Enzyme-linked immunosorbent assay for quantitative determination of capsular polysaccharide production in *Streptococcus pneumoniae* clinical isolates

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A simple, specific, sensitive and reproducible ELISA has been developed to quantify the level of CPS (capsular polysaccharide) production in supernatants of *Streptococcus pneumoniae* cell cultures. CPSs from *Strep. pneumoniae* have been widely used as vaccine antigens. The quantification method is based on two type-23F serotype-specific polyclonal antibodies: IgG, purified from sera of mice immunized with a pneumococcal type-23F CPS conjugate, used in the coating step, and a serotype-specific rabbit serum as the second antibody. Solutions of purified type-23F CPS were used as standards. The relationship between A_{492} and type-23F CPS concentration was linear over the range 1–310 ng/ml ($r = 0.989$), with 1 ng/ml as the lower limit of sensitivity. The specificity of ELISA was assessed because purified type-19F CPS and cell-wall polysaccharide samples were not detected after their evaluation by the ELISA described in the present study. Repeatability and intermediate precision of the assay were good, the coefficients of variation being 3 and 10% respectively. This ELISA allowed selection of an appropriate vaccine strain, for a natural polysaccharide vaccine, among several 23F pneumococcal clinical isolates and constituted a valuable analytical tool for *Strep. pneumoniae* fermentation and CPS purification follow-up.

Introduction

Streptococcus pneumoniae is an important cause of morbidity and mortality worldwide. This encapsulated Gram-positive bacterium can cause pneumonia, bacteraemia, meningitis and acute otitis media. The pneumococcal CPS (capsular polysaccharide) is a key virulence factor, and more than 90 immunologically different serotypes of pneumococci, based on the structure of the CPS, have been described [1]. Most human pneumococcal infections are caused by only 20 serotypes. *Strep. pneumoniae* CPSs are high-molecular-mass polymers whose function is to protect the microorganism against host immune response. CPSs are the main antigens used in both plain polysaccharide vaccines and those conjugated with carrier proteins [2].

Vaccination has been encouraged as a method for protection against emerging multidrug-resistant pneumococci. Currently available vaccines against this pathogen include a 23-valent plain CPS vaccine [3] and a 7-valent conjugate vaccine [4,5]. The plain vaccines are either poorly immunogenic or non-immunogenic in infants and approx. 50–90% effective in preventing invasive pneumococcal disease in older persons. Additionally, vaccine-induced protection declines after 3–5 years [6–8]. Unlike the plain vaccines, the conjugate vaccine is immunogenic in infants and able to induce immunological memory [9,10].

Strep. pneumoniae also produces a subcapsular CWPS (cell-wall polysaccharide) that is common to all pneumococci [11]. Despite the high immunogenicity of CWPS, anti-CWPS antibodies are not protective [12].

Key words: capsular polysaccharide, ELISA, fermentation, pneumococcal vaccine, serotype-specific rabbit serum, *Streptococcus pneumoniae*.

Abbreviations used: CPS, capsular polysaccharide; CWPS, cell-wall polysaccharide; mHSA, methylated human serum albumin; SSRS, serotype-specific rabbit serum; TSA, trypticase soy agar; TSB, trypticase soy broth.

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Bacterial CPS can be obtained by both chemical synthesis [13] and purification from bacterial cultures [14]. Routine characterization of the concentration, composition and chemical identity of polysaccharides has typically been confined to colorimetric assays specific for selected types of monosaccharides or substituents found in the polysaccharide repeating unit structure, the classic colorimetric assay based on hexose determination being one of the most used methods [15–17]. Other non-colorimetric methods have also been used to meet this goal, such as high-performance anion-exchange chromatography with pulsed amperometric detection, GLC and NMR spectroscopy, requiring highly purified samples and fairly expensive equipment [16,18,19]. Two methods for quantitative determination of CPS from *Salmonella typhi* Vi outer capsular polysaccharide, based on Vi optical properties, have been described [20]. Light-scattering rate nephelometry [21] has been also applied for polysaccharide determination. Other authors have described immunoassays to quantify CPS from *Haemophilus influenzae* [22,23] or from *Strep. pneumoniae* [24] in body fluids.

In the present study, we describe an ELISA for the quantitative determination of CPS production in cell culture supernatants of *Strep. pneumoniae* clinical isolates. Using solutions of purified type-23F CPS as standards, a simple, specific, sensitive and precise assay was developed. The development of such a method allows selection of candidate vaccine strains for the production of a natural polysaccharide vaccine, and also constitutes a valuable analytical tool for vaccine production follow-up.

Materials and methods

Pneumococcal strains

Strains 231, 232, 233, 234 and 235 of *Strep. pneumoniae* serotype 23F and strain 191 serotype 19F were recovered from children under 5 years old with meningitis. Typing was done by the capsular reaction with pooled type, or group antisera from Statens Seruminstitut (Copenhagen, Denmark).

Plain and conjugated CPSs

Plain CPS from *Strep. pneumoniae* serotypes 23F and 19F, used as standards and control respectively in the ELISA experiments, were a gift from The National Health Institute (Bilthoven, The Netherlands). CWPS was purchased from Statens Seruminstitut. Tetanus-toxoid conjugates of type-23F pneumococcal CPS, used for mice immunizations, were prepared from fractions of type-23F CPS with molecular masses between 1000 and 10000 Da that were conjugated through a spacer at the terminal end by maleimido–thiol coupling chemistry [13].

Mice pneumococcal type-23F CPS-specific IgG

Two groups of ten female 5–6-week-old Balb/c (H-2d) mice each were supplied by the Center for Laboratory Animals Production (Havana, Cuba). They were housed under controlled 12 h light/12 h dark cycles at 22 °C and were provided with water and food *ad libitum*. Mice were intraperitoneally inoculated with 1 μg (group 1) and 3 μg (group 2) of type-23F pneumococcal CPS conjugated with tetanus toxoid. Booster injections were administered 15 and 30 days after the first inoculation. Animals were bled on day 0 and 15 days after the last immunization. Sera were stored at −20 °C until use. Experiments with animals were carried out in accordance with the legal requirements of the national authority. For the characterization of mouse immune response, high-binding-polystyrene 96-well flat-bottomed microtitre plates (Costar, Cambridge, MA, U.S.A.) were coated, overnight at 4 °C, with a mixture of mHSA (methylated human serum albumin) and type-23F pneumococcal CPS, both diluted at 5 μg/ml in 0.05 M sodium carbonate buffer (pH 9.6) as previously described [25]. Serum samples were serially diluted in 3% (w/v) skimmed milk in PBS/T (PBS and 0.05%, v/v, Tween 20). Bound antibodies were detected with horseradish-peroxidase-conjugated goat anti-mouse IgG antibodies (Amersham), with the chromogen *o*-phenylenediamine and A_{492} values were read. All sera were analysed in duplicate. The maximal dilution at which the immune serum gave an absorbance value 2-fold higher than preimmune serum was regarded as the serum titre. Sera with titres higher than 1:200 were pooled and precipitated with $(NH_4)_2SO_4$, and the IgG fraction was purified by affinity chromatography using Protein A from *Staphylococcus aureus* (Amersham Biosciences) as described in [26]. To prepare mHSA, HSA was treated with methanolic HCl as reported in [27].

ELISA for quantitative determination of pneumococcal CPS

The assay used was a modification of that of Kim et al. [28]. High-binding-polystyrene 96-well flat-bottomed microtitre plates (Costar) were coated at 4 °C for 18 h with 0.05 ml of affinity-purified mice IgG specific for pneumococcal type-23F CPS at 10 μg/ml diluted in 0.05 M sodium carbonate buffer (pH 9.6). Plates were washed three times with PBS/T and once with PBS. After incubation with 3% (w/v) BSA in PBS/T (blocking solution) at 37 °C for 1 h, plates were incubated at 22 °C for 2 h with 0.05 ml of either purified type-23F CPS, used as standard, 2-fold serially diluted in PBS, starting at 5 μg/ml or supernatant samples from pneumococcal cell cultures diluted 1:50 and 1:100 in PBS. To assess the specificity of assay, after blocking, some wells were incubated with pneumococcal type-19F CPS or CWPS. After three washes, a 23F SSRS (serotype-specific rabbit serum) (Statens Seruminstitut) was added, diluted at 1:200

in blocking solution and incubated at 22°C for 2 h. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1:1000 in blocking solution, at 37°C for 1 h. Plates were washed and incubated at 22°C for 5 min with substrate solution containing the chromogen *o*-phenylenediamine (Sigma) at 0.5 mg/ml and 0.03% (v/v) H_2O_2 diluted in 0.1 M citric acid phosphate buffer (pH 5). Enzyme reactions were stopped with 25 μl of 1 M H_2SO_4. A_{492} was read using an automatic ELISA reader (Sens/Den Scan, Merck, Germany). All samples were assayed in duplicate and A_{492} was expressed as the mean value.

For standardization of adequate peroxidase-conjugated dilution, able to give the higher A_{492} values with lower background, several dilutions of conjugate, ranging from 1:500 to 1:10000, were assayed. Conjugate dilution of 1:1000 was selected for further work.

For determination of SSRS working dilution, several dilutions were previously evaluated using the ELISA described for characterization of mice immune response.

Shake flask experiments

A frozen stock (50 μl) of *Strep. pneumoniae* culture was used to inoculate a TSA (trypticase soy agar; Oxoid, U.K.) plate, supplemented with 7% (v/v) sheep blood and incubated in a candle jar at 37°C for 18 h. Cells were used to inoculate two 250 ml flasks containing 100 ml of TSB (trypticase soy broth) medium (Oxoid) at an attenuance (D_{620}) of 0.1. Flasks were incubated at 37°C and 50 rev./min for 3 h. Bacterial cultures from each flask were then used to inoculate a 250 ml flask containing 100 ml of fresh Hoeprich's medium [29] at a D_{620} of 0.1 and grown at 37°C and 50 rev./min for 8 h. Culture samples from both flasks were collected at 2, 4, 6 and 8 h after inoculation and centrifuged at 5600 ***g*** for 20 min. Supernatants from both bacterial cultures, regarded as samples A and B, were stored at −20°C until analysis. The purity of cultures was assessed by plating the samples on blood/agar plates. For each *Strep. pneumoniae* strain evaluated, this experiment was conducted in duplicate. The geometric mean of the A_{492} value for the four determinations conducted for each strain was used for analysis and graphics.

Bioreactor batch cultivation

Frozen stocks (50 μl) of *Strep. pneumoniae* cultures were used to inoculate TSA plates supplemented with sheep blood. Cells were inoculated in a 500 ml flask containing 250 ml of TSB and cultivated at 37°C and 100 rev./min for 3 h before inoculation of the bioreactor to obtain an initial D_{620} of 0.1. Batch cultures were carried out in a 6-litre bioreactor (Pierre Guerin Sas Division Biolafitte, Mauze-sur-le-Mignon, France) with automatic control of pH, temperature and shaking. The bioreactor, containing 3.5 litres of fresh Hoeprich's medium [29], was operated at 37°C and 100 rev./min for 5 h. pH was controlled at 7.6 by the addition of 5 M NaOH. Following inoculation, culture samples were collected every hour and centrifuged at 5600 ***g*** for 20 min. Supernatants were stored at −20°C until analysis. The purity of cultures was assessed by plating the samples on blood/agar plates.

CPS purification and analysis

Purification of *Strep. pneumoniae* 23F serotype CPS started with whole-culture precipitation with 1.5% (w/v) hexadecyl trimethylammonium bromide and centrifuged at 11000 ***g*** for 45 min. The cell-free supernatant was concentrated 10-fold using a tangential 30 kDa-cut-off membrane to eliminate low-molecular-mass contaminants. Sodium acetate at 5% (w/v) was added and pH adjusted to 5.5. Ethanol was added to a final concentration of 28% (v/v); the mixture was centrifuged at 11000 ***g*** for 45 min to eliminate nucleic acids and proteins. For polysaccharide precipitation, sodium acetate was added to a final concentration of 7.2% (w/v) and pH 6.8. Ethanol was then added to a final concentration of 60% (v/v). Polysaccharide was recovered by centrifugation at 11000 ***g*** for 45 min [30]. The CPS was diluted in 0.3 M sodium acetate (pH 6.9) and extracted three times with 0.25 vol. of saturated phenol with 0.5 M sodium acetate (pH 9.0). Extracted CPS was extensively dialysed against double-distilled water. Purified CPS was precipitated as described above. After centrifugation, pellet was dissolved in double-distilled water and CPS was freeze-dried. Polysaccharide content was determined by the phenol/sulphuric acid method for hexose quantification [17] using glucose as a standard. Protein contaminants were determined using the BCA (bicinchoninic acid) protein assay reagent kit (Pierce). Nucleic acid content was estimated by measuring A_{620} as previously described [31]. Phosphorus concentration was determined by the method of Chen et al. [32]. The molecular size of CPS and the distribution coefficient were determined in a Sepharose CL-4B (Amersham Biosciences, Sweden)-packed XK16/100 column, equilibrated with 0.2 M ammonium acetate using a differential index refractometer (Knauer, Berlin, Germany) and a UV detector (Uvicord; Amersham Biosciences). Polysaccharide identity was carried out by ^{1}H-NMR spectroscopy (900 MHz).

Data analysis

A_{492} for the 2-fold dilutions of polysaccharide standards, starting from 5.0 μg/ml, were plotted as the vertical variable against the polysaccharide concentration plotted as the horizontal variable. A linear fit was made for values within the range of concentration from 1 to 310 ng/ml and the calculated least-squares regression line was used as a standard curve. All curves and regression lines were controlled by examination of a graphic display and by calculation of correlation coefficients. The concentration of each sample was

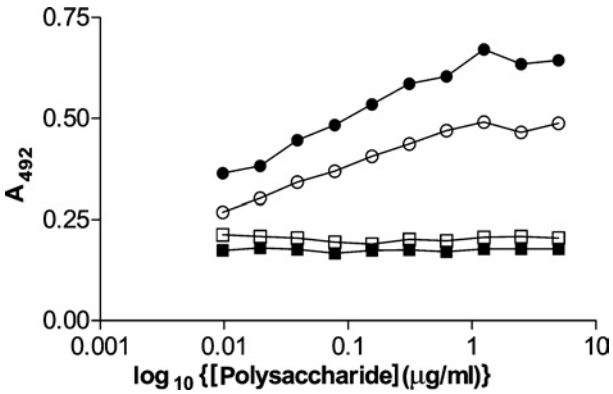

Figure 1 Relationship between A_{492} values and CPS concentration (μg/ml) for the ELISA technique described

The Figure shows ELISA reactivity of type-23F (circles) and type-19F (squares) polysaccharides. Dilutions of 1:200 (filled symbols) and 1:400 (open symbols) of 23F SSRS were assayed. The dilution selected for further experiments with SSRS was 1:200.

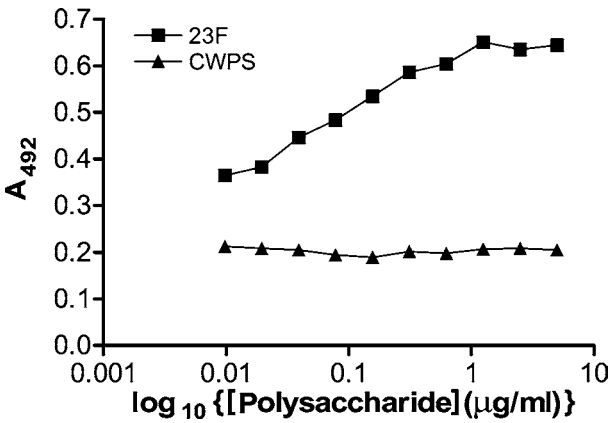

Figure 2 Specificity of ELISA developed for quantification of pneumococcal type-23F CPS

The Figure shows the reactivity of pneumococcal CWPS (triangles) and type-23F polysaccharide (squares) on the ELISA described. Only type-23F-specific CPS was detected, but not the CWPS ($P < 0.001$).

estimated by introducing the corresponding A_{492} value into the equation for standard curve, followed by multiplication for the sample dilution. Absorbance values for sample dilutions outside the linear range limits of curves were not considered.

Statistical analysis

The statistical significance of differences between A_{492} values measured for the assayed serotype and negative control was assessed by Dunn's multiple comparison test. This test was also used to evaluate the statistical significance of differences in CPS concentration on supernatant samples. $P < 0.05$ was considered statistically significant.

Results

ELISA for quantitative determination of type-23F pneumococcal CPS

Optimization of 23F SSRS working dilution At the beginning we were concerned about determination of adequate dilution of SSRS, for satisfactory ELISA signal in future CPS quantification experiments. Serial 2-fold dilutions of SSRS, starting from 1:50, were directly applied on to ELISA plates previously coated with a mixture of mHSA and *Strep. pneumoniae* type-23F CPS, both diluted at 5 μg/ml [25]. After evaluation by ELISA, maximal dilution that fulfils the cut-off criterion assumed of $A_{492} \geqslant 0.5$ was 1:400.

For CPS quantification ELISA, plates were coated with affinity-purified mouse IgG specific for type-23F CPS, diluted at 10 μg/ml and ten 2-fold serial dilutions, starting from 5 μg/ml, of polysaccharides from serotypes 23F and 19F were evaluated. SSRS was used at dilutions of either 1:200 or 1:400 (see Figure 1). Significant differences were detected between A_{492} values measured for 23F CPS and 19F CPS independently of the SSRS dilution assayed, with $P < 0.01$ and $P < 0.05$ for dilutions 1:200 and 1:400 respectively. The higher difference, between specific and non-related CPS, was detected when SSRS was diluted at 1:200 and thus was chosen as SSRS working dilution.

Specificity of the assay The CWPS and CPS are both linked to the *Strep. pneumoniae* cell-wall peptidoglycan [33]. Because of this, CPS purified from bacterial sources may contain significant amount of CWPS. The presence of CWPS common to all serotypes must be taken into account when specific CPSs are measured [34]. In order to evaluate the specificity of the assay, the invariant CWPS was evaluated. Figure 2 shows that only type-23F-specific CPS was detected, but not the CWPS ($P < 0.001$), showing the specificity of the assay.

Sensitivity, linearity and precision (repeatability and intermediate precision) A total of 15 solutions of 2-fold serially diluted purified type-23F CPS, starting from 5 μg/ml, were prepared and assayed by ELISA. As depicted in Figure 3, the relationship between absorbance and CPS concentration was linear over the range of 1–310 ng/ml ($r = 0.989$), and this relationship was reproducible in all experiments, with 1 ng/ml as the lower limit of sensitivity.

The precision of the ELISA system was considered at two levels: repeatability and intermediate precision. To evaluate the repeatability of the assay, supernatants from shake-flask cultures were quantified three times on the same day and variability observed was low, the coefficient of variation being 3%. Furthermore, the same supernatant sample was assayed several times using the described ELISA

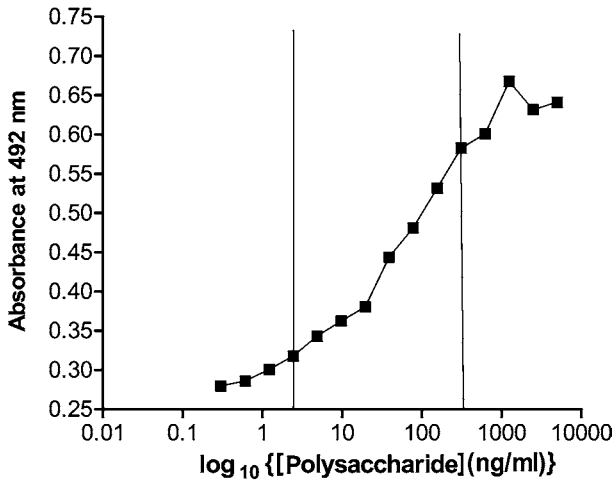

Figure 3 Linear range and lower limit of sensitivity of ELISA developed for quantification of *Strep. pneumoniae* CPS

The Figure shows that the relationship between A_{492} and antigen concentration was linear over the range 1–310 ng/ml ($r = 0.989$). The lower limit of sensitivity was 1 ng/ml.

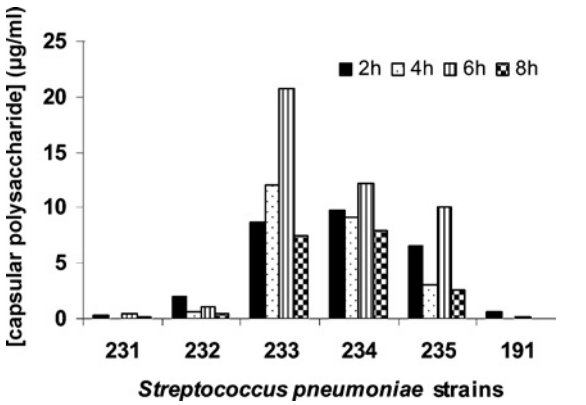

Figure 4 Quantification of CPS by ELISA in culture supernatants of *Strep. pneumoniae* type-23F clinical isolates

Five serotype 23F *Strep. pneumoniae* clinical isolates (strains 231–235) were grown for 8 h and culture samples were collected every 2 h. A type-19F strain (191) was used as a control. The Figure shows the geometric mean value of polysaccharide concentration measured in supernatants of the four determinations performed for each strain. The highest levels of polysaccharide production were detected after 6 h of growth for strain 233.

methodology, but on different days and by different analysts, to test the variability of the results within assays. The coefficient of variation of the result from those experiments was 10% and was considered as intermediate precision. An intermediate precision of approx. 10% suggests a high consistency of the technique, similar results being obtained independently of the analyst and laboratory conditions.

Applications of quantification ELISA

Selection of strains with highest level of CPS production Five *Strep. pneumoniae* serotype 23F clinical isolates grown for 8 h in flask and culture samples were collected every 2 h. To select the strain with the highest levels of CPS accumulation, the amount of CPS released to the medium by the cells of each strain was measured by the quantification ELISA described above (Figure 4). The highest levels of CPS production were found in culture supernatants corresponding to strains 233 and 234, with no significant difference detected between these two isolates ($P > 0.05$). Strains 231, 232 and 235 showed levels of CPS production similar to that detected in supernatant samples from type-19F control strain ($P > 0.05$). Differences between control strain and strains 233 and 234 were statistically significant ($P < 0.001$ and $P < 0.05$ respectively). Strain 233 was selected for further experiments of fermentation and purification of serotype 23F pneumococcal CPS.

Strep. pneumoniae fermentation processes follow-up Pneumococcal type-23F strain 233 was cultivated in a bioreactor. Two fermentation processes were carried out and culture samples were collected every hour after inoculation. No

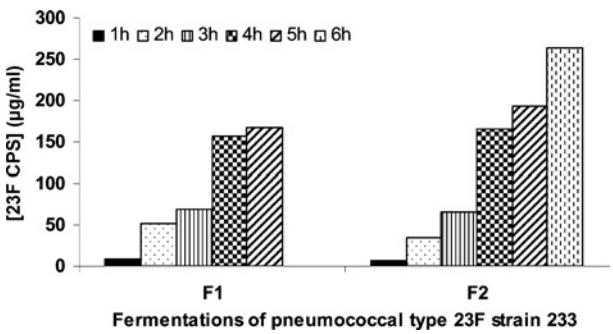

Figure 5 Kinetics of CPS expression of *Strep. pneumoniae* type-23F strain 233, cultured on a bioreactor

Two fermentation processes (F1 and F2) were carried out and culture samples were collected every hour after inoculation. Levels of CPS released to the medium by the pneumococcal cells were measured by the quantification ELISA. The highest levels of CPS expression were detected after 5 and 6 h of growth, for F1 and F2 respectively.

further increase in A_{620} was detected for fermentation 1 (F1) after 5 h of growth, so it was stopped, but fermentation 2 (F2) was stopped after 6 h. Figure 5 shows the levels of CPS released to the medium by the pneumococcal cells during the two fermentation processes, measured by the quantitative ELISA. The highest CPS expression levels were detected after 5 and 6 h of growth, for F1 and F2 respectively.

Bacterial cultures from both bioreactor batch growth experiments were centrifuged and supernatants were used to purify the 23F type CPS released to the medium by the *Strep. pneumoniae* cells. After purification, both purified CPS

samples showed phosphorus, protein and DNA contents in accordance with the WHO (World Health Organization) requirements for vaccine manufacturing. Analysis by ^{1}H-NMR demonstrated the chemical identity of purified CPS from both fermentations (results not shown).

Samples of 1 mg/ml, based on dry weight, were prepared using purified polysaccharide from both fermentations. Both samples were quantified using both the classic colorimetric assay, based on hexoses determination [17], and the quantification ELISA developed in the present study. The samples were quantified using two different methods in order to evaluate whether the two methods could bring significant equal results or not. Then, the closeness of agreement of the values obtained by both methods was considered as the recovery. After analysis of purified pneumococcal 23F type CPS either by the hexose determination assay or by the ELISA, sugar contents of 0.95 and 0.907 mg/ml respectively were detected for F1 samples. Sugar contents of 0.84 and 0.87 mg/ml respectively were detected for F2 samples. This result shows a good recovery between the two methods, with values of 95.47% (F1) and 103.57% (F2), and an average of 99.52%.

Discussion

CPSs have long been recognized as the major *Strep. pneumoniae* virulence factors. Host protection against invasive pneumococcal disease depends crucially on the presence of anti-CPS antibodies. Commercial vaccines available for the prevention of pneumococcal disease are composed of pneumococcal polysaccharide separately purified from the outer capsules of several pneumococcal strains [3,35] or pneumococcal conjugate vaccines, in which polysaccharides are covalently linked to carrier proteins [9].

Quantitative determination of saccharide component in both plain CPS and glycoconjugate vaccines is a key control test. Several methods have been developed to achieve this [15,17–21,36]. Immunoassays have also been described to measure levels of pneumococcal polysaccharide antigens in body fluids. In 1980, Nolan and Ulmer [24], for the measurement of *Strep. pneumoniae* type III CPS in cerebrospinal fluid in an experimental model of pneumococcal meningitis, described a single-antibody sandwich technique in which the globulin fraction of a pneumococcal type-specific antiserum was used to coat the solid phase before antigen attachment and to conjugate with the labelling enzyme, horseradish peroxidase. Also, an enzyme immunoassay for the quantification of pneumococcal C polysaccharide in the sputum of patients with presumptive pneumococcal pneumoniae was described. In this assay, samples of the sputum of patients were applied on to ELISA plates, previously coated with an anti-phosphocholine mouse IgG3, probed with an anti-phosphocholine rabbit serum and reactivity revealed with an alkaline-phosphatase-conjugated anti-rabbit IgG [37].

Kim et al. [28], investigating the relationship between cell surface carbohydrates, i.e. teichoic acid and CPS, in opaque and transparent *Strep. pneumoniae* colony phenotypes, described a capture ELISA to determine CPS production in sonicated cell fractions of cultured bacteria. First, they coated the ELISA plates with a commercial type-6B-specific rabbit antiserum from the Statens Seruminstitut and used purified type-6B CPS, at a known concentration, as a standard. Both standard and CPS in sonicated cell fractions were detected with a commercial mouse IgM monoclonal antibody from Statens Seruminstitut elicited against type-6A and type-6B CPS. In the present study we have developed several modifications to the ELISA described by Kim et al. [28] in order to see whether it could be applied for detection and quantification of CPS released to the media by cells of *Strep. pneumoniae* serotype 23F during growth. The main modifications introduced were the use, in the coating step, of a laboratory-made type-23F-specific IgG purified from sera of mice immunized with a type-23F polysaccharide–protein conjugate, and the use of the type-23F-specific rabbit antiserum from the Statens Seruminstitut as the second antibody. Good recovery, repeatability and intermediate precision, as well as the linearity of the method, were demonstrated. As low concentration as 1 ng/ml could be measured using the quantification ELISA described in the present paper, being at least as sensitive as other immunoassays developed to quantify bacterial polysaccharide antigens [22,37]. A limit of sensitivity of 1 ng/ml was reported for an ELISA developed to detect and quantify the capsular antigen of *H. influenzae* type b [22]. Other research [37] reported 2 ng/ml as a limit of sensitivity for an immunoassay designed to quantify pneumococcal C polysaccharide in sputum samples of patients.

The assay was found to be 23F-CPS-specific: decreases in A_{492} values were observed only when lower 23F-CPS concentrations were assayed, not when other non-specific, such as 19F CPS or CWPS, were evaluated. When curves for 23F were compared with curves for 19F and CWPS, significant differences were detected, yielding $P < 0.01$ and $P < 0.001$ respectively. It is known that most *Strep. pneumoniae* CPS preparations, including vaccines and reagents used to measure antibody responses, are contaminated with invariant CWPS, which is present in all serotypes. In fact, in addition to type-specific antibodies to pneumococcal CPS, most people also produce antibodies to CWPS, which may be detected in antibody assays due to the contamination of CPS antigens. To measure both anti-CPS antibody levels and opsonic activity of such antibodies, more accuracy is needed to remove by adsorption the CWPS antibodies [34,38,39]. In the present study, we demonstrated that

the CPS quantification ELISA developed here is able to detect type-23F-specific CPS, but not CWPS contaminants. Addition of decreasing quantities of CWPS does not cause further decreases in A_{492} values measured, reducing the possibilities of overestimation of specific CPS produced by the *Strep. pneumoniae* cells.

The specificity of the system was also proved when a pneumococcal CPS belonging to serotype 19F was evaluated and results similar to those obtained for CWPS were achieved. The future evaluation of CPS belonging to several serotypes, using the ELISA system developed here for quantification of type-23F CPS, could indicate the specificity of this system in detecting only serotype 23F *Strep. pneumoniae* strains. The development of an ELISA system specific for detection and quantification of CPS from strains belonging to other pneumococcal serotypes could raise the possibility of using this system for typing of *Strep. pneumoniae* isolates.

In the present study, we have described an ELISA system sensitive, specific and precise in detecting type-specific polysaccharide released from *Strep. pneumoniae* cells to the culture media, able to detect and quantify CPS from *Strep. pneumoniae* cell cultures belonging to serotype 23F. This technique is a simple test that can be performed in most laboratories, with readily available materials and without need for complex equipment. This system could also be applied to quantify CPS from strains belonging to other pneumococcal serotypes by using different combinations of mouse and rabbit sera. In fact, the methodology described in the present paper was used to quantify CPS from serotypes 18C, 1, 14 and 19F using home-made mouse IgG and commercial rabbit sera specific to these serotypes. Repeatability, sensitivity and specificity regarding the above serotypes CPS quantification were also assayed with similar results (results not shown). Purified CPSs from each serotype were used as standards. At present, these curves are being used to quantify the pneumococcal CPS production of *Strep. pneumoniae* cells to select adequate vaccine strains for CPS production, intended to be used in an anti-pneumococcal vaccine. Further experiments should be done in order to see whether the ELISA system described here could be applied for pneumococcal cell typing or diagnosis.

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