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Clinical evaluation of a disposable amperometric magneto-genosensor for the detection and identification of *Streptococcus pneumoniae*

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ABSTRACT

A disposable PCR-based amperometric magneto-genosensor for detection and identification of *Streptococcus pneumoniae* was evaluated. ROC curve analysis used to determine optimal signal cutoff values yielded a sensitivity of 91% and a specificity of 90%. The method was also tested for the direct detection of pneumococci in clinical samples.

The streptococci of the Mitis group (SMG) include the closely related species *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus pseudopneumoniae* and *Streptococcus oralis*, which possess significantly different pathogenic properties and antimicrobial susceptibility patterns. Differentiation between pneumococci and other SMG is clinically relevant and is typically done by phenotypic (e.g., optochin susceptibility testing and/or bile solubility assay) and/or serological methods (Lund and Henrichsen, 1978). The methods that require bacterial culturing are time-consuming, while immunochromatographic methods are rapid but present cross-reactivity problems in some patient groups (Blaschke, 2011).

To improve accuracy and speed diagnosis, new methods based on the polymerase chain reaction (PCR) have been developed (Harris et al., 2008; Rouphael et al., 2008; Sheppard, 2004; Smith et al., 2009). Several *S. pneumoniae*-specific markers have been proposed among which the most relevant are the *ply* and *lytA* genes, which encode the pneumolysin (Marriott et al., 2008) and the main autolysin (López and García, 2004), respectively.

Homologs of the *lytA* gene of *S. pneumoniae* have also been found in other SMG but characteristic features in the pneumococcal gene have allowed the design of a specific PCR assay (Llull et al., 2006; Obregón et al., 2002).

Electrochemical DNA biosensors, also called genosensors, are based on the integration of a sequence-specific probe and an electrochemical signal transducer, and offer an interesting detection method (Drummond et al., 2003; Lucarelli et al., 2008) which is easy to implement, with low instrumentation costs, and the possibility for accurate and sensitive detection in small sample volumes (Wei et al., 2010).

Magnetic beads (MBs) facilitate efficient target retrieval and concentration, reduce the assay time and favor higher sample throughput and automation (Chen et al., 2007). The use of MBs enhances sensitivity and reduces detection time in electrochemical genosensors (Bettazzi et al., 2013; Campuzano et al., 2011; Pedrero et al., 2012).

We present an evaluation of the performance of a recently described amperometric magneto-genosensor for the identification of *S. pneumoniae* (Campuzano et al., 2011) using a collection of microbial isolates and clinical samples in comparison with standard microbiological identification procedures. The sensor uses a specific DNA capture probe for the detection of a characteristic *lytA* single-stranded DNA fragment amplified by asymmetric PCR (aPCR) (Fig. 1). Both, the capture probe and the single-stranded amplicon are biotinylated. Streptavidin-modified MBs are loaded with the capture probe, the loaded beads are then hybridized with the aPCR product, and the hybrid molecules are labeled with streptavidin–peroxidase polymer (Strep-HRP). The modified MBs are captured by a magnetic field on the surface of gold screen-printed electrodes (Au/SPEs) and the peroxidase activity is measured as the amperometric response at −0.15 V upon TMB/H_2O_2 addition.

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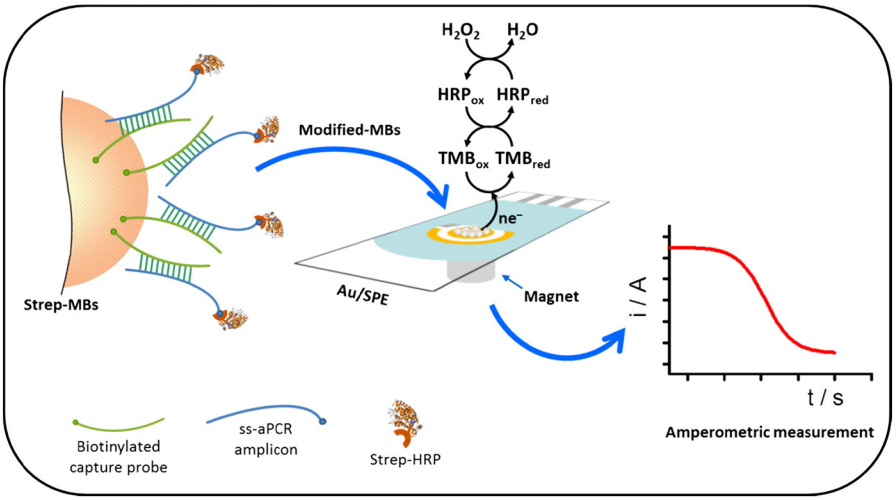

Fig. 1. Scheme of the amperometric detection of the pneumococcal *lytA* gene: magnetic beads (MBs) are loaded with capture probe and this is hybridized with *lytA* single-stranded aPCR product which is then labeled with streptavidin-HRP. MBs are captured by a magnetic field over the surface of an Au/SPE electrode. To determine the amount of aPCR product the peroxidase activity over the electrode is measured by amperometric detection of the reduction of TMB/H_2O_2.

Microbial isolates (n = 107) were identified by Vitek 2 (BioMérieux SA, Marcy l'Etoile, France) or Wider systems (Francisco Soria Melguizo SA, Madrid, Spain), MALDI-TOF mass spectrometry (Bruker Daltonics, GmbH, Bremen, Germany) and optochin test.

Clinical samples (n = 109) were obtained from skin, abscesses, sputum, purulence, blood, swabs taken from throat, ear or conjunctiva, nasal, tracheal or bronchial aspirates, pleural fluid and bronchoalveolar washes. All the samples were obtained in the Microbiology Department of Hospital La Paz, and had been anonymized previously, keeping only microbiological data.

DNA (from agar plate cultures or from clinical samples) was extracted using the UltraClean® Microbial DNA Isolation Kit (MoBio, Carlsbad, CA USA). When required, clinical samples were fluidized with 0.7% dithiothreitol (final concentration) before extracting DNA.

The magneto-genosensing strategy has been recently described (Campuzano et al., 2011). Briefly, streptavidin-coated MBs were loaded with the biotinylated DNA capture probe and hybridized with the amplicon generated by aPCR (biotinylated reverse to forward primer ratio of 8:1). The hybrid-attached beads were washed twice with 500 μL of Tris–HCl (pH 7.2) and labeled with streptavidin–peroxidase polymer. The enzyme-tagged hybrid-MB assemblies were washed five times (2 min each) with 500 μL of PBST (PBS containing 0.05% Tween®20), and once more with 500 μL of PBS (10 mM sodium phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5). The MBs were resuspended in 45 μL of PBS and magnetically captured on the surface of an Au/SPEs (220AT, 4-mm ∅, Dropsens, Spain) by placing a neodymium magnet under the working electrode surface. TMB-H_2O_2 K-Blue reagent solution (Neogen, Lexington, KY) in a ready-to-use format (K-Blue enhanced-activity substrate, also containing H_2O_2) was used as a substrate, and amperometric measurements were done with a single-channel amperometric detector (InBea Biosensores S. L., Madrid, Spain). Statistical analyses were done using SPSS 17.0. The continuous output obtained from amperometric measurements was transformed into a binary classification by ROC curve analysis. The optimal cutpoint value was determined as the point on the ROC curve closest to (0.1) and the Youden index (Perkins and Schisterman, 2006), which maximizes sensitivity and specificity.

Genosensor performance was tested with a series of 107 cultured microbes obtained from clinical samples. Seventy nine had been classified as *S. pneumoniae* (Sp) and twenty eight were non-pneumococcal isolates (designated as N-Sp): 25 streptococci [*Streptococcus anginosus* (2 strains), *Streptococcus gallolyticus* (1 strain), *Streptococcus intermedius* (3 strains), *S. mitis* (8 strains), *S. oralis* (4 strains), *Streptococcus pyogenes* (4 strains), *Streptococcus salivarius* (1 strain), *Streptococcus sanguinis* (1 strain) and *Streptococcus suis* (1 strain)], and three non-streptococcal microbes, i.e., *Staphylococcus lugdunensis*, *Enterococcus faecalis and Candida albicans*. All the samples were tested in duplicate. Two blanks were included in each PCR run to control reagent contamination and were also processed to determine the baseline of the amperometric measurements. Typically, the blanks (n = 102) gave signals around −100 nA. To minimize inter-assay variation the sample signals were normalized dividing them by their corresponding blanks. The average of the normalized ratios was 7.55 for Sp isolates and 1.15 for N-Sp species (Fig. 2A). An optimal cutoff value of 1.92 for the sample to blank ratio was estimated from ROC curve analysis (Fig. 2C). The same value was obtained with the ROC (0.1) and the Youden indexes. The area under the curve (AUC) was 0.94 [95% confidence interval (CI) 0.89–0.98]. Using a normalized value >1.92 as the threshold to identify *S. pneumoniae*, 72 out of 79 neumococci and 25 out of 28 non-neumococci were correctly identified. With this cutoff, the assay had a sensitivity of 91% (95% CI 85–97%) and a specificity of 90% (95% CI 79–100%) (Table 1). The seven false negative and the three false positive (two *S. mitis* and one *S. pyogenes*) results were tested and their identity was confirmed by *lytA* PCR and 16S rRNA gene pyrosequencing (data not shown).

The ability of the method to directly detect *S. pneumoniae* in complex clinical samples was tested. A series of 109 samples of diverse origins that had been previously used for microbiological diagnostic was collected (Table 2 and Fig. 2B). Total DNA was extracted and a fixed volume (5 μL) was used for aPCR of the *lytA* gene. *S. pneumoniae* had been identified in 60 samples. In 44 of them, the genosensor gave a positive identification, while in 16 the results were negative. The remaining 49 samples included 7 samples in which no growth was detected, 13 samples containing mixed saprophytic microbiota, and 29 samples in which some other pathogen had been identified (including several streptococci and staphylococci, *Pseudomonas* sp., *Acinetobacter baumannii*, *Escherichia coli, Enterobacter cloacae, Haemophilus influenzae, Moraxella catarrhalis, Stenotrophomonas maltophilia*, *Candida parapsilosis* and *C. albicans*). In 39 of these 49 samples the genosensor results were negative (≤1.92), while 10 samples were positive. Five of these were polymicrobial and four were sterile.

With the cutoff selected, both sensitivity and specificity were around 90%. PCR retesting and 16S gene pyrosequencing of conflicting samples