19A CPS; B: Before the adjustment of pH; 3.5~6.0: adjusted pH). Antibody used was 19A CPS Ab.

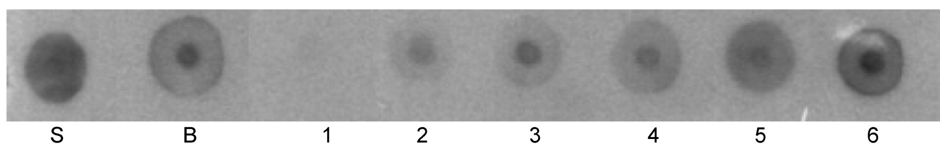


**Fig. 2.** Dot blot of precipitants on each ethanol concentration.
Primary antibody was used the antisera against 19A capsular polysaccharide. (S: ATCC 19A CPS; B: Before ethanol precipitation; 1: precipitant in final ethanol concentration 30%; 2: precipitant from ethanol concentration 30~40%; 3: precipitant from ethanol concentration 40~50%; 4: precipitant from ethanol concentration 50~60%; 5: precipitant from ethanol concentration 60~70%; 6: precipitant from ethanol concentration 70–80%). Antibody used was 19A CPS Ab.

**Table 3.** The 19A capsular polysaccharide, protein, nucleic acid, and purity in product purified by the simplified process.

| Procedure | CPS amount (mg/l) | Yield (%) | Protein/CPS (%) | Nucleic acid/CPS (%) | Purity Protein/nucleic acid (%) |
|---|---|---|---|---|---|
| New* | 31.32±3.11 | 75±1.08 | 0.22±0.1 | 2.25±0.5 | 99.78±0.1/97.45±0.5 |

*Procedure was the simplified process invented in our study.
Cultivation and purification processes were repeated four times and then all values calculated.

(the CPS sum of the three ethanol precipitations; 50−60%, 60−70%, and 70−80%) with no detectable amount of protein and 2.01 mg of nucleic acid in CPS (prepared from a 1 l culture). The fractionated CPS was further dialyzed to remove accidentally incorporated small carbohydrate (data not shown). Consequently, by using the optimized process, the final pure 19A CPS was quantified to 31.32 ± 3.11 mg/ 1 l culture (75% recovery) by HPLC and the CPS was contaminated with 0−69.6 μg protein (99.78% purity) and 0.8 mg nucleic acid (97.45% purity, Table 3).

To date, the purification of type 19A CPS from both cells and culture supernatant has been done by an 8-step or 16-step purification procedure that involves ultrafiltration and filtering steps, with the final CPS containing purity less than 2% protein or nucleic acid over the limit of contaminant in the WHO series [2, 17−19]. The presently developed protocol represents an alternative simplified purification procedure, which is achieved in three steps: pH adjustment to 4.5 after cell lysis with sodium deoxycholate treatment and boiling, fractionation with 50−80% ethanol, and dialysis. Reduction of pH causes a precipitation of soluble protein and other soluble components such as DNA, RNA, and cellular debris [16]. Presently, CPS was prepared using fractionation with 50−80% ethanol for the additional removal of nucleic acid. Serotype 3 CPS, which ranges in size from 280−1,200 kDa and which has a structure of [→3)-βD-GlcUA-(1→4)-βD-Glc(1→], precipitates in 30−50% ethanol [12]. Type 19F CPS, whose structure is [→4)-βD-ManpNAc-(1→4)-αD-Glcp-(1→2)-αL-Rhap-(1-PO4-)→], similar to that of type 19A CPS, [→4)-βD-ManpNAc-(1→4)-αD-Glcp-(1→3)-αL-Rhap-(1-PO4-)→], but which is larger (370~1,000 kDa), can be prepared using 40−65% ethanol [8]. Since type 19A CPS has a smaller size (100−270 kDa) and an overall negative charge due to the constituent phosphate residues, it is probably soluble in up to 50% ethanol [12]. Thus, the differing compositions, linkages, and sizes of CPS require different purification regimens [4, 8]. The presently developed procedure can be completed in fewer than 48 h, which is superior to other procedures that require over 72 h [2, 20].

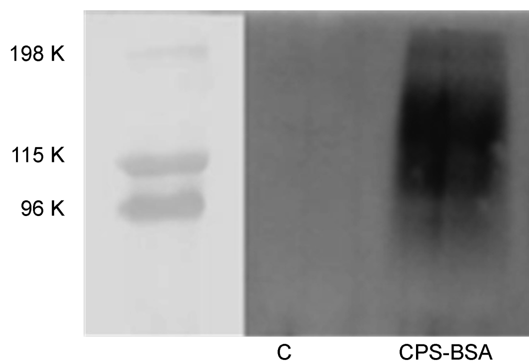


**Fig. 3.** Western blot analysis for the conjugation of the purified 19A CPS to BSA.
C, Purified CPS before the conjugation; CPS-BSA, Conjugated CPS to BSA. Antibody used was 19A CPS Ab.

Protection against encapsulated bacteria is mediated by Ab raised against CPS. Most polysaccharides are, however, weak immunogens, which induce mainly IgM Abs and little, if any, memory response [20]. Infants, whose immunological systems are not fully developed and are therefore most in need of protection against infectious organisms, respond poorly to these antigens and are thus susceptible to infections from encapsulated bacteria. A covalently linked protein-to-polysaccharide antigen converts the polysaccharide to a T-cell dependent antigen, which can induce an anamnestic response, with class switching and, importantly, also induce Ab responses in infants [1]. We conjugated the CPS to BSA for investigating the possibility to conjugate with vaccine carrier protein. Purified 19A CPS was successfully conjugated to BSA, as confirmed by Western blotting (Fig. 3). Purified CPS was not transferred to the membrane, but the conjugated CPS was successfully transferred to the membrane. The conjugated CPS size ranged from 100 to 180 kDa, indicating the hydrolysis of the polysaccharide during periodates oxidation. Since different-sized CPS may influence the efficiency of the immune response, the conjugation of BSA with different-sizes of type 19A CPS is in progress. The conjugation of type 19A CPS with carrier protein such as FlaB is in progress.

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## References

1. Alonso de Velasco, E., A. F. Verheul, G. H. Veeneman, L. J. Gomes, J. H. van Boom, J. Verhoef, and H. Snippe. 1993. Protein-conjugated synthetic di- and trisaccharides of

pneumococcal type 17F exhibit a different immunogenicity and antigenicity than tetrasaccharide. *Vaccine* **11:** 1429−1436.

2. Arnold, F. 1998. Alcohol-free pneumococcal polysaccharide purification process. US Patent 5714354.
3. Avery, O. T. and R. Dubos. 1931. The protective action of a specific enzyme against type Iii pneumococcus infection in mice. *J. Exp. Med.* **54:** 73−89.
4. Bednar, B. and J. P. Hennessey. 1993. Molecular size analysis of capsular polysaccharide preparations from *Streptococcus pneumoniae*. *Carbohydr. Res.* **243:** 115−130.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248−254.
6. Brian, B., T. Lee, J. A. Lotvin, M. E. Ruppen, and P. F. Charbonneau. 2006. Separation of contaminants from *Streptococcus pneumoniae* polysaccharide by pH manipulation. US Patent 0228381.
7. Bruyn, G. A. and R. van Furth. 1991. Pneumococcal polysaccharide vaccines: Indications, efficacy and recommendations. *Eur. J. Clin. Microbiol. Infect. Dis.* **10:** 897−910.
8. Cano, F. J. S. V., J. S. C. O. Kuo, and M. V. R. V. Querry. 1980. Purification of pneumococcal capsular polysaccharides. US Patent 4242501.
9. Choi, E. H., S. H. Kim, B. W. Eun, S. J. Kim, N. H. Kim, J. Lee, and H. J. Lee. 2008. *Streptococcus pneumoniae* serotype 19A in children, South Korea. *Emerg. Infect. Dis.* **14:** 275−281.
10. Dagan, R., M. Isaachson, R. Lang, J. Karpuch, C. Block, and J. Amir. 1994. Epidemiology of pediatric meningitis caused by *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis* in Israel: A 3-year nationwide prospective study. *J. Infect. Dis.* **169:** 912−916.
11. Ghebregzabher, M., S. Rufini, B. Monaldi, and M. Lato. 1976. Thin-layer chromatography of carbohydrates. *J. Chromatogr.* **127:** 133−162.
12. Katzenellenbogen, E. and H. J. Jennings. 1983. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 19A. *Carbohydr. Res.* **124:** 235−245.
13. Musher, D. M. 1992. Infections caused by *Streptococcus pneumoniae*: Clinical spectrum, pathogenesis, immunity, and treatment. *Clin. Infect. Dis.* **14:** 801−807.
14. Myers, C. and A. Gervaix. 2007. *Streptococcus pneumoniae* bacteraemia in children. *Int. J. Antimicrob. Agents* **30S:** S24−S28.
15. Pelton, S. I., H. Huot, J. A. Finkelstein, C. J. Bishop, K. K. Hsu, and J. Kellenberg. 2007. Emergence of 19A as virulent and multidrug resistant pneumococcus in Massachusetts following universal immunization of infants with pneumococcal conjugate vaccine. *Pediatr. Infect. Dis. J.* **26:** 468−472.
16. Skov-Sorensen, U. B., J. Blom, A. Birch-Andersen, and J. Henrichsen. 1988. Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen in pneumococci. *Infect. Immun.* **56:** 1890−1896.
17. World Health Organisation. 2000. Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines. *WHO Tech. Rep. Ser.* **897:** 27−56.
18. World Health Organization (2005). Recommendations for the production and control of pneumococcal conjugate vaccines. *WHO Tech. Rep. Ser*. **927:** 64−98.
19. World Health Organization. 2004. Recommendations for the production and control of meningococcal group C conjugate vaccines. *WHO Tech. Rep. Ser*. **924:** 102−128.
20. Yuan, Y. T., M. G. Ruppen, W. M. Sun, L. S. Chu, J. U. N. Simpson, J. C. O. H. Patch, J. K. V. C. Moran, and P. N. C. Fink. 2008. Shortened purification process for the production of capsular *Streptococcus pneumoniae* polysaccharide. US Patent WO/2008/118752.