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# Purification and characterisation of anti-pneumococcal capsular polysaccharide IgG immunoglobulins

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ABSTRACT

**Objectives:** The production of reference materials for quantifying pneumococcal antibody concentrations relies upon large scale vaccination. An alternative simple, reproducible protocol has been developed for the affinity purification of 23 serotype anti-pneumococcal capsular polysaccharide (PCP) IgG immunoglobulins.

**Design & Methods:** The purification protocol utilised IgG fractionation, capsular polysaccharide (CPS) adsorption, and affinity chromatography using Pneumovax®-Sepharose. Purification efficiency and method reproducibility were assessed by comparison of 4 batches of anti-PCP IgG. Immunoglobulin composition was determined using nephelometry and functionality was evaluated using VaccZyme™ ELISAs.

**Results:** Anti-PCP IgG preparations were ≥95% pure by SDS-PAGE analysis with no contaminating IgA or IgM immunoglobulins or IgG antigen specific antibodies towards *haemophilus influenzae b*, *diphtheria* toxoid or *tetanus* toxoid. The predominant IgG subclass in the preparation was IgG2.

**Conclusions:** This novel purification procedure produced highly specific anti-PCP IgG preparations that compared well to both Lot 89SF and 007sp international serum standards and could be used as an alternative method for the production of reference materials.

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## 1. Introduction

Historically, assays for quantifying pneumococcal antibody concentrations relied upon the use of the reference material Lot 89SF. Lot 89SF was produced using pooled serum specimens from 17 adults immunized with Pneumovax® [1]. Lot 89SF has now been superseded by a new reference serum, 007sp, which was developed using pooled plasma samples from 262 adults immunized with Pneumovax® [2]. For both Lot 89SF and 007sp no additional purification steps were employed.

Large scale vaccination of healthy volunteers requires questionnaires to prove medical validity and considerable co-ordination in order to obtain the material. In addition to IgG, in response to Pneumovax® IgM and IgA will also be produced [3]. The binding of all three antibodies to their target epitopes and opsonisation activities will be heavily influenced by the presence of each antibody presumably through epitope competition and steric interaction [3,4]. This may therefore affect estimation of the IgG concentrations if IgA and IgM are not first removed. We hypothesised that with the provision of alternative starting material and the use of a simple purification procedure we could produce a highly purified preparation of anti-pneumococcal capsular polysaccharide IgG antibodies.

The current study reports a simple alternative method for the generation of IgG anti-pneumococcal reference material that does not require a large scale vaccination programme and is free from antigen specific IgA and IgM. This methodology uses a 3-step column chromatography protocol to produce PCP IgG-specific antisera and allows quantification of the PCP-IgG for standardisation purposes.

## 2. Materials and methods

### 2.1. Purification procedures

#### 2.1.1. Preparation of CPS-Sepharose

Thirty milligrams of *Streptococcus* CPS (Statens Serum Institut, Copenhagen, Denmark) were reconstituted to 2 mg/ml in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH adjusted to 8.1 with 0.5 M sodium borate) and incubated with 15 ml of N-hydroxysuccinimide (NHS)-activated Sepharose (GE Healthcare, Amersham, UK) for 20 h at room temperature. The resulting matrix was poured into a gravity-flow column (Bio-Rad, California, USA) and the unbound eluate collected. The unreacted active groups on the material were blocked for 2 h with 0.2 M Tris pH 8.0 and then alternately washed for 3 cycles with 0.1 M

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Tris pH 8.5 containing 0.5 M NaCl, and 0.1 M sodium acetate pH 4.5 containing 0.5 M NaCl.

*2.1.2. Preparation of Pneumovax-Sepharose*

A total of 3.5 mg of each of the 23 Pneumovax serotypes (Sanofi Pasteur, Lyon, France) were pooled, concentrated to 15 ml, and buffer exchanged into 0.5 M NaCl (Centricon plus 70, 10 kDa cut-off membrane, EMD Millipore, Massachusetts, USA). Sodium acetate (0.5 ml of 1 M, pH 5.5) was added to the polysaccharides and allowed to oxidise with 10 mM sodium periodate for 1 h in the dark. Excess periodate was quenched with 1% glycerol and removed by desalting on a Hiprep Sephadex G25 column (GE Healthcare), equilibrated in 50 mM potassium phosphate 150 mM NaCl pH 7.4. The oxidised polysaccharides were then mixed with 10 g of epoxy-1,6-diaminohexane (EAH)-Sepharose 4B (GE Healthcare) for 1 h at room temperature. Reductive amidation using sodium-cyanoborohydride (Sigma-Aldrich, Missouri, USA) was achieved with 40 mg initially and a subsequent addition of 10 mg, for 2 days at room temperature. 20 mg of sodium borohydride was then added to quench the reaction. The Pneumovax-coupled Sepharose (10 ml) was washed and blocked as described for the CPS coupled Sepharose.

*2.1.3. Pooled donor serum*

Healthy donor plasma (typically 5–10 donors) was obtained from expired stock (Bio Products Ltd., Elstree, UK). The plasma was pooled, preserved with 0.099% Sodium Azide, 5 mg/ml EACA, 1 mM EDTA, 2 mM Benzamidine HCl, clotted and filtered to produce serum. All procedures were performed at 4 °C.

*2.1.4. Purification of anti-PCP IgG immunoglobulins*

IgG immunoglobulins were precipitated from pooled donor serum (2 L) with 40% ammonium sulphate and purified using DE52 anion-exchange chromatography (loading buffer: 10 mM sodium phosphate pH 7.0) [5]. Adsorption and affinity chromatography were performed sequentially using CPS- and Pneumovax-linked Sepharose, respectively (loading buffer: 25 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, 0.1% EACA, 0.01% benzamidine, 0.05% *v*/v sodium azide, pH 7.0; elution buffer: 0.1 M glycine, pH 3.0 containing 10% v/v glycerol and 0.05% v/v Proclin preservative). Elution fractions were neutralised with 10% volume 2 M Tris, 1.5 M NaCl, pH 8.0, dialysed against fully preserved PBS buffer (25 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, 0.1% EACA, 0.01% benzamidine, 0.05% *v*/v sodium azide, pH 7.0) and stored at −80 °C.

Two batches of the PCP IgG preparation (1 A and 1B) were purified from the same pool of serum, and two further independent batches were prepared from separate pools of sera (batches 2 and 3).

*2.2. Analytical procedures*

The efficiency of CPS-Sepharose coupling was determined by dot blot using standard procedures. Analysis of the starting material and unbound material was accomplished with a biotinylated HAA lectin probe and visualised with Streptavidin peroxidase (both Sigma, UK). Dot blots probed with human anti-PCP antisera were used to confirm retention of activity following coupling of Pneumovax to Sepharose. The blot was probed with PCP-positive human antisera (1:250), followed by anti-IgG-HRP (1:1000), and visualisation with AEC.

The concentration of the PCP polysaccharides was estimated using the Phenol-Sulphuric acid procedure [6] against a calibrator composed of an equimolar mixture of glucose, galactose and N-acetyl-glucosamine. Protein concentration was measured by the Bicinchoninic acid assay (BCA) procedure (Gamma Globulin Std., Thermo-Pierce, UK).

Concentrations of IgG, IgA, IgM and IgG subclasses were determined using commercially available nephelometric assays (The Binding Site Group Ltd., Birmingham, UK) analysed using a BN™II analyser (Siemens Healthcare Ltd., Marburg, Germany). To assess functionality and the presence of cross-reactive antibodies, the purified IgG preparations were tested in the following VaccZyme™ ELISAs according to manufacturer's instructions: PCP IgG, PCP IgA, PCP IgM, Tetanus toxoid IgG, Diphtheria Toxoid IgG and Haemophilus influenzae type b IgG (The Binding Site Group Ltd).

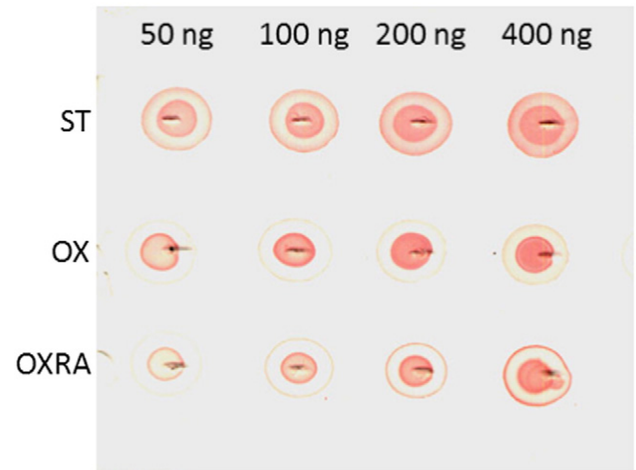


**Fig. 1.** Dot blot of Pneumovax polysaccharides: before (ST), after oxidation with 10 mM periodate (OX) and subsequent reductive amination (OXRA).

SDS-PAGE was conducted using 4–12% NUPAGE® gels (Thermofisher Scientific Inc., Massachusetts USA) with a pH 6.0 MOPS running buffer as described in the manufacturer's instructions. Silver staining of the gels was accomplished using the BioRad silver stain kit.

Serotype specific ELISAs, similar to those previously reported (without 22F absorption [2]) and Luminex technology [7] were employed to determine the relative concentration of serotypes in all batches.

*2.3. Ethical Considerations*

This project did not require ethical approval. Remnants of specimens collected for routine diagnostic testing were used in accordance with the Declaration of Helsinki.

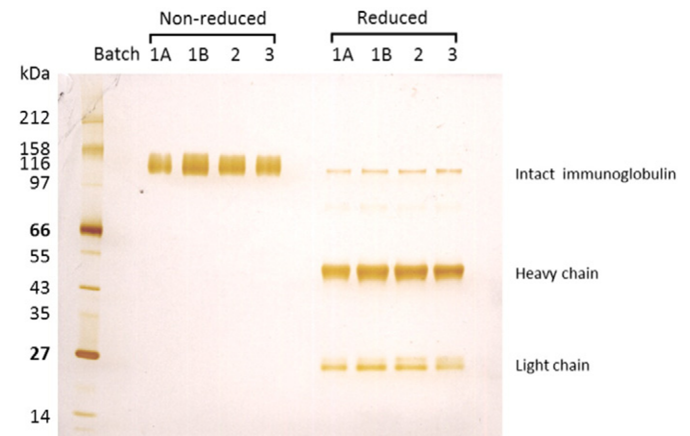


**Fig. 2.** SDS-PAGE analysis of four different affinity purified anti PCP IgG immunoglobulin batches. 1 μg total protein per batch (1 A, 1B, 2 and 3) was loaded into each lane and separated under reduced or non-reduced conditions as per Materials and Methods. Under non-reduced conditions (left hand panel), only one protein band was observed corresponding to an intact immunoglobulin. The mobilities of the immunoglobulin were similar in each batch. Under reduced conditions (right hand panel), three protein bands were observed corresponding to intact immunoglobulin, heavy and light chains. The mobilities of intact immunoglobulins, heavy and light chains were comparable between each batch.

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