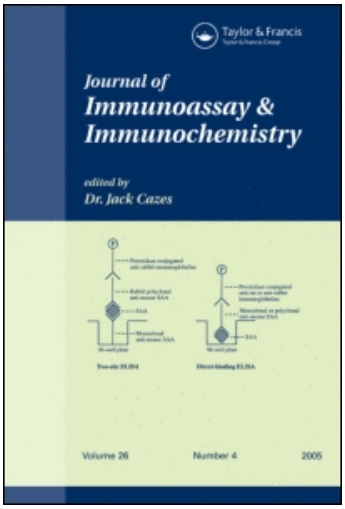

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A SPECIFIC AND ULTRASENSITIVE CHEMILUMINESCENT SANDWICH ELISA TEST FOR THE DETECTION AND QUANTITATION OF PNEUMOLYSIN

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ABSTRACT

A chemiluminescent sandwich ELISA test has been developed for the detection and quantitation of pneumolysin. The test is based on a mouse monoclonal as the capture antibody and on rabbit polyclonal IgGs as detection antibodies, in combination with an anti-rabbit IgG alkaline phosphatase conjugate. The estimated detection limit of the purified recombinant toxin in phosphate-buffered saline with 0.05% Triton X-100 is around 5 pg ml^{-1}, with averaged intra- and inter-assay variation coefficients of 7% and 13.5%, respectively.

The assay has been applied to the quantitation of pneumolysin in pneumococcal isolates, providing, for the first time, a direct measurement of the amount of the toxin produced by different strains; a variation has been found in their pneumo-

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lysin content. The test is highly specific as no other purified toxins or human pneumonia- or meningitis-associated bacteria yielded false-positive results. This specific and highly sensitive method could help in the diagnosis of human infections.

INTRODUCTION

Streptococcus pneumoniae, highly adapted to colonizing the human upper respiratory tract, is a leading cause of community-acquired pneumonia, otitis media, sinusitis, and meningitis.(1,2)

The diagnosis of pneumococcal infection currently relies on the isolation of the pneumococcus, but this takes no less than 48 hours and can be unsuccessful because of the spontaneous or antibiotic-induced lysis of the bacteria. Gene amplification diagnostic procedures, being highly specific and sensitive, are rather laborious and require specialized knowledge and laboratory equipment;(3-13) results are not known in less than three hours either. At present, the most advantageous alternative diagnostic methods are immunoassays based on the detection of capsular or cell wall components in biological specimens, although they are not absolutely specific for the pneumococcus.(14-23) As diagnostic tests, ELISA analyses are fast and well-established routine laboratory procedures.

Pneumolysin (PLY) is a 53-kDa toxic protein characteristically produced by the pneumococcus; it is not secreted, but liberated, by autolysis. PLY is a member of a family of antigenically related cholesterol-binding pore-forming bacterial thiol-activated toxins (TACYs); all these toxins are lytic for eukaryotic cells.(24, 25) Suilysin (SLY), expressed by *Streptococcus suis*, has been shown to share the highest sequence identity and also the closest phylogenetic relationship with PLY (26).

The gene encoding PLY exhibits very limited sequence variability. The toxin has been found in almost all the pneumococcal strains isolated so far.(27) Its immunodetection has recently been shown to allow the identification of the so called "atypical" pneumococcal isolates as well as the characterization of related *Streptococcus mitis* strains harboring the PLY gene.(28-30)

No detailed study on the extent of PLY production by different bacterial strains has been reported so far; variations in the production of PLY have been inferred from the observed hemolytic activities of the strains under investigation.(27, 30-33)

The present work was undertaken with the aim of developing a useful immunodetection test for routine clinical practice. We herein describe a capture ELISA which is superior, in terms of specificity, to the currently

applied diagnostic tests of pneumococcal infection based on the detection of capsular components. We have also explored the suitability of this ELISA to quantitate the amount of PLY produced by different pneumococcal isolates.

EXPERIMENTAL

Antigens

Recombinant PLY was expressed and purified as previously reported.(28) The protein purity of the final preparations in 10 mM phosphate-buffered saline (PBS), pH 7.3, was assessed by SDS-PAGE and Coomassie brilliant blue R-250 staining; the total protein concentration was colorimetrically estimated applying the Bio-Rad (Bradford) protein assay. Streptolysin O (SLO), α-hemolysin from *Staphylococcus aureus* and α-hemolysin from *Escherichia coli* were purchased from Sigma. Recombinant purified listeriolysin O (LLO) was kindly provided by Dr Siegfried Weiss, GBF, Germany.

Antibodies

The generation and reactivity characteristics of anti-PLY rabbit IgG and the PLY-7 mouse monoclonal antibody have already been described.(28, 34) The antibody 1.4G8.66 is a mouse IgG1, kappa monoclonal generated against human pepsinogen C.(35)

Bacterial Strains

Reference bacterial strains were obtained from collections. Clinical isolates of *Streptococcus agalactiae, Haemophilus influenzae,* and *Neisseria meningitidis* were from the Servicios de Microbiología, Hospital Central de Asturias, Oviedo; the source and/or characteristics of the other strains used in the present study have already been defined.(28)

Sandwich ELISA

Flat-bottomed polystyrene Combiplate 8 White Breakable (Labsystems) plates were coated with 1 μg/100 μL per well of capture anti-

body in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were incubated at 37°C for 6 h.

This solution was then poured off and wells were filled with 200 μL of blocking buffer, prepared according to the instructions of the manufacturer of the ELISA-Light™

Chemiluminescence Detection System (Tropix)

The plates were then incubated at 37°C for 1 h and overnight at 4°C. After four washings with 300 μL per well of PBS, with an additional 0.1% Tween 20, 100 μL per well of the samples to be assayed were added, and incubated at 37°C for 1 h with shaking.

Once washed six times as above, 100 μL per well of anti-PLY rabbit IgG (1:500) in blocking buffer were incubated at 37°C for 30 min with shaking.

After washing, the wells were filled with 100 μL of anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma), 1:5,000, in blocking buffer, and incubated at 37°C for 30 min with shaking.

Following washing four times as before, and one more time with assay buffer (ELISA-Light™ Chemiluminescence Detection System, Tropix), the plates were loaded on a Luminoskan RS (Labsystems) luminometer and then the assay wells were automatically filled with 100 μL of ready-to-use substrate/enhancer solution (0.4 mM CSPD® with 1X Sapphire-II™) (Tropix) and incubated for 10 min.

A regression line for recombinant PLY was set after measuring five triplicates of doubling dilutions of the antigen (500, 250, 125, 62.50, and 31.25 pg mL^{-1}). The detection limit of the assay was defined from the precision of the zero dose estimates according to the formula: mean of zero dose estimates + 2.8 x SD (standard deviation).(36)

The reproducibility of measurements was evaluated from high, middle, and low controls. Concentrations were estimated with reference to the calibration curve. Intra-assay coefficients of variation were determined from 15 replicates of each control. The inter-assay precision was evaluated from 30 determinations made on different plates and days.

Quantitation of PLY in Pneumococcal Lysates

Pneumococcal strains were grown as lawns on blood agar plates at 37°C for 24 h. From these, cell suspensions were prepared in PBS, pH 7.3.

Viable counts were made from 1:10 serial dilutions in PBS by plating in duplicate on blood agar.

Lysates were prepared from aliquots (900 μL) of the dilutions used for viable counts as already described.(28) Briefly, bacterial suspensions were pelleted by centrifugation. Then, pellets were resuspended in 900 μL of PBS with 0.05% Triton X-100, pH 7.3, and incubated at 37°C for 30 min. After centrifugation, supernatants were stored at -20°C until assay in ELISA for PLY detection. Determinations were made in triplicate.

To accurately quantitate the concentration of PLY in bacterial lysates, only those dilutions which rendered measurements falling within the assayed linear range of the standard curve were taken into consideration. In the case of high PLY-producing strains, the amount of the toxin contained in 1 x 10^7 colony forming units (CFU) was extrapolated from those data.

Specificity Assays

When available, purified toxins were tested; otherwise, non-pneumococcal strains were treated as the pneumococcal ones. *H. influenzae* and *N. meningitidis* strains were grown on chocolate agar. Lysates from 1 x 10^7 CFU were assayed in ELISA in triplicate.

Statistical Analyses

These were carried out using the Statistica program (StatSoft).

RESULTS

Optimization of the Sandwich ELISA Test

Based on the reactivity properties of the monoclonals we had generated, we selected the PLY-7 one as the capture antibody of our indirect ELISA sandwich test.

Prior to the chemiluminescent assay, the optimal concentration and characteristics of the detecting and enzyme-conjugated antibodies were established by means of conventional colour ELISA determinations. We assayed horseradish peroxidase and alkaline phosphatase conjugates from different sources, and various substrates (OPD, TMB, pNPP, and the AMPAK™ detection system from DAKO A/S). In our hands, the lowest detection limit observed (100 pg mL^{-1}) was reached with the alkaline-phos-

phatase/ pNNP system. Then, seeking to increase the sensitivity of the detection of PLY, we next applied the chemiluminescent variant as described in the EXPERIMENTAL. Section

As PLY is not secreted, we considered that the chances for detection of this antigen in biological samples would be higher if the pneumococci present in these were lysed and/or permeabilized. Thus, several formulations of lysing solutions which allowed bacterial lysis and/or permeabilization, along with PLY detection, were assayed. We found that the suspension of bacteria in PBS, added with 0.05% Triton X-100, for 30 min at 37°C, satisfied both requirements; the presence of 0.05% Triton X-100 in those diluents did not decrease PLY-detection sensitivity in ELISA.

ELISA Performance

Figure 1 shows a representative titration curve of purified recombinant pneumolysin, diluted in PBS with 0.05% Triton X-100. Doubling dilutions of PLY, from 500 to 31.25 pg mL^{-1}, were tested. As shown, the assay is clearly linear within this range of concentrations. The averaged detection limit of the assay was 2.34 ± 1.52 pg mL^{-1}.

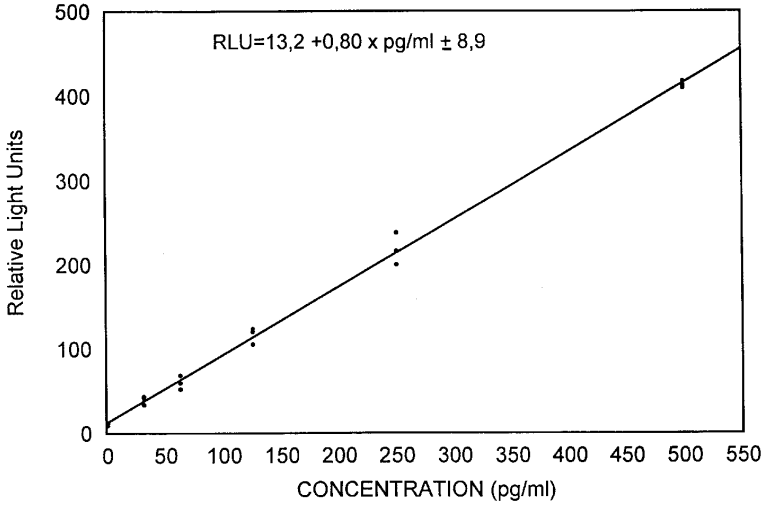

Figure 1. Representative ELISA standard curve for recombinant PLY.

Table 1. Precision Data for Purified Recombinant PLY Detection

	High Control 450 pg mL^{-1}	Middle Control 150 pg mL^{-1}	Low Control 50 pg mL^{-1}
Intra–assay (n = 30)			
Mean	413.8	164.2	67.5
SD[1]	31.9	11.0	5.0
CV (%)[2]	7.70	6.68	7.38
Inter-assay (n = 30)			
Mean	471.0	187.2	71.4
SD	68.5	20.4	10.6
CV (%)	14.55	10.87	14.83

[1] SD: Standard deviation.
[2] CV: Coefficient of variation.

Table 2. Production of PLY by Pneumococcal Strains

Bacterial Strain	ng mL^{-1} [a]
Reference strains	
Streptococcus pneumoniae 993 ATCC 33400 Type 1	0.6
Streptococcus pneumoniae NCTC 7466 Type 2	43.7
Streptococcus pneumoniae GB05 Type 3	181.5
Non-typeable isolates	
2/97	1392.7
5/97	286.3
15/98	86.2
Atypical isolates	
AT5	232.4
AT7	0.1
AT10	0.3
AT12	1.3
AT16	232.5
AT18	0.04
AT20	0.06
Isolates from healthy carriers	
P-428	91.2
P-809	30.0
P-95/38924	365.5

[a]Referring to 1 x 10^7 CFU.

Then, 450, 150, and 50 pg mL^{-1} were chosen as controls to establish the precision of the assay. Intra-batch coefficients of variation (CVs) ranged (Table 1) from 6.68 to 7.70; inter-assay CVs fell between 10.87 and 14.83.

Study of the Content of PLY in Different Pneumococcal Isolates

Table 2 shows the content of PLY referring to 1 x 10^7 CFU of several pneumococcal strains. A variation in the amount of PLY produced by the isolates tested was observed.

Specificity of the Test

The ELISA did not show any false-positive measurements when 1.4G8.66, a PLY-irrelevant isotype-matched mouse monoclonal, was assayed as the capture antibody instead of PLY-7.

The test was also negative with 1μg of commercialy available purified SLO, (-hemolysin from *S. aureus*, α-hemolysin from *E. coli* or purified recombinant LLO.

No positive signals were recorded either when lysates from 1 x 10^7 CFU of supposed producers of suilysin (*S. suis* NCTC 10234), streptolysin O (a clinical isolate of *S. pyogenes*), listeriolysin O (*Listeria monocytogenes* serogroup 4b CECT 935), ivanolysin (*Listeria ivanovii* serogroup 5 CECT 913) or perfringolysin O (two different clinical isolates of *Clostridium perfringens)* were subjected to the test.

Similar lysates from *S. salivarius* subsp. *salivarius* ATCC 7073, *S. aureus* Cowan 1 ATCC 12598, *E. coli* ATCC 25922, clinical isolates of the viridans group of streptococci, *S. agalactiae*, *H. influenzae*, and *N. meningitidis*, were also negative.

Lysates from *S. mitis* ATCC 33399 and *S. sanguis* ATCC 10556 gave signals equivalent to 0.06 and 0.03 ng of PLY mL^{-1}, respectively.

DISCUSSION

Immunodetection of pneumococcal components for diagnostic purposes is especially useful when patients have received prior antibiotic therapy which leads to a remarkable decrease in microbial burden and consequently culture recovery of the pneumococcus may become negative.

Previous epitope mapping analyses(34) showed, at that time, that the epitope recognized by the antiPLY-7 monoclonal was only present in PLY

but not in perfringolysin O or listeriolysin O; it had also been observed that this monoclonal had the highest relative affinity to PLY among the various monoclonals tested (data not shown). Therefore, we selected the PLY-7 monoclonal as the capture antibody for the sandwich ELISA we planned to develop. On the other hand, the anti-PLY polyclonal rabbit IgG we had generated(28) had been shown to clearly recognize PLY but faintly recognize other TACYs when lysates from pneumococcal and non-pneumococcal strains were subjected to Western blot analyses.

In terms of analytical sensitivity, the PLY-based chemiluminescent ELISA we have hereby described does measure concentrations of recombinant PLY in PBS $\geq$ 5.5 pg mL^{-1} ($\cong$ mean detection limit + 2 SD); the linearity range extends at least up to 500 pg mL^{-1}, the highest concentration tested. Intra- and interassay coefficients of variation were around 7 and 13.5%, typical of immunoassays conducted over long periods of time.(37)

The amount of pneumolysin produced by various pneumococcal isolates was previously and indirectly inferred from their hemolytic activities.(27, 30-33) We have directly quantitated, for the first time, the actual PLY content of different pneumococcal strains. On the one hand, and in accordance with former studies, we have observed a great diversity in this production among isolates, including the so called "atypical" pneumococci.(28, 30) Lysates from strains ATCC 33400 Type 1, AT7, AT18 and AT20 have been previously shown to be partially or not hemolytic, as well as PLY-mediated agglutination negative;(28) the relatively low production of PLY ($\leq$ 100 pg ml^{-1} from 1 x 10^7 CFU as measured by our ELISA) by these pneumococcal isolates, is in agreement with those observations.

On the other hand, it was reported that noncapsulated strains isolated from carriers were negative or had low pneumolysin activity;(32) nevertheless, we have found strains either non-typeable or isolated from healthy carriers which produced significant amounts of pneumolysin. Therefore, pneumolysin production does not seem to be necessarily correlated with the capsular and/or the carrier phenotype of pneumococcal isolates.

It has been reported that suilysin, expressed by *S. suis*, is very similar to PLY.(26) *S. suis* causes infectious diseases mainly in pigs and occasionally in humans. The gene encoding SLY has been shown to be absent in about 37% of all S. *suis* field strains assayed. As for PLY, based on the hemolytic activity of the strains tested, a remarkable variation has been found in its production. The *S. suis* lysate we have assayed for the present study under the aforedescribed experimental conditions did not yield significant signals in our ELISA; as we have not had available purified SLY, we cannot presently provide any data on the extent of the detection of SLY versus PLY. Apart from this, our test neither identifies purified SLO or LLO nor other thiol-activated toxins presumably present in lysates from producer strains.

No positive results were observed either from lysates from other human pneumonia- or meningitis-associated bacteria (*H. influenzae* and *N. meningitidis*, respectively). However, from lysates of the *S. mitis* and *S. sanguis* strains hereby assayed, we obtained signals in the range of those measured from the lowest PLY-producing pneumococcal isolates. The presence of a functional PLY gene has been very recently reported in a number of bacterial isolates phenotypically and genetically identified as *S. mitis*;(30) these isolates were found to produce variable amounts of pneumolysin according to their hemolytic activities. Clinical strains from *S. sanguis* were not investigated in that study. Bearing all this in mind, the assessment of the production of PLY by different streptococci warrants further detailed analysis.

Altogether, we may state that we have developed a specific and highly sensitive ELISA for the detection and quantitation of PLY. This methodology —fast, easy to perform and currently available in clinical laboratories— could help in the diagnosis of human infections; at present, we are exploring this possibility. In the same line, the detection of pneumolysin by Western blot in sputum has already been probed;(38) PLY could even be detected in culture-negative samples from patients receiving antibiotics, but with proven recent pneumococcal infection. These preliminary clinical observations highlight the diagnostic usefulness of pneumolysin.

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