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Dot Blot Enzyme Immunoassay (EIA) Antibody Analysis Directed against Differentially Extracted Antigens Derived from Whole Cell Proteins of *Neisseria meningitidis* and Non-pathogenic Species in Meningococcal Vaccinated, Healthy and Other Disease Individuals

Nor Hafeeda Rosdan[1, 2,*], Zainoodin Sheik Abdul Kader[2], Nor Hazwani Ahmad[2]

[1] Faculty of Health Sciences, Universiti Teknologi Mara Cawangan Pulau Pinang, Kampus Bertam, 13200 Pulau Pinang, Malaysia
[2] Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200 Pulau Pinang, Malaysia

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ABSTRACT

Invasive meningococcal disease (IMD) is a rare but potentially life-threatening illness caused by *Neisseria meningitidis* invading the bloodstream. Early identification is crucial, as the disease progresses rapidly within the first few hours. Diagnosis relies on clinicians recognising common clinical symptoms since laboratory tests may not be rapid enough. Although commercial serological tests are available, they have drawbacks such as cost and the need for trained personnel. Most people have natural immunity to meningococcal disease due to consistent exposure to meningococci, other Neisseria species, and related genera that share common antigens. Researchers are exploring the mechanisms to develop more effective vaccines. Therefore, this study aimed to modify and evaluate Dot Blot EIA on nitrocellulose membrane of differentially extracted antigens derived from whole cell protein of *N. meningitidis* and non-pathogenic *Neisseria* species for serodiagnosis. Furthermore, this study aimed to investigate the humoral immune response patterns against meningococcal vaccinated, healthy and other diseases individuals. Additionally, investigate the potential for cross-reactivity with other Neisseria species or Moraxella genera. Analysis of the humoral immune response patterns revealed that most vaccinated individuals had IgG antibodies, as well as some presence of IgM and IgA antibodies. Although Men A and Men B of WCP and CSP did not show detectable antibodies in any of these types, their presence was revealed through the use of three different extraction methods. This demonstrates the sensitivity of our Dot Blot EIA assay. Conversely, the other disease group primarily exhibited IgM antibodies. Both the healthy group and individuals with other diseases may also have IgG and IgA antibodies present. Interestingly, some results from the healthy individuals showed a similar humoral immune response pattern as the vaccinated group. The presence of all antibody types in closely and distantly related species suggests the development of natural immunity. These commensal bacteria may share epitopes with pathogenic bacteria, leading to the production of cross-reactive antibodies and immune responses. Ultimately, further studies are necessary to fully validate and establish the potential of the Dot Blot EIA that may offer valuable insights into the immune response to meningococcal disease and vaccine effectiveness.

* *Corresponding author.*
E-mail address: hafeeda0480@uitm.edu.my

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1. Introduction

IMD, or invasive meningococcal disease, is an extremely rare event in which the meningococcal pathogen enters the circulatory system after invading the mucosal epithelium. The meningococci can cross the blood–brain barrier and spread to the meninges. The highest incidence of meningococcal disease is often in infants younger than one year, with most cases occurring in children under five years and adolescents [1,2]. The initial symptoms of meningococcal meningitis can be identical to those of a viral infection such as the flu [3], with one-third of patients presenting with the classic triad of fever, meningismus, and altered mental status. Although the classic petechial rash is a more specific feature, it appears on average 13 to 22 hours after onset in 45 to 65% of cases [3,4]. As a result, prompt diagnosis and treatment of the disease might be challenging. Within the first few hours of the onset of symptoms, the condition can progress rapidly from bacteraemia or meningitis to life-threatening septic shock syndrome or meningitis. Rapidly identifying bacteria causing meningitis is crucial, as delays in treatment increase the mortality rate.

However, available laboratory tests may not be rapid enough to diagnose the disease accurately. Due to the lack of a rapid diagnostic test, the diagnosis depends on the attending clinician recognising common clinical symptoms. If meningococcal disease is suspected, treatment should be started immediately, with microbiological tests only useful for disease confirmation. Therefore, the gold standard for bacteriologic infection identification is the organism's culture; however, early antibiotic therapy may decrease its sensitivity. Another drawback is the time it takes to get results [5]. Gram stain smears of cerebrospinal fluid (CSF) can provide rapid preliminary tools, making it a fast and accurate test. However, the sensitivity varies considerably [6]. CSF antigen detection assays can also be helpful but have a high rate of false negatives. Polymerase chain reaction (PCR) analysis of blood samples has shown promise, with 91% sensitivity and 76% specificity. However, it is not widely available, requires highly skilled personnel and may not be timely, making it more useful for surveillance than as a clinical tool. Therefore, the development of simple, convenient, sensitive and inexpensive rapid tests for meningococcal disease, which could be used in all low-, middle- and high-income countries is still desirable.

Serology remains an important tool, especially with the increased usage of common antibiotics prior to hospital admission [7]. It is a non-cultural diagnostic approach to meningococcal disease that has been used increasingly while the number of culture-diagnosed cases has gradually decreased [8]. Serological tests can also offer rapid diagnosis because of their speed in producing the results, can be available within hours or minutes, are technologically simple, and have low training requirements. Many commercial serological tests are available worldwide for detecting bacterial meningitis or meningococcal meningitis. However, some of the commercially available serological tests have a number of disadvantages, including high cost, the need for trained personnel to operate the equipment, the inability to detect all etiological agents, cross-reactivity with other bacterial species, the failure to detect species serotypes, the need of large samples of volumes, and the need for electricity [9]. In light of this, the "Defeating Meningitis by 2030" roadmap includes the development of rapid diagnostic tests that can be used at all levels of care, including those with limited resources [9].

Despite the challenges, most people have innate immunity against meningococcal disease due to their consistent exposure to meningococci, other *Neisseria* species (particularly *Neisseria lactamica*), and related genera that share common antigens [10]. *Neisseria* and *Moraxella* genera are commonly found in the human upper respiratory tract. *N. lactamica*, a non-pathogenic Neisseria species, shares antigenic similarities with *N. meningitidis*. Previous research has demonstrated that individuals who carry *N. lactamica* develop immunity against *N. meningitidis* [11]. As a result, some researchers are

eager to investigate the underlying causes of this natural immunity, as these findings could potentially improve future efforts to develop vaccines.

Vaccines are critical in preventing IMD. Currently, three licenced conjugate vaccines target meningococcal serogroups A, C, W, and Y. Each vaccine contains capsular polysaccharides from each of the four serogroups, each conjugated to a carrier protein. MenACWY-D (Menactra®) [12], MenACWY-CRM197 (Menveo®) [13], and MenACWY-TT (Nimenrix®) [14] are among the quadrivalent conjugate vaccines. MenC-TT (Neis-Vac-CTM), MenC-CRM197 (Menjugate®), and MenA-TT (MenAfriVac) are all monovalent meningococcal conjugate vaccines that target a single serogroup. Hib-MenC-TT (Menitorix®) is a combination conjugate vaccine that contains both MenC conjugated to TT and *Haemophilus influenzae* type b. While vaccines based on capsular polysaccharides for serogroup B meningococcal are ineffective, vaccines targeting conserved subcapsular antigens such as MenB-FHbp (Trumenba®, bivalent rLP2086) and 4CMenB (Bexsero®, MenB-4C) have become available in recent years [15].

The Serum Bactericidal Antibody (SBA) assay is a surrogate measure for evaluating the effectiveness of meningococcal vaccines during development and regulatory approval processes. Due to limited sample sizes, the SBA assay has become an important tool for measuring the ability of antibodies to destroy bacteria in the presence of complement, known as complement-mediated killing via the classical immune response pathway [16]. Establishing a correlation between SBA titer results (with a benchmark titer of ≥ 4) and protection against invasive meningococcal disease (IMD) has been pivotal, relying on human complement for validation. However, acquiring and standardizing human complement is complex, which has led to the use of baby rabbit complement as a common alternative. Despite these adaptations, the World Health Organization (WHO) recommends that the evaluation of new vaccines, especially those incorporating serogroup C or A conjugate components, should employ SBA assays. The guideline suggests a human complement threshold of ≥ 4 and a baby rabbit complement threshold of ≥ 8 for serogroups including W and Y [17]. However, discrepancies between results from rabbit (rSBA) and human (hSBA) assays have become evident over time, with variations not only due to the complement source but also other assay conditions. While a study highlighted a decent alignment between rSBA and hSBA titers for serogroup C, this correlation did not hold for serogroups A and Y, where rSBA often indicated higher titers than hSBA. These distinctions underscore the necessity to recognize that specific rSBA and hSBA assays might diverge significantly, impacting the interpretation of protection levels against various meningococcal strains [15].

The Dot Blot test, also called dot-blot ELISA and dot immunobinding assay, is a technique where an antigen is directly applied onto a nitrocellulose (NC) membrane. This membrane acts as a solid surface for detecting antibodies. It involves sensitising nitrocellulose membranes with particular antigens and exposing them to primary (serum) and secondary antibodies marked with peroxidase to create a colour reaction [18]. The dot blot enzyme immunoassay (dot EIA) has been reported to be equal to, or more sensitive than, the ELISA plate in the detection of *N. meningitidis* serogroup A using monoclonal antibodies and works across a wide range of antigen-antibody ratios [19,20]. In addition, no electronic equipment is required to read the results [21]. Therefore, this study aimed to modify and evaluate Dot Blot EIA on nitrocellulose membrane of differentially extracted antigens derived from whole cell protein of *N. meningitidis* and non-pathogenic *Neisseria* species for serodiagnosis. Furthermore, this study aimed to investigate the humoral immune response patterns against meningococcal vaccinated, healthy and other diseases. In addition, explore the potential for cross-reactivity with other *Neisseria* species or *Moraxella* genera.

2. Methodology

2.1 Bacteria Culture Preparation

The Microbiology and Parasitology Laboratory in Kubang Kerian, Kelantan has provided two clinical isolates from meningitis cases in Hospital Universiti Sains Malaysia (HUSM). Along with this, a stock culture of *Neisseria cinerea* was also obtained from the same laboratory at HUSM. In addition, four reference strains of *N. meningitidis* serogroup A (ATCC13077), B (ATCC13090), C (ATCC13102), Y (ATCC35561), *Moraxella catarrhalis* (ATCC25238) and *Neisseria sicca* (ATCC 9913) were acquired from Thermo Scientific.

2.2 Extraction of the Whole Cell Protein (WCP), Cell Surface Protein (CSP), and Surface Depleted Whole Cell Protein (sdWCP)

A modified protocol, as described by Horvath and Riezman [22], was used to extract capsule polysaccharide (CSP) and whole cell proteins (WCP) from various strains of *Neisseria meningitidis*, including serogroups A, B, C, and Y, as well as clinical strains *M. catarrhalis*, N. sicca, and *N. cinerea*. The strains were initially cultured on sheep blood agar at 37°C in a 5% CO2 atmosphere for 48 hours. After this time, colonies grown on the agar were transferred to a tube containing a sample preparation buffer (SPB) that contained Tris, 2% sodium dodecyl sulfate (SDS), and glycerol at pH 6.8. This mixture was then boiled for 5 minutes and centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant obtained from this process was mixed with double its volume of ice-cold ethanol to precipitate the proteins overnight at -20°C. The precipitated protein pellet was then dissolved in 10mM Tris at pH 7.4 containing phenylmethanesulfonylfluoride (PMSF), a protease inhibitor, and stored at -20°C.

To extract CSP, the culture suspensions of the various strains were treated with glycine-HCl at a pH of 2.0. This was incubated at room temperature for 15 minutes, then centrifuged at 10,000 x g for 10 minutes at 4°C to separate the whole cells. The supernatant, containing the CSP, was collected, and its pH was adjusted to 7.4 before adding double its volume of absolute ice-cold ethanol for overnight protein precipitation at -20°C. The CSP was then retrieved through centrifugation at 10,000 x g for 10 minutes at 4°C, with the pellet being dissolved in Tris-PMSF and stored at -20°C.

The pellet previously set aside from CSP extraction was solubilized in SPB containing Tris, 2% SDS, and glycerol at pH 6.8, with the addition of ß-mercaptoethanol to achieve a final concentration of 10% v/v. Following boiling for 5 minutes, it was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant from this centrifugation was then treated with double its volume of ice-cold ethanol to precipitate the protein. This precipitated sdWCP protein was dissolved in Tris-PMSF and stored at -20°C.

2.3 Serum Samples

This preliminary investigation examined three categories of human serum samples alongside a single positive control. The collection of serum was approved by the Human Research Ethics Committee USM (HREC). The first group consisted of 12 serum samples collected from vaccinated pilgrims returning from Umrah or Hajj. Before embarking on Umrah or Hajj, these individuals were required to receive the mandatory quadrivalent meningococcal conjugate vaccine ACWY. In addition, 58 samples of human serum were collected from healthy staff, students, and regular blood donors at the Advance Diagnostic Laboratory (ADL) of AMDI in Bertam, Kepala Batas. Furthermore, 20 serum samples were collected from patients suffering from various non-meningococcal conditions. These

sera were collected from AMDI patients suffering from a variety of diseases, such as pneumonia, typhoid fever, tuberculosis, melioidosis, and cancer. Meanwhile, the control sera used in this study were Rabbit anti-Neisseria meningitidis, a polyclonal antibody commercially purchased from Bio-Rad, USA. This polyclonal antibody was stored and preserved at -20°C. Prior to being used, this polyclonal antibody was diluted in a 1:300 ratio.

2.4 Optimisation of Antigen for the Development of Dot Blot EIA Test

The concentrations of 620 g/mL, 420 g/mL, 160 g/mL, and 80 g/mL were prepared and spotted on 0.2 m membrane strips (Bio-Rad Laboratories, USA) for titration. To block the unbound sites and reduce non-specific binding, 5% skim milk in 1XTris buffer saline (TBS) was incubated for one hour. The membrane strips were then incubated with primary antibody serum of known healthy positive, serum vaccinated positive, and serum healthy negative (as negative control) diluted at a ratio of 1:100. The membrane strips were washed with the same blocking buffer three times for one minute each. The membrane strips were incubated for an hour with IgM, IgG, and IgA secondary antibodies. IgM and IgA antibody isotypes were diluted at a ratio of 1:1000. In contrast, IgA antibody was diluted at a ratio of 1:2000. The membrane strips were washed three times with TBS that contained 5% skim milk. The membrane strips were then incubated for 10 minutes with an AP conjugate (NBT-BCIP, alkaline phosphatase substrate kit from Bio-Rad, USA) for optimum colour development. The reaction was stopped by rinsing for ten minutes with distilled water. In order to develop a dot EIA test for the serodiagnosis of meningococcal infection, the two lowest concentrations that produce visible colour intensity were selected.

2.5 The Dot Blot EIA Test

A piece of nitrocellulose membrane (NC) with a pore size of 0.20 µm (Bio-Rad, USA) was cut into small strips measuring 19 mm by 18 mm and 10 mm by 18 mm to fit the 21- small compartment storage box. The strips were further subdivided into small squares measuring 4 mm by 4 mm. The WCP, sdWCP and CSP antigens were serially diluted and one microliter of each antigen was spotted onto the center of the divided small square membrane. Two spots each were spotted on the membrane for each WCP and sdWCP, while CSP had only one spot on the membrane. The spotted NC membrane was allowed to air dry. The NC membrane was blocked for 1 hour with tris buffered saline (TBS, pH 7.5) containing 5% non-fat skim milk (TBS-SM) with gentle agitation. After blocking, the membranes were washed in 5% TBS-SM 3 times at 5-minute intervals. All fresh sera were diluted 1:100 in TBS and stored at 4°C. 500 µL of serum samples were added to each storage container compartment and incubated for 1 hour with gentle shaking. The serum samples were aspirated, and strips were subjected to 3 times wash with 1 mL of 5% TBS-SM for 5 minutes each. The washing solution was removed, and 500 µL of diluted alkaline phosphate (AP) conjugated goat anti-human immunoglobulin M, G and A (KPL ReserveAP^{TM}) was added to the storage well of the respective compartments and incubated with gentle shaking for 1 hour. The optimal dilution for the secondary antibody was 1: 1000 for Anti-human IgM and IgA, and 1:1000 for anti-human IgG was diluted at 1: 2000. Upon completion of incubation, the secondary antibody was aspirated, and the membrane washed in TBS-SM as described above. The test strip was then ready for colour development, which was visualised using a chromogenic substrate detection system (NBT-BCIP, Bio-Rad alkaline phosphatase substrate kit, USA). Immediately before use, the chromogenic substrate solutions nitroblue tetrazolium (Solution A), 5-bromo-4-chloro-3-indolyphophate (Solution B) were each mixed with AP colour development buffer according to the manufacturer's recommended ratio and 1 mL

was added to each compartment. The strips were incubated for 10 minutes at room temperature with gentle agitation until the colour development was complete. The development of colour was stopped by removing the AP conjugate substrate, followed by washing with distilled water for 10 minutes with gentle agitation. A positive reaction appeared as the dark blue colour spot on the strips.

2.6 Application for Antigenicity Study Against Individual Healthy Serum, Vaccinated, Other Diseases, and Polyclonal Rabbit Anti N. Meningitidis

One microliter of nine antigens derived from WCP, sdWCP and CSP of *N. meningitidis* serogroups A, B, C, and Y, two clinical isolates, *M. catarrhalis, N. sicca, and N. cinerea* were spotted onto a nitrocellulose membrane (NC) and allowed to air dried for 10 minutes. Spotted antigens were blocked in TBS containing 5% skim milk for 1 hour with gentle agitation to avoid an unspecific reaction. After blocking, the membranes were washed three times in 5-minute intervals with TBS containing 5% skim milk to remove any antibodies that had not bound during the reaction. The membranes were then subjected to conjugation with serum from 3 categories of subjects (meningococcal vaccinated, healthy and blood donor, and other diseased patients) diluted in TBS 1:100, while polyclonal antibody diluted 1:300 in TBS for 1 hour. After aspirating off the sera, the membranes were rewashed as described previously. The washing step was performed three times, each for 5 minutes. Alkaline phosphatase-conjugated with anti-human IgM, IgA, and IgG at dilutions of 1:1000 and 1:2000, respectively, was prepared in TBS for secondary antibody incubation. The diluted alkaline phosphatase-conjugated anti-human IgM, IgG and IgA isotypes were added and incubated for 1 hour at room temperature. Following incubation, the secondary antibody was aspirated before being washed three times in TBS, as described above. The membrane strips were then incubated for 10 minutes with AP conjugate (NBT-BCIP, Bio-Rad alkaline phosphatase substrate kit, USA) for optimal colour development, and the reaction was stopped by washing with distilled water for 10 minutes. All of the steps were carried out at room temperature. The colour intensity was assessed visually. The positive and negative were interpreted by comparing to positive control (polyclonal antibody). Results were interpreted while the test strips were still wet. The experimental workflow for the development of Dot Blot EIA is presented in Figure 1.

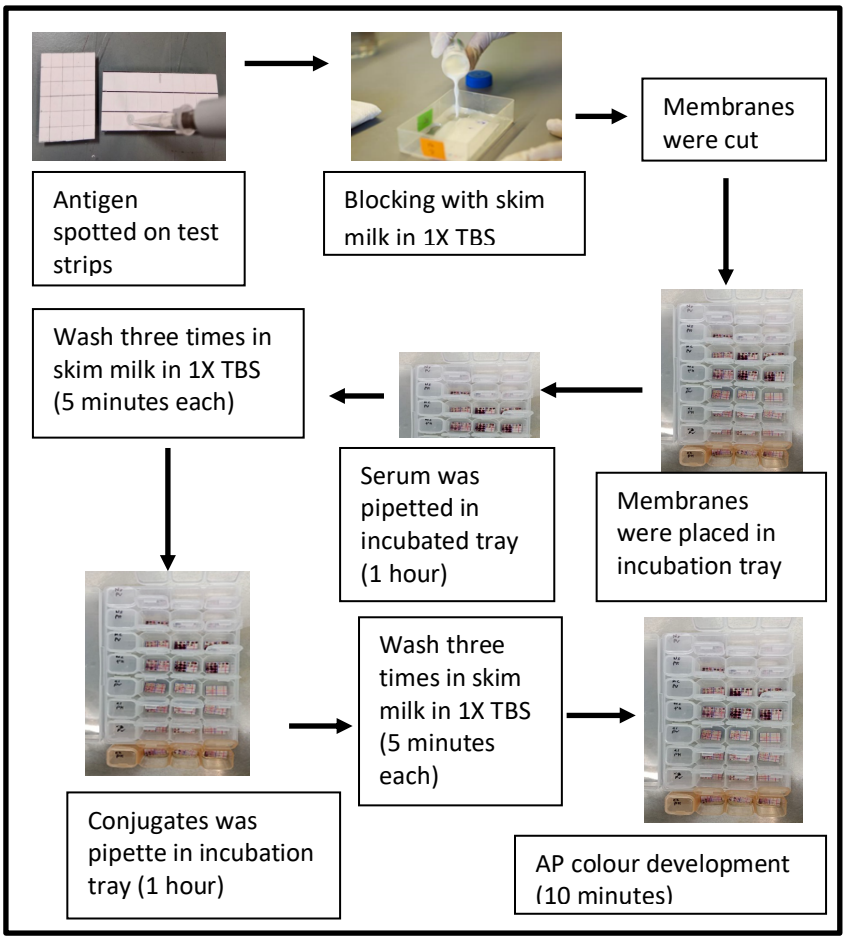

Fig. 1. Flowchart illustrating the workflow of the experimental procedures for dot blot EIA test development

3. Results

3.1 Optimisation of Dot Blot EIA Test

The results for four different antigen concentrations for each differentially extracted Men A, B, C, Y, clinical isolates 1 and 2, *M. catarrhalis*, *N. sicca*, and *N. cinerea* are displayed in Figures 2 to 4. Using the dot blot EIA method, serial dilutions of WCP, sdWCP, and CSP antigens derived from *N. meningitidis,* and non-pathogenic species were dotted onto nitrocellulose (NC) strips and detected. To determine the optimisation, three known serum samples were diluted in 1: 100: serum healthy positive (SH+), serum vaccinated positive (SV+), and serum healthy negative (SH-). The cut-off concentrations of the spotted antigens were determined by visually comparing the colour intensities produced by each serum against the serially diluted antigens. These results highlight the complex cocktail of proteins present for differentially extracted Men A, B, C, and Y, clinical isolates 1 and 2, *M. catarrhalis*, *N. sicca*, and *N. cinerea*, which appeared highly immunogenic and sufficient for use in the serodiagnosis of meningococcal disease infection. Based on the dot blot EIA test results, two different ranges of antigen concentrations (320 µg/mL and 160 µg/mL) were selected for further use in detecting the response of antibody isotypes IgM, IgG, and IGA in three groups of serum and polyclonal antibody as a positive control.

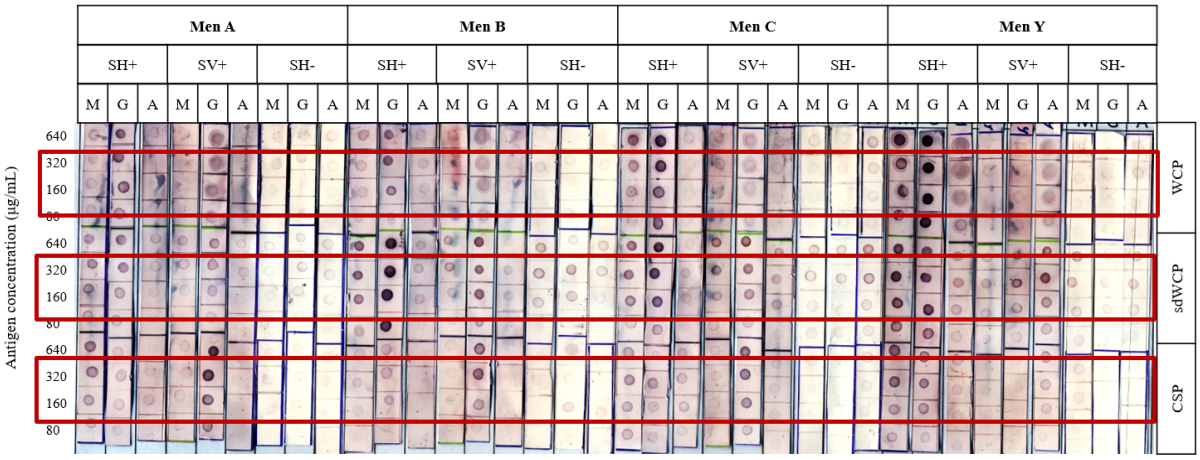

Fig. 2. Optimisation of the dot blot EIA test for detecting IgM, IgG and IgA antibody isotypes derived from Men A, B, C and Y antigens.

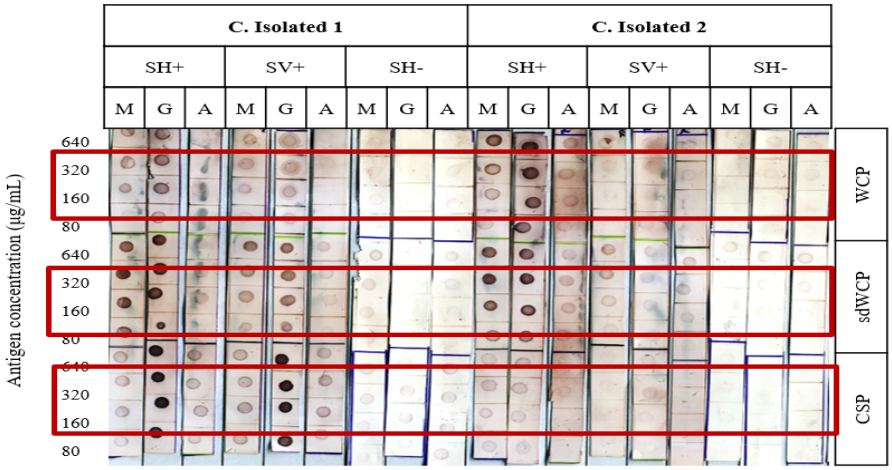

Fig. 3. Optimisation of the dot blot EIA test for detecting IgM, IgG and IgA antibody isotypes derived from clinical isolated 1 and 2 antigens

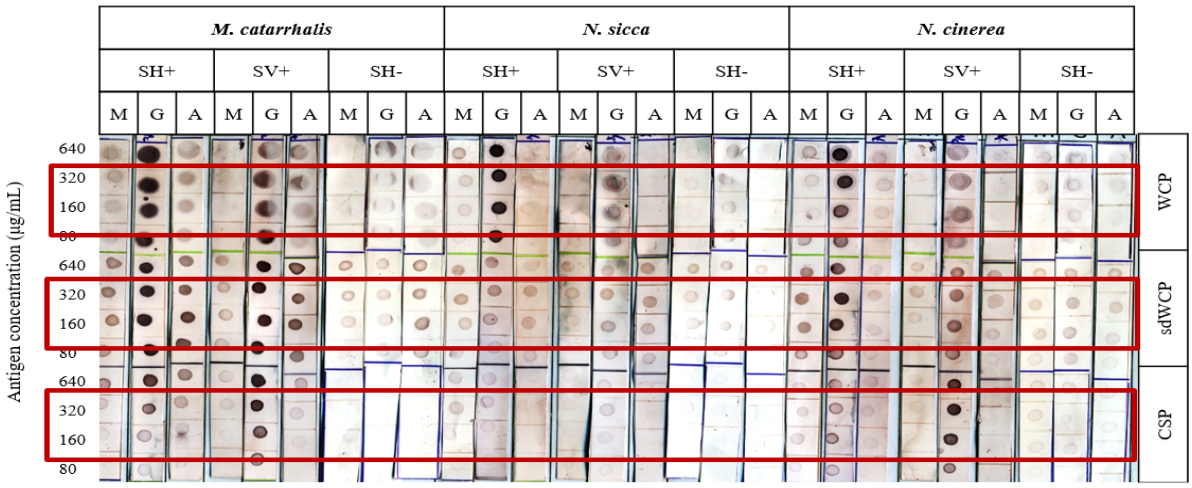

Fig. 4. Optimisation of the dot blot EIA test for detecting IgM, IgG and IgA antibody isotypes derived from *M. catarrhalis*, *N. sicca* and *N. cinerea*

3.2 Determination of the Positive Results Dot Blot EIA Test

A standardised dot blot EIA test was conducted to examine the humoral immune response in different subject groups. These groups included vaccinated Hajj or Umrah pilgrims, healthy individuals, as well as individuals with other diseases. The test used different antigens extracted from *Neisseria meningitidis* (serogroup A, B, C, and Y), clinically isolated (1 and 2), *N. sicca, N. cinerea*, and distantly related *M. catarrhalis*. The test identified three distinct antibody isotypes present in the serum samples - IgM, IgG, and IgA. The test defined a positive result as having an intensity that was equal to or greater than the intensity of the positive control. Figure 5 and Table 1 depict the positive results of the test.

As indicated in Table 2, the vaccinated group exhibited predominantly IgG antibodies in their humoral immune response pattern, alongside some presence of IgM and IgA antibodies in their blood samples. All participants in the vaccinated group had received the quadrivalent conjugate meningococcal A, C, W, and Y (MenACWY) vaccine before embarking on their Hajj or Umrah pilgrimage [23].

Conversely, the healthy and other disease groups mainly displayed IgM antibodies in their humoral immune response pattern (as demonstrated in Tables 3 to 4). Nevertheless, there were also detectable IgG and IgA antibodies in the blood samples. It is plausible that some serum samples from the healthy subjects showcased a comparable humoral immune response pattern as the vaccinated group, potentially owing to prior vaccination with MenACWY or prior infection with meningococcal bacteria, resulting in antibody production.

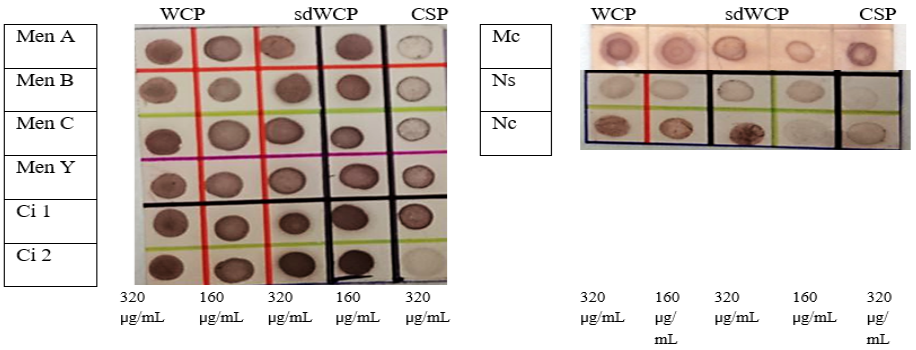

Fig. 5. IgG polyclonal antibody dotting results on different concentrations for differentially extracted proteins versus different antigens

Table 1
Summary of IgG polyclonal results. Positive results are marked as "P". Negatives are marked as "N"

Subject	Category	Org.	WCP	sdWCP	CSP
Polyclonal antibody	Species specific	Men A	P	P	N
		Men B	P	P	P
		Men C	P	P	P
		Men Y	P	P	P
		Ci 1	P	P	P
		Ci 2	P	P	N
	Closely related	N. s	P	N	P
		N. c	P	P	P
	Distantly related	M. c	P	N	P

Table 2

Summary of dot blot EIA test positive results IgM, IgG and IgA for vaccinated Hajj/Umrah pilgrimage individual serum, positive results marked as "P" and negative marked as "N". Pathogenic Neisseria were categorised into species specific, clinical isolates for unknown *N. meningitidis*, closely related *Neisseria (N. sicca* and *N. cinerea)* and distantly related (*M. catarrhalis*)

(W:Whole cell protein, sd: surface depleted Whole cell protein and C:Cell surface protein)

No.	Category	Org.	IgM			IgG			IgA			No.	IgM			IgG			IgA		
			W	sd	C	W	sd	C	W	sd	C		W	sd	C	W	sd	C	W	sd	C
V1	Species	Men A	N	P	N	N	P	N	N	P	N	V7	N	N	N	P	P	N	N	P	N
	Specific	Men B	N	P	N	N	P	N	N	P	N		N	N	N	P	P	N	N	P	N
		Men C	P	P	N	N	P	P	N	P	N		N	N	N	P	P	P	P	P	P
		Men Y	N	N	N	P	P	P	N	N	N		N	N	N	P	P	P	P	N	P
	Clinical	Ci 1	N	P	N	N	P	N	N	P	N		N	P	N	P	P	P	N	P	N
	Isolated	Ci 2	N	P	N	P	P	N	N	P	N		N	N	N	P	P	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	N	N	N		N	N	N	P	N	N	P	P	N
	Related	N. c	N	P	N	P	N	N	P	N	N		N	N	N	P	P	N	P	P	N
	Distantly	M. c	P	P	N	P	P	P	P	P	P		N	P	N	P	P	P	P	P	P
V2	Species	Men A	N	P	N	P	P	N	N	P	N	V8	P	P	P	N	P	N	N	P	N
	Specific	Men B	N	N	N	P	P	N	N	P	N		N	P	N	N	P	N	N	P	N
		Men C	N	N	N	P	P	P	N	N	N		N	P	N	N	N	P	N	P	N
		Men Y	N	N	N	P	P	P	N	N	N		P	P	N	N	N	N	N	N	N
	Clinical	Ci 1	N	P	N	P	P	P	N	P	N		N	P	N	P	P	N	N	N	N
	Isolated	Ci 2	N	P	N	P	P	P	N	P	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	N	N	N	P	N	N	N	N	N		P	P	N	P	N	N	N	N	N
	Related	N. c	N	N	N	P	P	N	P	N	N		P	P	N	N	N	N	P	N	N
	Distantly	M. c	P	P	N	P	P	P	P	P	P		P	P	N	P	P	N	P	P	N
V3	Species	Men A	N	N	N	P	P	N	N	P	N	V9	P	P	P	P	P	N	N	P	N
	Specific	Men B	N	N	N	P	P	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	P	P	P	P	P	P		P	P	N	P	P	P	N	P	N
		Men Y	N	N	N	P	P	N	N	N	N		P	P	P	P	P	P	N	P	N
	Clinical	Ci 1	N	N	N	P	P	P	N	P	N		P	P	N	P	P	N	P	P	N
	Isolated	Ci 2	N	N	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	P	P	N		P	P	N	P	P	N	N	N	N
	Related	N. c	N	N	N	P	N	N	P	P	N		P	P	P	P	P	N	P	N	N
	Distantly	M. c	N	P	N	P	P	P	P	P	P		P	P	P	P	P	P	P	P	N
V4	Species	Men A	P	P	N	P	P	P	N	P	N	V10	P	P	P	P	P	N	N	N	N
	Specific	Men B	N	P	N	P	P	P	N	P	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	P	P	P	P	N	N		P	P	P	P	P	N	N	N	N
		Men Y	P	P	N	P	P	P	P	N	N		P	P	P	P	P	P	N	P	N
	Clinical	Ci 1	P	P	N	P	P	P	P	P	N		P	P	N	P	P	N	P	N	N
	Isolated	Ci 2	P	P	N	P	P	P	P	P	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	P	N	N		P	P	P	P	P	N	P	P	N
	Related	N. c	P	P	N	P	N	N	P	N	N		P	P	P	P	P	N	N	N	N
	Distantly	M. c	P	P	P	P	P	P	P	P	P		P	P	P	P	P	P	P	P	N
V5	Species	Men A	N	P	N	P	P	N	P	P	N	V11	P	P	N	P	P	N	P	P	N
	Specific	Men B	N	P	N	P	P	N	N	P	N		N	N	N	P	P	N	N	N	N
		Men C	N	N	N	P	P	P	P	P	P		N	N	N	P	P	N	N	N	N
		Men Y	N	N	N	P	N	N	P	N	N		N	P	N	P	P	N	N	P	N
	Clinical	Ci 1	N	P	N	P	P	P	N	P	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	N	N	N	P	P	P	N	P	N		N	P	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	P	P	N
	Related	N. c	N	N	N	P	N	N	N	N	N		P	P		P	P	P	P	N	N
	Distantly	M. c	N	N	N	P		P	P	P	P		P	P	P	P	P	N	P	P	N
V6	Species	Men A	N	P	N	P	P	N	N	P	N	V12	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	P	P	N	N	P	N		N	N	N	N	N	N	N	N	N
		Men C	P	P	P	P	P	P	P	P	P		N	N	N	P	N	N	N	N	N

		Men Y	P	P	N	P	P	P	P	N	N		N	N	N	P	N	N	N	N	N
	Clinical	Ci 1	P	P	N	N	P	N	N	P	N		N	N	N	N	P	N	N	N	N
	Isolated	Ci 2	N	P	N	N	P	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	P	P	N		P	P	N	P	N	N	N	P	N
	Related	N. c	P	P	N	P	N	N	P	P	N		N	N	N	P	N	N	N	N	N
	Distantly	M. c	P	P	P	P	P	P	P	P	P		P	P	N	P	P	N	P	P	N

Table 3

Summary of dot blot EIA test positive results IgM, IgG and IgA for healthy individual serum, positive results marked as "P" and negative marked as "N". Pathogenic Neisseria were categorised into species specific, clinical isolates for unknown N. meningitidis, closely related Neisseria (N. sicca and N. cinerea) and distantly related (M. catarrhalis)

(W:Whole cell protein, sd: surface depleted Whole cell protein and C:Cell surface protein)

No.	Category	Org.	IgM			IgG			IgA			No.	IgM			IgG			IgA		
			W	sd	C	W	sd	C	W	sd	C		W	sd	C	W	sd	C	W	sd	C
H1	Species	Men A	P	P	N	N	N	N	N	N	N	H4	N	N	N	N	N	N	N	N	N
	Specific	Men B	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men C	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Clinical	Ci 1	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	P	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	P	P	N	P	P	N	P	N	N		N	N	N	P	N	N	N	N	N
	Distantly	M. c	P	P	P	P	P	P	P	P	P		N	N	N	P	P	P	P	P	P
H2	Species	Men A	N	P	N	N	N	N	N	N	N	H5	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Isolated	Ci 2	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	P	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	P	P	N	P	N	N		N	N	N	P	N	N	P	N	N
	Distantly	M. c	N	N	N	P	P	P	P	P	P		N	N	N	P	P	P	P	P	P
H3	Species	Men A	N	N	N	N	N	N	N	N	N	H6	N	P	N	N	P	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	P	N	N	N	N	N		P	P	N	P	N	N	N	N	N
	Related	N. c	N	N	N	P	N	N	P	N	N		P	P	N	P	N	N	N	N	N
	Distantly	M. c	N	N	N	P	P	P	P	P	P		N	N	N	P	P	P	N	N	N
H7	Species	Men A	N	P	N	N	P	N	N	N	N	H12	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men C	N	N	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	N	N	N	P	P	N	N	N	N		N	N	N	N	N	N	P	P	N
	Clinical	Ci 1	N	P	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	N	P	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	P	P	N	P	P	N
H8	Species	Men A	N	P	N	N	P	N	N	P	N	H13	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	P	P	N	N	N	P		P	P	P	N	N	N	N	N	N
		Men C	P	P	P	N	N	N	N	N	P		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	P	N	N		N	N	N	N	N	N	N	N	N

	Clinical	Ci 1	P	P	N	P	P	N	N	P	N		P	P	N	N	N	N	N	N	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
	Closely	N. s	P	N	N	P	N	N	N	N	N		P	N	N	P	N	N	N	N	N
	Related	N. c	N	P	N	P	P	N	N	N	N		N	N	N	P	P	N	P	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	P	P	N	P	P	N
H9	Species	Men A	N	N	N	N	N	N	N	P	N	H14	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	P	N	N	P	N	N		N	N	N	N	P	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	N	P	N	P	P	N
H10	Species	Men A	N	N	N	N	N	N	N	P	N	H16	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		N	N	N	N	P	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	N	N	N	P	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	P	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	P	P	N	N	N	N
H11	Species	Men A	N	N	N	N	N	N	N	N	N	H17	P	P	N	N	P	N	N	P	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	P	N	N	N	N	N		N	N	N	P	N	N	P	N	N
	Related	N. c	N	N	N	P	P	N	N	N	N		N	N	N	P	P	N	P	N	N
	Distantly	M. c	N	N	N	N	P	N	N	N	N		N	N	N	P	P	N	P	P	N
H18	Species	Men A	N	N	N	N	N	N	N	N	N	H25	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	N	P	N	N	N	N	N	N	N		N	P	N	N	N	P	N	N	P
	Isolated	Ci 2	N	P	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	P	P	P	N	N	N	N	N	N		N	P	N	P	N	N	N	N	N
	Related	N. c	P	P	P	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Distantly	M. c	P	P	N	N	P	N	N	P	N		N	P	N	P	P	N	P	P	N
H19	Species	Men A	N	P	P	N	N	N	N	N	N	H26	P	N	P	N	N	N	N	N	N
	Specific	Men B	N	P	P	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	P	P	P	N	N	N	N	N	N		N	P	P	N	P	P	N	N	P
	Isolated	Ci 2	P	P	P	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	P	P	P	N	N	N	N	N	N		P	P	P	P	N	N	N	N	N
	Related	N. c	P	P	P	N	N	N	N	N	N		P	P	P	P	N	N	N	N	N
	Distantly	M. c	P	P	N	N	P	N	N	N	N		P	P	N	P	P	N	P	P	N
H20	Species	Men A	P	P	P	N	N	N	N	N	N	H27	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men C	N	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	P	P	N	N	N	N	N	N	P		N	P	P	N	P	P	N	N	N
	Isolated	Ci 2	P	P	P	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	P	P	P	N	N	N	N	N	N		P	P	P	P	P	N	N	N	N

	Related	N. c	P	P	P	N	N	N	N	N	N		P	P	P	P	P	N	N	N	N
	Distantly	M. c	P	P	N	N	P	N	N	P	N		N	N	N	P	P	N	N	P	N
H21	Species	Men A	N	P	P	N	N	N	N	N	N	H28	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		P	P	N	N	N	N	N	N	N
		Men C	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	P	P	P	N	N	N	N	N	N		N	P	P	N	N	P	N	N	P
	Isolated	Ci 2	P	P	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	P	P	P	N	N	N	N	N	N		P	P	P	P	N	N	N	N	N
	Related	N. c	P	P	P	N	N	N	N	N	N		P	P	N	P	N	N	N	N	N
	Distantly	M. c	P	P	N	P	P	N	N	P	N		P	P	N	P	P	N	P	P	N
H22	Species	Men A	N	P	P	N	N	N	N	N	N	H29	N	P	N	N	N	N	N	N	N
	Specific	Men B	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	P	P	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	P	P	N	N	N	N	N	N	N		N	P	N	N	P	P	N	N	N
	Isolated	Ci 2	P	P	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Closely	N. s	P	P	P	N	N	N	N	N	N		P	P	N	P	N	N	N	N	N
	Related	N. c	P	P	P	N	N	N	N	N	N		P	P	P	P	N	N	N	N	N
	Distantly	M. c	N	P	N	N	P	N	N	N	N		P	P	N	P	P	N	N	P	N
H23	Species	Men A	N	N	N	N	N	N	N	N	N	H30	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	N	P	N	N	N	N	N	N	N		N	P	N	N	N	P	N	N	P
	Isolated	Ci 2	N	P	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	N	N	N		N	P	N	P	N	N	N	N	N
	Related	N. c	P	P	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Distantly	M. c	N	P	N	N	N	N	N	N	N		N	P	N	P	P	N	P	P	N
H24	Species	Men A	N	N	N	N	N	N	N	N	N	H31	P	N	P	N	N	N	N	N	N
	Specific	Men B	N	N	N	P	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	N	N	N	P	N	N	N	N	N		N	P	N	N	N	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	P	N	N	N		N	P	P	N	P	P	N	N	P
	Isolated	Ci 2	N	N	N	P	P	N	N	N	N		N	P	N	N	P	N	N	N	N
	Closely	N. s	N	P	N	P	N	N	N	N	N		P	P	P	P	N	N	N	N	N
	Related	N. c	N	N	N	P	P	N	N	N	N		P	P	P	P	N	N	N	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	N
H32	Species	Men A	P	N	N	P	P	N	N	P	N	H37	N	N	N	N	N	N	N	N	N
	Specific	Men B	P	N	N	P	P	N	N	P	N		N	P	N	P	P	N	N	N	P
		Men C	P	P	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	N	P	N		N	P	N	P	P	N	N	N	N
	Clinical	Ci 1	N	P	P	P	P	P	N	P	N		N	P	N	P	P	P	N	P	P
	Isolated	Ci 2	P	N	N	P	P	N	N	N	N		N	N	N	P	N	N	N	N	N
	Closely	N. s	P	N	N	N	N	N	P	N	N		N	N	N	N	N	N	N	N	N
	Related	N. c	N	N	N	P	N	N	P	P	N		N	N	N	N	N	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	P	P	N	P	P	N
H33	Species	Men A	P	P	N	P	P	N	N	N	N	H38	N	N	N	N	P	N	N	N	N
	Specific	Men B	N	P	N	P	P	P	N	N	N		N	N	N	P	P	N	N	N	N
		Men C	N	N	N	P	P	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	P	N
	Clinical	Ci 1	P	P	P	P	P	P	N	N	P		P	P	N	P	P	P	N	N	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	N	N	P	N	N	P	P	N		P	P	N	P	P	N	N	P	N
	Related	N. c	N	N	N	P	P	N	P	P	N		P	P	P	P	P	P	N	P	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		P	P	P	P	P	P	P	P	N
H34	Species	Men A	P	P	N	N	P	N	N	N	N	H39	N	P	N	N	P	N	P	P	N

	Specific	Men B	P	P	P	N	P	N	N	N	N		N	P	N	P	P	N	P	P	N
		Men C	N	N	N	N	N	N	N	N	N		P	P	P	P	P	N	P	P	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	P	P	N
	Clinical	Ci 1	P	P	P	P	N	P	N	N	N		P	P	P	P	P	P	P	P	P
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	P	P	N
	Closely	N. s	P	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	P	N
	Related	N. c	N	N	N	P	N	N	P	N	N		N	N	N	P	N	N	P	P	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	P	P	N	P	P	N
H35	Species	Men A	N	N	N	N	N	N	N	N	N	H40	P	P	N	P	P	N	N	P	N
	Specific	Men B	N	P	N	N	P	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	P	N	P	N	P	N
		Men Y	N	P	N	N	P	N	N	N	N		P	P	N	P	P	P	N	P	N
	Clinical	Ci 1	N	P	N	N	P	P	N	N	N		P	P	P	P	P	N	N	N	P
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	P	P	N		P	P	N	P	P	N	N	P	N
	Related	N. c	N	N	N	P	N	N	N	N	N		P	P	N	P	P	P	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	N	N		P	P	N	P	P	P	P	P	N
H36	Species	Men A	N	N	N	P	P	P	N	P	P	H41	N	P	N	P	P	N	N	P	N
	Specific	Men B	N	P	N	P	P	P	N	P	P		N	N	N	P	P	N	N	N	N
		Men C	N	N	N	P	P	P	N	N	N		N	N	N	P	N	P	N	P	N
		Men Y	N	P	N	P	P	N	N	N	N		N	N	N	P	P	P	N	P	N
	Clinical	Ci 1	N	P	N	P	P	P	N	P	P		N	P	P	P	P	N	N	N	P
	Isolated	Ci 2	N	N	N	P	P	P	N	P	P		N	N	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	P	N	N		P	P	N	P	N	N	N	P	N
	Related	N. c	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	P	N	N
	Distantly	M. c	N	N	N	N	N	N	P	N	N		P	P	N	P	P	N	P	P	N
H42	Species	Men A	P	P	N	N	P	N	N	P	N	H47	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	P	P	N	P	N	N	P	N		N	P	N	N	P	N	N	N	N
		Men C	N	P	N	N	P	N	N	P	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	N	P	N	N	P	N		N	N	N	N	N	N	P	P	N
	Clinical	Ci 1	P	P	P	N	P	P	N	N	P		N	N	N	N	P	P	N	P	N
	Isolated	Ci 2	P	P	N	N	P	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	P	N	N	N	N	N		P	N	N	P	N	N	N	N	N
	Related	N. c	P	P	N	P	P	N	P	N	N		N	N	N	P	P	N	P	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		N	N	N	P	P	N	P	P	N
H43	Species	Men A	P	P	N	P	P	N	N	P	N	H48	N	P	N	N	P	N	N	N	N
	Specific	Men B	N	P	N	P	P	P	N	N	N		P	P	N	P	N	N	N	N	N
		Men C	N	N	N	P	P	P	N	N	N		P	P	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	P	P	N		P	P	N	N	P	N	N	P	N
	Clinical	Ci 1	P	P	P	P	P	P	N	N	P		P	P	N	N	P	N	N	P	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	P	N	N	N	N	N		P	N	N	P	N	N	N	N	N
	Related	N. c	P	P	N	P	P	P	P	N	N		N	P	N	P	P	N	N	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	N
H44	Species	Men A	N	P	N	N	P	N	N	N	N	H49	N	P	N	N	P	N	N	N	N
	Specific	Men B	N	P	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
		Men C	N	N	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Clinical	Ci 1	P	P	P	P	P	P	N	N	N		N	P	N	N	P	P	N	N	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	P	N	P	P	N	P	P	N		P	P	N	P	N	N	P	P	N
	Related	N. c	P	P	N	P	P	N	N	N	N		N	P	N	P	P	N	P	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	P	N	P	P	N	P	P	N
H45	Species	Men A	N	P	N	N	P	N	N	N	N	H50	P	P	N	P	P	N	N	N	N
	Specific	Men B	N	N	N	P	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	P	N	N	N	N	N		N	N	N	P	N	N	N	N	N
		Men Y	N	P	N	P	P	N	N	P	N		P	P	N	P	P	N	N	N	N

	Clinical	Ci 1	P	P	N	P	P	N	N	N	N		P	N	N	P	P	N	N	P	N
	Isolated	Ci 2	N	N	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	N	N	N	N	N	N	N	N		P	P	N	P	N	N	P	P	N
	Related	N. c	N	P	N	P	P	N	P	N	N		P	P	N	P	P	N	P	P	N
	Distantly	M. c	N	P	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	N
H46	Species	Men A	N	P	N	N	P	N	N	N	N	H51	P	P	P	N	P	N	N	P	N
	Specific	Men B	N	N	N	P	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
		Men Y	N	P	N	N	P	N	N	P	N		P	P	N	N	P	N	N	P	N
	Clinical	Ci 1	N	P	N	N	P	N	N	N	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	N	N	N	N	P	N	N	N	N		P	P	N	N	P	N	N	N	N
	Closely	N. s	P	N	N	N	N	N	N	N	N		P	P	N	P	N	N	N	N	N
	Related	N. c	N	P	N	N	P	N	N	N	N		N	P	N	P	P	N	N	P	N
	Distantly	M. c	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	P	P	N
H52	Species	Men A	P	P	N	P	N	N	N	N	N	H56	P	P	N	P	P	N	N	N	N
	Specific	Men B	N	P	N	N	P	N	N	N	N		P	P	P	P	P	P	P	N	N
		Men C	N	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	P	N
	Clinical	Ci 1	N	P	P	P	P	P	N	N	N		P	P	P	P	P	P	N	P	P
	Isolated	Ci 2	N	P	N	P	P	N	N	N	N		P	P	N	P	P	N	P	P	N
	Closely	N. s	P	P	N	P	N	N	P	P	N		P	P	N	P	P	N	P	P	N
	Related	N. c	N	P	N	N	P	N	P	P	N		P	P	N	P	P	N	P	P	N
	Distantly	M. c	P	P	N	N	P	N	P	P	N		P	P	N	P	P	N	P	P	N
H53	Species	Men A	P	P	N	P	P	N	N	N	N	H57	N	N	N	N	P	N	N	N	N
	Specific	Men B	N	P	N	N	P	N	N	N	N		N	N	N	N	P	N	N	N	N
		Men C	N	P	N	N	P	N	N	N	N		P	N	P	P	N	P	P	N	N
		Men Y	N	P	N	N	P	N	N	P	N		N	N	N	P	P	N	N	P	N
	Clinical	Ci 1	P	P	P	P	P	P	N	P	N		N	P	N	N	P	N	N	P	P
	Isolated	Ci 2	N	P	N	P	P	N	N	N	N		N	P	N	P	P	N	P	N	N
	Closely	N. s	P	P	N	P	P	N	P	N	N		P	P	N	P	N	N	N	N	N
	Related	N. c	N	P	N	N	P	N	P	N	N		N	P	N	N	P	N	P	P	N
	Distantly	M. c	P	P	N	N	P	N	P	P	N		N	P	N	N	P	N	P	P	N
H54	Species	Men A	P	P	N	N	N	N	N	N	N	H58	N	P	N	P	P	N	N	N	N
	Specific	Men B	N	P	N	N	P	N	N	P	N		N	P	N	P	P	P	P	N	N
		Men C	N	P	N	N	N	N	N	P	N		N	N	N	P	P	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	P	P	N
	Clinical	Ci 1	P	P	P	P	P	P	N	P	P		P	P	P	P	P	P	N	P	P
	Isolated	Ci 2	P	P	N	P	P	N	P	N	N		P	P	N	P	P	N	P	P	N
	Closely	N. s	P	N	N	P	N	N	N	N	N		P	P	N	P	N	N	P	N	N
	Related	N. c	N	P	N	P	P	N	P	P	N		P	P	N	N	P	N	P	P	N
	Distantly	M. c	P	P	N	N	P	N	P	P	N		P	P	N	N	P	N	P	P	N
H55	Species	Men A	P	P	N	P	P	N	N	N	N										
	Specific	Men B	N	P	N	P	P	N	P	N	N										
		Men C	N	P	N	P	P	N	P	N	N										
		Men Y	P	P	N	P	P	N	N	P	N										
	Clinical	Ci 1	P	P	P	P	P	P	N	P	P										
	Isolated	Ci 2	P	P	N	P	P	N	P	N	N										
	Closely	N. s	P	P	N	P	P	N	P	N	N										
	Related	N. c	P	P	N	P	P	N	P	P	N										

Table 4

Summary of dot blot EIA test positive results IgM, IgG and IgA for other diseases individual serum, positive results marked as "P" and negative marked as "N". Pathogenic Neisseria were categorised into species specific, clinical isolates for unknown N. meningitidis, closely related Neisseria (N. sicca and N. cinerea) and distantly related (M. catarrhalis)

(W:Whole cell protein, sd: surface depleted Whole cell protein and C:Cell surface protein)

No.	Category	Org.	IgM			IgG			IgA			No.	IgM			IgG			IgA		
			W	sd	C	W	sd	C	W	sd	C		W	sd	C	W	sd	C	W		
D1	Species	Men A	P	P	N	N	N	N	N	N	N	D7	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	N	N	N	N	N	N		N	P	N	P	P	N	N	P	N
		Men C	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	N	N	N	N	N	N		N	N	N	P	P	N	N	P	N
	Clinical	Ci 1	P	P	N	N	N	N	N	N	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	P	P	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Closely	N. s	P	P	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	P	P	N	N	N	N	P	N	N		N	N	N	P	P	N	N	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		N	P	N	N	N	N	P	P	N
D2	Species	Men A	N	P	N	N	N	N	N	N	N	D8	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		P	P	N	N	P	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	P	P	N		P	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	N	N	N	P	N	N		N	N	N	N	P	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		N	N	N	N	P	N	N	N	N
D3	Species	Men A	P	P	N	N	P	N	N	P	N	D9	N	P	N	N	N	N	N	N	N
	Specific	Men B	P	P	N	P	P	N	N	P	N		P	P	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	P	P	N		N	N	N	P	P	N	N	N	N
	Clinical	Ci 1	P	P	N	P	P	N	N	P	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	P	P	N	P	P	N	P	N	N		N	N	N	P	N	N	N	N	N
	Closely	N. s	P	P	N	P	N	N	P	N	N		N	N	N	P	N	N	P	N	N
	Related	N. c	P	P	N	P	P	N	P	P	N		N	N	N	P	P	N	P	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		N	N	N	N	P	N	P	P	N
D4	Species	Men A	N	N	N	N	N	N	N	N	N	D10	N	P	N	P	P	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
		Men Y	N	N	N	N	N	N	N	P	N		P	P	N	P	P	P	N	N	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	P	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	P	N
	Closely	N. s	P	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
	Related	N. c	N	N	N	N	N	N	N	N	N		P	P	P	P	P	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	N	N	N		P	P	N	P	P	N	P	P	N
D5	Species	Men A	N	N	N	N	N	N	N	N	N	D11	N	N	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	N	N	N	N	N	N	N	N	N		N	N	N	N	P	N	N	P	N
	Clinical	Ci 1	P	N	N	N	N	N	P	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Related	N. c	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Distantly	M. c	N	N	N	N	N	N	P	P	N		N	N	N	P	P	N	P	P	N
D6	Species	Men A	N	N	N	N	N	N	N	N	N	D12	N	P	N	N	P	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	P	N	N

		Men Y	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	P	N
	Clinical	Ci 1	N	N	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
	Isolated	Ci 2	N	N	N	N	N	N	N	N	N		N	P	N	P	P	N	N	N	N
	Closely	N. s	N	N	N	N	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Related	N. c	N	N	N	N	N	N	N	N	N		N	N	N	P	P	N	N	N	N
	Distantly	M. c	N	N	N	N	N	N	N	P	N		N	N	N	P	P	N	P	P	N
D13	Species	Men A	P	P	N	N	N	N	N	N	N	D17	N	N	N	N	P	N	N	N	N
	Specific	Men B	N	P	N	N	P	N	N	N	N		N	N	N	N	P	N	N	P	N
		Men C	P	N	N	N	N	N	N	N	N		N	N	N	N	P	N	N	N	N
		Men Y	P	P	N	P	P	N	P	P	N		N	N	N	N	N	N	N	P	N
	Clinical	Ci 1	P	P	N	P	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	P	P	N	P	N	N	N	N	N		N	N	N	N	N	N	N	P	N
	Closely	N. s	P	P	N	P	N	N	N	N	N		P	P	N	P	P	N	P	P	N
	Related	N. c	P	P	N	P	P	N	N	N	N		N	N	N	P	N	N	P	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	P
D14	Species	Men A	N	P	N	N	P	N	N	N	N	D18	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	P	N	N	P	N	N	N	N		N	P	N	N	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	N	P	N	N	N	N
	Clinical	Ci 1	P	P	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	N	N	N	N	N	N
	Closely	N. s	P	P	N	P	P	N	P	P	N		N	N	N	P	N	N	P	P	N
	Related	N. c	N	P	N	P	P	N	N	N	N		N	P	N	P	N	N	N	N	N
	Distantly	M. c	P	P	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	N
D15	Species	Men A	N	P	N	N	N	N	N	P	N	D19	N	P	N	N	N	N	N	N	N
	Specific	Men B	N	N	N	N	N	N	N	N	N		N	P	N	N	P	N	N	N	N
		Men C	N	N	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	P	N	N	N	N	N	N	P	N		N	P	N	N	P	N	N	N	N
	Clinical	Ci 1	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Isolated	Ci 2	P	P	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N
	Closely	N. s	N	P	N	P	N	N	N	N	N		N	P	N	P	N	N	N	N	N
	Related	N. c	N	P	N	P	N	N	N	N	N		N	N	N	P	N	N	N	N	N
	Distantly	M. c	N	N	N	P	P	N	P	P	N		P	P	N	P	P	N	P	P	N
D16	Species	Men A	P	P	N	N	N	N	N	N	N	D20	N	N	N	P	N	N	N	N	N
	Specific	Men B	P	P	N	N	P	N	N	N	N		P	P	N	P	P	N	N	N	N
		Men C	P	P	N	N	N	N	N	N	N		N	P	N	N	N	N	N	N	N
		Men Y	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Clinical	Ci 1	N	P	N	N	N	N	N	N	N		P	P	N	P	P	N	N	N	N
	Isolated	Ci 2	P	P	N	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Closely	N. s	P	P	P	P	P	N	N	N	N		P	P	N	P	P	N	N	N	N
	Related	N. c	P	P	N	P	P	N	N	N	N		P	P	P	P	P	N	N	N	N
	Distantly	M. c	P	P	P	P	P	P	N	N	N		P	P	N	P	P	N	P	P	N

3.2 Distribution Number of Positive Results for IgM, IgG, and IgA of Differentially Extracted Proteins Against Vaccinated, Healthy, and Other Diseases

The study assessed the presence of different antibody isotypes against various extracted antigens from species specific, closely and distantly related species. The distribution of positive results detected in WCP, sdWCP, and CSP results for each antibody isotype was tabulated in Table 5(a) to (c). The collected samples included twelve samples obtained from meningococcal vaccinated pilgrims who had performed the Hajj or Umrah (Table 5(a)), fifty-eight serum samples obtained from healthy individuals (Table 5(b)), and another twenty samples obtained from patients with other diseases (Table 5(c)). Results indicated that WCP and sdWCP had the highest percentage of positive results,

ranging from 92% to 100%, in meningococcal vaccinated individuals for IgG and IgA isotypes. IgM isotype was also detected in significant numbers across all antigens and serum groups. However, CSP had the lowest proportion of positive outcomes and even registered negative values (0%).

Among species specific, Men A, Men B, and Men Y exhibited the highest detected IgG isotype results in WCP and sdWCP. The clinical isolate 1 led to 100% detection of IgG antibodies isotype also in vaccinated subjects. Among closely related species, *N. cinerea* showed the highest presence of IgG isotype in WCP, while *M. catarrhalis* had the predominant IgG and IgA isotypes detected (100%) in WCP and sdWCP. In the healthy subjects' group, the highest antibody isotype detected was IgM in sdWCP of Men Y and clinically isolated 1, around 72% and 76%, respectively. In closely related species, IgM in WCP of *N. sicca* was 69%. In contrast, the highest detected antibody isotype in distantly related species was IgG in sdWCP, which was 95% for *M. catarrhalis*.

Moreover, in the other disease groups, Men A and Men B of sdWCP had the highest detected antibodies isotype, ranging between 60 – 62%. Approximately 67% of IgM in WCP was detected in clinical isolate 2. Among closely related species, the highest detected antibody isotype was IgG in WCP at 70%, whereas for *M. catarrhalis*, the highest was IgG in sdWCP at 95%.

Table 5
Distribution number of positive results (%) of differentially extracted proteins for each species specific (Men A, B, C and Y), clinically isolates 1 and 2, closely related (*N. sicca and N. cinerea*) and distantly related spp. (*M. catarrhalis*) among (a) vaccinated, (b) healthy, and (c) other disease subjects

(a) VACCINATED HAJJ/ UMRAH PILGRIMAGE SUBJECTS										
		WCP			sdWCP			CSP		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
		Positive results (%)								
Species	Men A	5 (42)	9 (75)	1 (8)	9 (75)	11 (92)	10 (83)	3 (25)	0 (0)	0 (0)
Specific	Men B	0 (0)	9 (75)	0 (0)	7 (58)	11 (92)	7 (58)	0 (0)	0 (0)	0 (0)
	Men C	4 (33)	9 (75)	5 (42)	5 (42)	10 (83)	7 (58)	2 (17)	9 (75)	4 (33)
	Men Y	5 (42)	11 (92)	4 (33)	6 (50)	9 (75)	3 (25)	2 (17)	7 (58)	1 (8)
Clinical	Ci 1	5 (42)	9 (75)	3 (25)	10 (83)	12 (100)	9 (75)	0 (0)	3 (25)	0 (0)
Isolated	Ci 2	3 (25)	9 (75)	1 (8)	8 (67)	11 (92)	4 (33)	0 (0)	3 (25)	0 (0)
Closely	N. s	9 (75)	7 (58)	5 (42)	9 (75)	3 (25)	6 (50)	1 (8)	0 (0)	0 (0)
Related	N. c	6 (50)	11 (92)	9 (75)	7 (58)	5 (42)	4 (33)	2 (17)	1 (8)	0 (0)
Distantly	M. c	9 (75)	12 (100)	12 (100)	11 (92)	12 (100)	12 (100)	5 (42)	9 (75)	7 (58)
(b) HEALTHY SUBJECTS										
		WCP			sdWCP			CSP		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
		Positive results (%)								
Species	Men A	15 (26)	11 (19)	1 (2)	33 (57)	26 (45)	11 (19)	7 (12)	1 (2)	1 (2)
Specific	Men B	8 (14)	24 (41)	4 (7)	35 (60)	29 (50)	5 (7)	3 (5)	5 (9)	3 (5)
	Men C	9 (16)	19 (33)	3 (5)	25 (43)	17 (29)	5 (9)	4 (7)	5 (9)	1 (2)
	Men Y	23 (40)	24 (41)	6 (10)	42 (72)	31 (53)	18 (31)	0 (0)	2 (3)	0 (0)
Clinical	Ci 1	24 (41)	24 (41)	1 (2)	45 (76)	34 (59)	16 (28)	17 (29)	27 (47)	19 (33)
Isolated	Ci 2	25 (43)	27 (47)	6 (10)	39 (67)	35 (60)	4 (7)	2 (3)	3 (5)	1 (2)
Closely	N. s	40 (69)	38 (66)	13 (22)	32 (55)	7 (12)	11 (19)	9 (16)	0 (0)	0 (0)
Related	N. c	23 (40)	41 (71)	26 (45)	34 (59)	33 (57)	12 (21)	9 (16)	3 (5)	0 (0)
Distantly	M. c	22 (40)	43 (74)	44 (76)	29 (50)	55 (95)	49 (84)	2 (3)	8 (14)	5 (7)
(c) OTHER DISEASES SUBJECTS										
		WCP			sdWCP			CSP		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
		Positive results (%)								
Species	Men A	4 (20)	2 (10)	0 (0)	13 (65)	5 (25)	2 (10)	0 (0)	0 (0)	0 (0)
Specific	Men B	4 (20)	5 (25)	0 (0)	12 (60)	10 (50)	3 (15)	0 (0)	0 (0)	0 (0)
	Men C	3 (15)	1 (5)	1 (5)	5 (25)	3 (15)	0 (0)	0 (0)	0 (0)	0 (0)

	Men Y	8 (40)	8 (40)	2 (10)	9 (45)	11 (55)	8 (40)	0 (0)	1 (5)	0 (0)
Clinical	Ci 1	12 (60)	8 (40)	1 (5)	11 (55)	8 (40)	4 (20)	1 (5)	0 (0)	0 (0)
Isolated	Ci 2	8 (67)	9 (45)	1 (5)	9 (45)	7 (35)	2 (10)	2 (10)	0 (0)	0 (0)
Closely	N. s	10 (50)	14 (70)	6 (30)	9 (45)	5 (25)	4 (20)	1 (5)	0 (0)	0 (0)
Related	N. c	6 (30)	14 (70)	5 (25)	9 (45)	11 (55)	1 (5)	2 (10)	0 (0)	0 (0)
Distantly	M. c	10 (50)	15 (75)	16 (80)	11 (55)	17 (85)	17 (85)	1 (5)	1 (5)	1 (5)

4. Discussion and Conclusions

The evaluation of the effectiveness of meningococcal vaccines relies on two primary serological assays, namely the Enzyme-Linked Immunosorbent Assay (ELISA) and the Serum Bactericidal Assay (SBA) [24]. Among these, the SBA is considered the gold standard for determining vaccine efficacy. However, it has some significant limitations, such as being time-consuming and labor-intensive, and requiring skilled personnel for colony counts and data analysis. Due to these limitations, it is essential to find more efficient and rapid data analysis methods in clinical trials and vaccine research [25]. Scientists and researchers are exploring alternative approaches to improve the SBA assay or seek other assays that provide quick and reliable results while maintaining accuracy and sensitivity. Developing more user-friendly and time-efficient methods for analysing vaccine efficacy is crucial for advancing vaccine development and improving public health responses to meningococcal infections.

In light of these challenges, this study introduces a modified Dot Blot Enzyme ImmunoAssay (EIA) as a multiplex immunoassay capable of simultaneously detecting antibodies against 30 species-specific antigens, clinical isolates, and 15 antigens from both closely and distantly related species in small serum volumes. This advancement not only proves to be more informative but also conserves time and serum, marking a significant improvement over traditional methods.

The humoral immune response, which encompasses a complex network of antibodies with distinct specificities and functions, is crucial for combating infections both directly, through pathogen neutralization and indirectly, by assisting the innate immune system in pathogen clearance [26]. Humoral immunity is the arm of the immune system that becomes active when the body becomes infected, producing various antibody isotypes and subclasses Following activation, mature naive B cells undergo Immunoglobulin (Ig) heavy chain class switching, transitioning from IgM and IgD to IgG, IgE, or IgA, thus enhancing their capacity to eliminate specific pathogens [27].

Utilizing this method, the study evaluates antigen-specific IgM, IgG and IgA antibody isotypes in serum samples from meningococcal vaccinated Hajj or Umrah, healthy, and other diseases. The polyclonal antibody results, shown in Figure 5 and Table 1, served as a positive control for comparison with all groups of serum samples tested. The findings reveal considerable interindividual variability in antibody detection, likely attributable to the diverse antigenicity of different Neisseria species and strains, as well as varying previous exposures and individual capacities to mount an antigen-specific humoral immune response.

The results reveal a clear pattern of humoral immune responses in vaccinated individuals, showing a strong immune response characterized by the presence of antibodies such as IgG in WCP, sdWCP, and CSP from pathogenic *N. meningitidis* and non-pathogenic Neisseria spp. This response is higher compared to healthy individuals and those who have other diseases, as shown in Tables 2 to 4. This is because the production of serum immunoglobulin G (IgG) antibodies is considered the primary mechanism by which meningococcal vaccines prevent invasive disease. Following the initial vaccination dose, IgM antibody formation usually precedes IgG antibody formation. However, the IgG response becomes more robust with subsequent vaccinations. In a study conducted by Ruijne *et al.,* [28], total IgG levels significantly increased in both young and older infants, with 98% of participants showing elevated IgG levels in the OMV ELISA after completing the primary vaccination

schedule. Similarly, Vermont *et al.,* [29] evaluated the avidity maturation and IgG isotype distribution of antibodies after vaccination with a meningococcal B outer membrane vesicle (OMV) vaccine. Their findings revealed the presence of IgM, IgG, and IgA antibodies in the pre- and post-vaccination sera of 134 healthy toddlers (ages 2 to 3 years), consistent with the established patterns of humoral immune responses to vaccination. Notably, IgM is the initial isotype generated in response to an infection or vaccination, providing early protection. IgG is the primary isotype that offers long-term protection, while IgA is produced in mucosal surfaces and aids in protecting these regions against infection.

This study highlights the enhanced sensitivity of the dot blot EIA assay, which is further supported by our observations on species-specific antigens among Men A, Men B, and Men Y. Significantly, the IgG of sdWCP and IgG of WCP displayed the highest number of positive detections (Table 5), demonstrating the assay's effectiveness. Although Men C did not exhibit the highest number of positive detections, the findings revealed a relatively higher number of positive detections in IgG of sdWCP, indicating a broad efficacy across different meningococcal serogroups. Additionally, the presence of IgM and IgA antibody isotypes for all species-specific antigens was observed, underscoring the assay's comprehensive detection capabilities. Even though, some species-specific antigens showed a 0% detection rate initially, the application of three different extraction methods led to the identification of antibodies, further validating the method's ability to enhance sensitivity.

Supporting these findings, Broderick *et al.,* [30] have demonstrated the significant impact of the MenACWY vaccine in increasing the production of antibodies against *N. meningitidis* serogroups C and Y. Specifically, the vaccination resulted in higher serum IgG concentrations, functional bactericidal antibodies, and an increased proportion of individuals who seroconvert after receiving the vaccine, thereby providing evidence of the vaccine's effectiveness. However, it's noteworthy that this study did not investigate the presence of antibodies for Men A and Men W, highlighting a gap in the research. Complementing this, Anderson *et al.,* [31] discovered significant increases in total antibody levels to both *N. meningitidis* group A and C polysaccharides after one month of vaccination with conjugate vaccine in adults. This vaccine not only increased IgG, IgA, and IgM antibodies against both polysaccharides but also led to a significant increase in serum bactericidal titre among all groups of vaccine recipients, further corroborating the effectiveness of vaccination strategies against meningococcal diseases.

In this study, a notable concern arises from the discovery that vaccinated individuals possessed antibodies against Men B, although nearly all vaccinated participants received only the quadrivalent conjugate MenACWY vaccine. This suggests that the antibodies may be a result of previous infections rather than vaccination. Additionally, the presence of antibodies to Men B could be due to pharyngeal colonization by commensal *Neisseria* spp. which can induce bactericidal antibodies against meningococcus [32].

Several studies have examined into the presence of meningococcal antibodies in healthy individuals. Specifically, Apicella *et al.,* [33] revealed that normal human serum's bactericidal antibodies primarily target lipopolysaccharides, identifying an antigenic site shared across a wide array of gram-negative bacteria. They also noted that the IgM antibody's bactericidal activity against oligosaccharides (LOS) was significant, underscoring the role of IgM in combating pathogens. Building on these findings, the present study adds new dimensions to our understanding. It demonstrates that the sdWCP of Men Y notably shows the highest number of positive detections for the IgM antibody isotype among healthy individuals. Conversely, for other antigens of WCP and sdWCP for Men A, B, and C, the numbers of positive detections for both IgM and IgG antibodies are approximately equivalent, indicating a broader immune response that is not restricted to a single type of antibody.

Moreover, Newcombe *et al.,* [34] expanded on this topic by investigating the protein targets of natural protective immunity. They found both pan-reactive antigens, which are recognized by most sera, and subject-specific antigens, suggesting a diverse immune response among individuals. Similarly, Litt *et al.,* [35] compared antibody levels in serum samples from children recovering from meningococcal disease to those from uninfected children. Intriguingly, they found no differences for 22 to 23 antigenic proteins, which could suggest that the antibody reactivity observed in samples from healthy children may stem from asymptomatic colonization rather than from infection. This supports the idea that exposure to the bacteria, even without causing disease, can lead to the development of antibodies.

Moreover, it was observed that the humoral immune responses of the other disease group were comparable to those of healthy individuals. The highest levels of IgM were noted for Men A, B, and C of sdWCP, while for Men Y of sdWCP, IgG was the dominant antibody isotype. It is noteworthy, however, that the overall number of positive responses for all antibody isotypes in the other disease group was lower compared to healthy individuals. Nonetheless, the existence of antibodies that cross-react with *Neisseria* spp. antigens were apparent, as shown in the humoral immune response pattern illustrated in Table 4. The positive results summarized in Table 5c support the presence of cross-reactive antibodies in the other disease group with meningococcal antigens.

Besides that, clinical isolates 1 and 2 yielded similar results to the species-specific isolates in terms of humoral immune responses and positive detections for all serum groups tested, including vaccinated, healthy, and other disease subjects. However, it is important to note that the main limitation of these clinical isolates is the unknown species of pathogenic *N. meningitidis.* This uncertainty raises the possibility that these wild types may belong to the same species as the species-specific isolates studied or represent different strains not included in the research. Despite this limitation, it is worth mentioning that the highest number of positive detections was observed among vaccinated subjects, specifically for IgG of sdWCP, with a detection rate of 100%. This result suggests that the sdWCP antigen in vaccinated individuals induced a robust IgG immune response. While the humoral immune responses and positive detections in clinical isolates 1 and 2 are similar to species specific results, the lack of species identification introduces ambiguity in the interpretation of the findings.

On the other hand, the presence of all three antibody isotypes (IgM, IgG, and IgA) in WCP and SdWCP of closely and distantly related species, such as *N. sicca*, *N. cinerea*, and *M. catarrhalis*, provides evidence of natural immunity development. This suggests that the immune system has been exposed to these antigens and has produced a variety of antibodies to combat potential infections. The high degree of antigenic similarity between phylogenetically related commensal and pathogenic bacteria supports the hypothesis that natural immunity against pathogens in most adults is due to repeated colonisation by commensals during childhood and adolescence [36]. These commensal bacteria share epitopes with pathogenic bacteria, resulting in the production of cross-reactive antibodies and immune responses. This hypothesis is supported by numerous studies that have shown serological and cell-mediated cross-recognition between respiratory commensals and pathogens, particularly in cases of commensal streptococci and *Streptococcus pneumoniae* [36]. This cross-recognition further strengthens the idea that exposure to commensal bacteria contributes to the development of immunity against related pathogens. In addition, studies by several researchers reported the sharing of major outer membrane cross-reactive antigens among commensal *N. lactamica* spp., *M. catarrhalis*, and pathogenic *N. meningitidis* [16,37,38]. Clearly, this finding supports significant antigenic similarity between these diverse bacterial species, which can result in cross-reactive immune responses in individuals exposed to these bacteria.

This study has further discovered that all serum samples contained IgA antibody isotypes, regardless of whether the subjects were vaccinated, healthy, or suffering from other diseases. Interestingly, most of the IgA antibodies were found to be directed against the *M. catarrhalis* antigen, rather than *N. meningitidis* species-specific, clinical isolates, or closely related spp. antigens. Since *Neisseria* spp. and *M. catarrhalis* are mucosal pathogens, secretory IgA plays a crucial role in protecting mucosal surfaces by neutralizing bacteria and viruses and inhibiting their attachment to epithelial cells. According to Twigg [39], IgA is the predominant immunoglobulin in the upper respiratory tract, while IgG is found in smaller amounts. However, in healthy individuals, IgA was less commonly found than IgM and IgG in serum. Nonetheless, vaccination led to an increase in the number of IgA antibodies, as observed in previous studies by Zhang *et al.,* [40] and Zhang and Finn [41]. These studies demonstrated that meningococcal polysaccharide-based vaccines can induce the production of mucosal IgA and IgG antibodies in young adults and adolescents, thus highlighting the potential roles of IgA and IgG in protecting against vaccination.

Overall, the present study is a preliminary investigation that involved a limited number of samples due to the rarity of meningococcal meningitis cases in Malaysia. Further research is required to include positive samples from meningococcal cases to assess the specificity and sensitivity of the dot blot EIA assay. Despite the limitations, the study has demonstrated the potential of the dot blot EIA as a valuable tool, which could be used along with the SBA assay to evaluate vaccine efficacy. Additionally, the dot blot EIA has the potential to replace ELISA in testing the immune responses to vaccination.

The World Health Organization [24] has highlighted the controversy between the antibody titres measured by SBA and ELISA assays and their association with meningococcal disease protection. The discrepancy between the results and interpretations of these two standardised serological assays raises questions regarding their ability to provide clear and unambiguous data for making reliable decisions regarding vaccine licensing and public health recommendations. Therefore, exploring alternative serological methods, such as the dot blot EIA, may offer valuable perspectives and contribute to a better understanding of the immune response to meningococcal disease and the efficacy of vaccines. However, more comprehensive studies will be crucial to validate and establish the full potential of the dot blot EIA as a reliable method for vaccine evaluation and immune response assessment.

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