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Research paper

A fluorescent multiplexed bead-based immunoassay (FMIA) for quantitation of IgG against *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* protein antigens

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ABSTRACT

Streptococcus pneumoniae, *Haemophilus influenzae*, and *Moraxella catarrhalis* are pathogens commonly associated with infectious diseases in childhood. This study aimed to develop a fluorescent multiplexed bead-based immunoassay (FMIA) using recombinant proteins for the quantitation of serum IgG antibodies against these bacteria. Eight pneumococcal proteins (Ply, CbpA, PspA1, PspA2, PcpA, PhtD, SP1732-3 and SP2216-1), 3 proteins of *H. influenzae* (NTHi Protein D, NTHi0371-1, NTHi0830), and 5 proteins of *M. catarrhalis* (MC Omp CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1, MC_RH4_4730) were used to develop the FMIA. Optimal coupling concentrations for each protein, comparison of singleplex and multiplex assays, specificity, reproducibility, and correlation to ELISA for six pneumococcal antigens were determined for validation. FMIA was then used to analyze acute and convalescent paired serum samples of 50 children with non-severe pneumonia. The coupling concentrations varied for different antigens, ranging from 1.6 to 32 μg of protein/million beads. Correlation between singleplexed and multiplexed assays was excellent, with $R \geq 0.987$. The FMIA was specific, reaching >92% homologous inhibition for all specificities; heterologous inhibition ≥20% was found only in six cases. The assay was repeatable, with averages of intra-assay variation ≤10.5%, day-to-day variation ≤9.7% and variation between technicians ≤9.1%. Comparison with ELISA for pneumococcal antigens demonstrated good correlation with R ranging from 0.854 (PspA2) to 0.976 (PcpA). The samples from children showed a wide range of antibody concentrations and increases in convalescent samples. In conclusion, the FMIA was sensitive, specific, and repeatable, using small amounts of recombinant proteins and sera to detect

Abbreviations: AOM, acute otitis media; CAP, community-acquired pneumonia; CbpA, choline binding protein A; CV, coefficient of variation; EDC, 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl; FMIA, fluorescent multiplexed bead-based immunoassay; MFI, Mean Fluorescence Intensity; NTHi, nontypeable *H. influenzae*; OMP CD, outer membrane protein CD; PBS, phosphate buffered saline; PcpA, pneumococcal choline binding protein A; PCV, pneumococcal conjugate vaccines; PhtD, pneumococcal histidine triad protein D; Ply, pneumolysin; PspA, pneumococcal surface protein A; R-PE, R-phycoerythrin; RT, room temperature; Sulfo-NHS, Hydroxysulfosuccinimide

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antibodies against *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. The methodology would be suitable for studies investigating etiological diagnosis and in experimental vaccine studies.

1. Introduction

Streptococcus pneumoniae, *Haemophilus influenzae*, and *Moraxella catarrhalis* are pathogens commonly associated with respiratory tract infections in childhood, such as community-acquired pneumonia (CAP) and acute otitis media (AOM) (Casey et al., 2010; Rudan et al., 2008). Nevertheless, the accurate etiologic diagnosis of infection by these agents is difficult, and most serological tests can only detect a limited number of strains of each pathogen, reinforcing the need for new assays that recognize the immune response to these bacteria regardless of, for example, the serotypes or variations in other surface structures. Also, as no licensed vaccines have been introduced for nontypeable *H. influenzae* (NTHi) and *M. catarrhalis*, and the ones available against *S. pneumoniae* do not provide coverage against all serotypes (Rodgers and Klugman, 2011), new antigens have been increasingly studied as vaccine candidates. Therefore, the development of serological assays for these pathogens based on specific and conserved antigens for each species is warranted.

Several pneumococcal protein antigens have been well characterized and investigated for the development of new vaccines (Principi and Esposito, 2011) and have also been increasingly used in serological assays. Among them, pneumolysin (Ply), choline binding protein A (CbpA), pneumococcal surface protein A (PspA), pneumococcal choline binding protein A (PcpA) and pneumococcal histidine triad protein D (PhtD) are promising antigens for both purposes. Ply is a highly conserved cytotoxin released during autolysis that interacts in many ways with the host immune system (Tai, 2006; van der Poll and Opal, 2009). CbpA (Brooks-Walter et al., 1999; Tai, 2006; van der Poll and Opal, 2009) and PspA (Briles et al., 2000; Crain et al., 1990; Croney et al., 2012; Tai, 2006; van der Poll and Opal, 2009) are also present in most strains of *S. pneumoniae* and play several roles in the pathogenesis of pneumococcal infection. PcpA is a choline binding protein present in the majority of *S. pneumoniae* virulent strains involved in adherence to epithelial cells (Khan et al., 2012). Similarly, PhtD is a highly conserved surface protein from the Pht family (Adamou et al., 2001), that has been recently recognized as an adhesin (Khan and Pichichero, 2012).

Among the protein antigens of *H. influenzae*, protein D is an important vaccine candidate, as it is a conserved virulence factor present in all strains (Poolman et al., 2000). Further, it has been reported that protein D as a carrier in pneumococcal conjugate vaccine (PCV) provides protection against AOM caused by *H. influenzae* (De Wals et al., 2009). Several adhesins have also been identified in *M. catarrhalis* that would be suitable as diagnostic targets or as vaccine candidates, such as the outer membrane protein CD (OMP CD), an adhesin that also has other functions in pathogenesis, and Msp22, a surface lipoprotein (Murphy and Parameswaran, 2009; Saito et al., 2013; Smidt et al., 2013). Although there is no currently licensed vaccine using all the aforementioned proteins (except for the use of protein D as a carrier in one of the PCVs), these antigens merit further investigation.

To date, the quantitation of antibodies to these pathogens relies mostly on the use of ELISA, which is recognized as a specific and sensitive technique (Korppi et al., 2008). Nevertheless, when the evaluation of the immune response to multiple antigens is required, ELISA becomes considerably time-consuming and expensive. Additionally, the performance of one test per analyte demands a large volume of serum, which is usually hard to obtain when dealing with pediatric patients. In this setting, the use of a multiplexed assay allows the detection of antibodies against several antigens simultaneously with high sample throughput and reduction in the amount of serum needed, which represent important advantages when performing seroepidemiological studies or experimental vaccine trials. In this context, we describe in detail the development and validation of a multiplexed immunoassay using Luminex xMAP® Technology with recombinant proteins for the detection and quantitation of antibodies directed to *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*.

2. Materials and methods

2.1. Reagents

The presence of antibodies against *S. pneumoniae* was investigated using eight distinct recombinant pneumococcal protein antigens: Ply, CbpA, PspA family 1 (PspA1), PspA family 2 (PspA2), PcpA, PhtD, SP1732-3, and SP2216-1. Antibodies directed to *H. influenzae* were assayed using the recombinant proteins NTHi Protein D, NTHi0371-1, and NTHi0830. NTHi0371-1 is a fragment of the protein heme/hemopexin utilization protein A (gene hxuA) representing the first domain of the mature protein which includes the hemagglutinin activity domain. The protein NTHI0830 is the outer membrane antigenic lipoprotein B. *M. catarrhalis* antibodies were investigated using the recombinant protein antigens MC Omp CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1, and MC_RH4_4730 (Smidt et al., 2013). The combination of antigens was chosen with the purpose of providing a good coverage of the bacterial etiological agents causing respiratory tract infections and based on the information available on the immune response to antigens from these pathogens.

For the development of this fluorescent multiplex bead-based immunoassay (FMIA), a truncated PcpA (Posfay-Barbe et al., 2011) and PhtD (Seiberling et al., 2012) were kindly supplied by Sanofi Pasteur (Sanofi Pasteur S.A., Marcy L'Etoile, France); SP1732-3 and SP2216-1 (Giefing et al., 2008), NTHi Protein D, NTHi0371-1, NTHi0830, MC Omp CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1, and MC_RH4_4730 were from Andreas Meinke (Valneva, Vienna, Austria). The antigens Ply (Ogunniyi et al., 2001), CbpA (Orihuela et al., 2009), and PspA1 (UAB055) (Brooks-Walter et al., 1999; Shaper et al., 2004) were kindly supplied by Elaine Tuomanen (St. Jude Children's Research Hospital, Memphis, TN, USA) and PspA2 (UAB099)