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Short communication

Synergistic Effect of Coadministration of *Haemophilus influenzae* and *Neisseria meningitidis* Vaccines on Immune Responses to Meningitis in Mouse Model

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Abstract: Meningitis is a severe disease associated with death in children under five with highest rates of infections under age of one. Vaccines for *Neisseria meningitides* and *Haemophilus influenza* are used to prevent the main causative agents of meningitis. Administration of *H. influenzae* type b (Hib) vaccine is recommended at 2, 4 and 6 months with a booster dose at 18 months. *N. meningitidis* has two commercially available vaccines, the pure polysaccharide is recommended at 24 months meanwhile the protein-conjugated vaccines at 12 months. We sought in this study to examine if coadministering the vaccines for the two main meningitis causing bacteria might be synergistic as a preliminary step towards the possibility of shuffling immunization schedule. So, we coadministered Hib vaccine with commercially available vaccines either quadrate (ACWY) polysaccharide meningococcal (Men) or conjugated meningococcal (Nim) vaccines in Balb/C mice (n = 6/group) and compared to each vaccine administered separately and controls. Thirty-five days post immunization, we measured specific antibodies titers. Hib vaccine increased Men antibody titers significantly for serotypes Y and W. When Hib vaccine was coadministrated with Nim, antibody titer for Y, W and A significantly increased. For serotype C, there was no significant difference in antibody titers among immunized groups. As for effect of meningococcal vaccines on Hib, Men significantly increased Hib antibody titers while Nim had no effect. Collectively, our data suggested that coadministration of Hib and Men or Nim vaccines was safe and had synergistic effect on immune responses elicited to both vaccines. Further studies are needed before immunization schedule modifications. Such immunization schedule recommendation should provide better protection against this life-threatening disease in young children.

Keywords: *Haemophilus influenza*; *Neisseria meningitidis*; meningitis; vaccine efficacy evaluation; immunization schedule.

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1. Introduction

Meningitis is an important cause of morbidity and mortality for children less than 5 years especially in developing countries [1,2]. Meningitis can be caused by viral or bacterial agents [3,4]. Different types of bacteria can cause meningitis such as *Streptococcus pneumonia, Haemophilus influenza* and *Neisseria meningitides* [5,6].

N. meningitidis have been divided into 13 serogroups A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z. Serogroups A, B, C, W135 and Y cause more than 90 % of the invasive disease worldwide [5,7]. It is frequently associated with severe neurological sequelae even with use of antibiotics [8–10]. The epidemiology of bacterial meningitis varies substantially by capsular group. Group A meningococcal capsule causes large epidemics which represent the most serious public health issue caused by *N. meningitidis* [5,9–11]. The percentage of group C isolates responsible for endemic meningococcal disease varies by country. Until year 2000 capsular group W135 was a relatively uncommon cause of invasive disease worldwide [12,13]. In 2000–2001, a large number of pilgrims from the meningitis belt of Africa gathered at the Hajj. Given the high incidence of A and W135 strains meningitis in "the meningitis belt" of sub-Saharan Africa, Hajj pilgrimage 2000-2001 years had outbreaks of groupW135 disease reported in Saudi Arabia [14]. Capsular group X and W135 strains also cause substantial rates of disease in Africa [15]. Capsular group Y strains cause all of the major meningococcal clinical syndromes [16].

Another organism responsible for meningitis disease especially in children less than 5 years old is *H. influenzae* [17]. There are six identifiable types of *H. influenza,* a through f and other non-identifiable types called nontypeable. The *H. influenza* that most people are familiar with is *H. influenza* type b, or Hib [18].

Both microorganisms spread person-to-person by direct contact or through respiratory droplets [5, 6]. *H. influenzae* and *N. meningitidis* replicate in the blood stream resulting in bacteremia within hours after infection [19]. Several vaccines are available for both agents. Vaccines against *N. meningitidis* are in form of polysaccharide vaccine (Men), recommended from 24 months and conjugate vaccine (Nim) from 12 months [20]. *H. influenzae* type b polysaccharide-protein conjugate vaccine (Hib) is compulsory at 2, 4, and 6 months with a booster dose at 18 months [20]. Since meningitis causes morbidity among children under five especially in developing countries, coadministering these two vaccines might prove useful if given around 12 months or 24 months of age. Thus preventing death among children under five. Therefore, in the current study, we aimed to examine the possible synergistic effect of coadministration of meningococcal vaccines and *H. influenzae* vaccines on immune responses in an animal model. In addition, the safety of vaccine coadministration was assessed.

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2. Materials and Methods

2.1. Mice

Female Balb/C mice (6 weeks-old, ≥15 g of weight) were purchased from the animal facility of Theodor Bilharz Research Institute (Giza, Egypt). Animals were housed in animal house of National Organization for Research and control of biological (NORCB) (Agouza, Giza, Egypt).Mice were housed in a controlled environment: 22±3°C, 55±5% humidity, and 12 hours light/dark cycles. Animals were provided with a standard laboratory diet and water ad libitum. The mice were adapted to their environment for at least one week before starting the experiment. Research was conducted in compliance with the principles and recommendations of the Guide for the Care and Use of Laboratory Animals [21,22]. All animal studies were approved by the research ethics committee at Faculty of Pharmacy, Cairo University, protocol# MI1818.

2.2. Vaccines

Three licensed vaccines were used in this study:

a- Act-Hib® (Sanofi-Pasteur SA, France) containing 10 µg of purified polyribosylribitol phosphate capsular polysaccharide of *H. influenzae* covalently bound to tetanus protein per 0.5 ml

b- Mencevax® (Men) (GlaxoSmithKline (GSK), United Kingdom) containing 50 µg of each *N. meningitidis* meningococcal polysaccharide serogroups A, C, W-135 and Y per 0.5ml

c- Nimenrix® (Nim) (GSK, United Kingdom), a tetravalent meningococcal polysaccharide conjugated vaccine consisting of 5µg *N. meningitidis* capsular polysaccharides A, C, W-135 and Y each coupled to tetanus toxoid as a carrier protein per 0.5ml.

2.3. Experimental design of Mice immunization

Groups of Balb/C mice (n = 6 mice /group) were immunized intra-muscularly at days 0, 14 and 28 with 0.1ml dose containing 5µg of the following vaccines:

Group1: Mencevax®, (*N. meningitides* pure polysaccharide vaccine)
Group 2: Nimenrix®, (*N. meningitides* polysaccharide protein conjugated vaccine)
Group 3: Act-hib®, (*H. influenzae* vaccine)
Group 4: Act-hib® coadministrated with Mencevax®,
Group 5: Act-hib® coadministrated with Nimenrix® and
Group6: saline as negative control group.

At end of the experiment, day 35 from first immunization, blood was collected and sera were separated by centrifugation for 5 minutes at 2500 rpm. Separated sera were stored at -20°C until testing for antibodies responses.

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2.4. Assessment of vaccine safety

Different groups of immunized mice were observed for any signs of ill health or mortality. Ill health assessment included daily checks for swelling or redness of injection-site, animal lethargy or decreased grooming. This was done as an assessment of the safety of vaccine coadministration following European pharmacopoeia 2.6.9: testing for toxicity of immunosera and vaccines for human use section [23].

2.5.Analysis of specific antibody responses by ELISA

Sera specific antibody (IgG) levels elicited in mice groups against *H. influenza* and meningococcal ACWY antibodies levels were evaluated using ELISA.

In *H. influenza* ELISA, plates were coated with 1μg/ml purified polyribosylribitol phosphate (PRP) of Hib (National Institute for Biological Standards and Control (NIBSC), London, UK) in 1% 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Thermo Fisher Scientific, Massachusetts, USA).

For assaying meningococcal ACWY antibody (IgG) levels, plates were activated by adding 100 μl of 2 μg/ml poly L-lysine (Sigma-Aldrich, USA) in carbonate buffer at 37°C for 2 hours following previously detailed method[24]. After washing, plates were coated with 100μg/ml polysaccharide (NIBSC, London, UK) in PBS and incubated at 2-8°C overnight.

For both *H. influenza* and meningococcal antibodies titers were measured using anti-mouse IgG peroxidase (Sigma-Aldrich, USA) and chromogenic substrate solution (TMB)(Sigma-Aldrich, USA). Absorbance was measured at 450/620 nm by ELISA plate reader. Detailed methodology of ELISA explained in appendix A.

2.6. Statistical analysis

Data represented as mean absorbance ± standard error of the mean (SEM) of three experiments. All data were processed statistically and graphed using Graph Prism 6.05 software (California, USA). We used unpaired student t-test for comparison between single and combined vaccine groups. P values < 0.05 were considered significant. Statistically significant differences between groups were defined as * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$.

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3. Results

3.1- Safety assessment

Men, Nim, Act-hib are registered vaccines with high safety margins when used alone or when coadministered with other vaccines [2,3,4]. In our study we assessed the general safety of pairs of coadministered vaccines. We observed immunized mice for any signs of illness or mice mortality for 7 days after each injection[1]. We found that animals immunized with any combination of the two vaccines showed no signs of ill health nor death.

3.2- Evaluation of antibody levels to meningococcal ACWY when coadministered with Hib vaccine

In general coadministered hib increased the antibody level of some serotypes of Men vaccine and had no interference in other serotypes. When hib was coadministered with Men, antibody titer of meningococcal serotype Y increased coadministrated groups compared to the mencevax group ($P<0.001$) (Figure 1-A). Similarly, the antibody titer of serotype W in mencevax increased significantly after the coadministrated with haemophilus vaccine ($P <0.01$) (Figure 1-B). Hib had no interference as no significant effect on the antibody response for serotype A nor C after coadministration (Act-hib-Men group) (Figure 1-C and 1-D).

When Hib vaccine is coadministered with Nim different serotypes were increased. For mice immunized with Nim compared to mice immunized with (Act-Hib with Nimenrix); antibody titer of meningococcal serotype Y increased in coadministrated groups($P< 0.0001$) (Figure 1-A). Similarly, the antibody titer of serotype W in Nim increased significantly after the coadministrated with haemophilus vaccine ($P=0.03$) (Figure 1-B). Hib increase the effect on the antibody response for serotype A after coadministration (Act-hib-Nim group) ($P=0.04$) (Figure 1-C). For meningococcal serotype C, there was no significant difference in Nim compared to the coadministrated groups (Figure 1-D).

3.3- Evaluation of antibody levels to *H. influenza* when coadministered with Men or Nim vaccines

The antibody against *H. influenza* in Act-hib group was compared to Act-hib with mencevax group we found there was a significant effect on the antibody response after coadministration ($P=0.003$) (Figure 2). While in case of the Nimenrix vaccine had no effect on antibody titer of *H. influenza* without negative effects on antibody responses after the coadministration in Act-hib group compared to Act-Hib with Nimenrix group (Figure 2).

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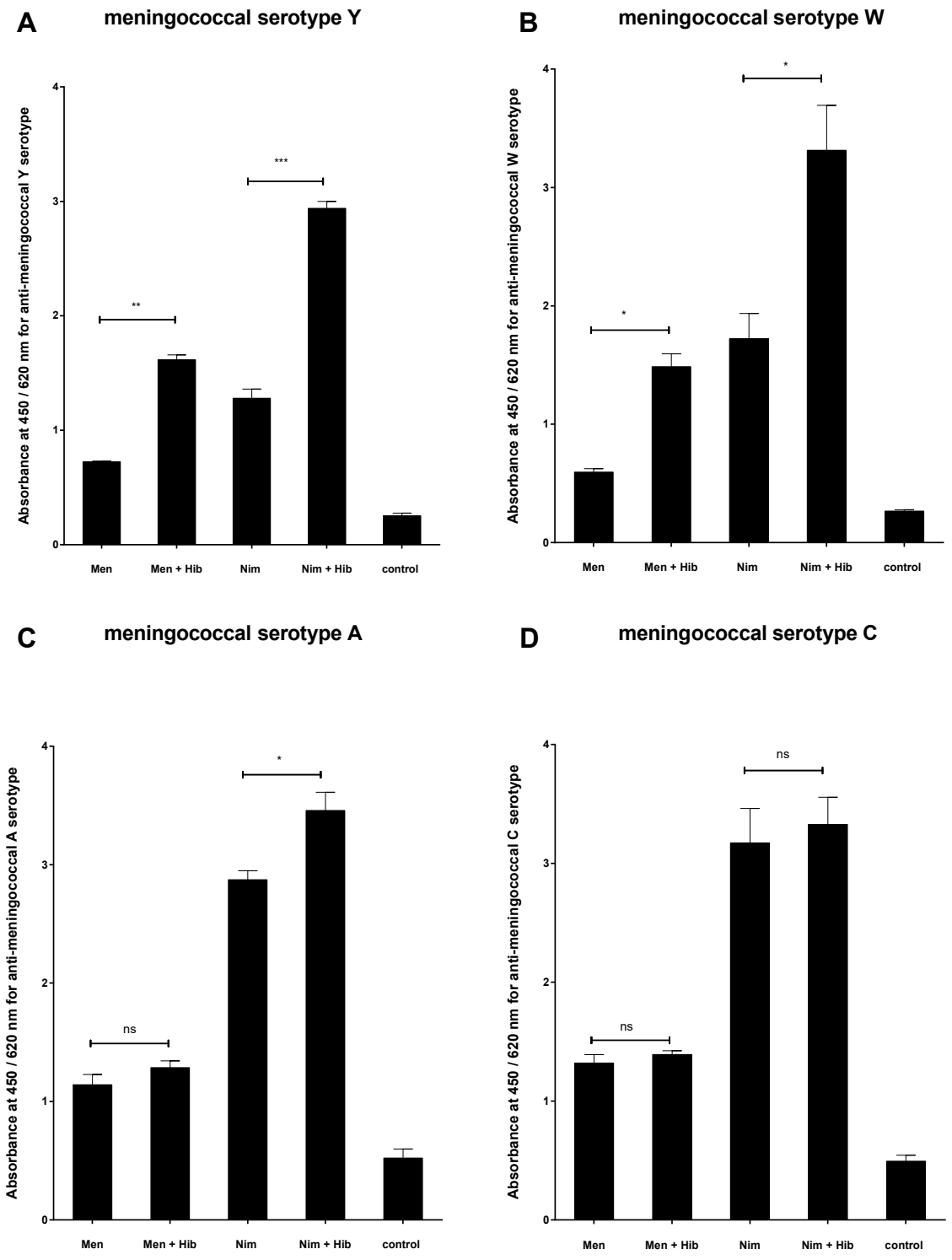

Figure 1. Evaluation of antibody response measured as absorbance values for meningococcal serogroups ACWY after vaccine coadministration. Balb /C mice (n=6 per group per experiment) were immunized intramuscularly with *N. meningitidis* polysaccharide vaccine mencevax (Men), or N. meningitidis conjugated vaccine nimenrix (Nim), or with *H. influenza* type B vaccine (act-hib) with either Men or Nim on days 0, 14, and 28. The antibody titer was measured on day 35 for mice immunized. A control group was injected with saline at each time point. Each bar represent mean ± standard error of the mean (SEM) of three experiments. Statistical analysis was performed using unpaired student t-test for comparison between single and combined vaccine groups.

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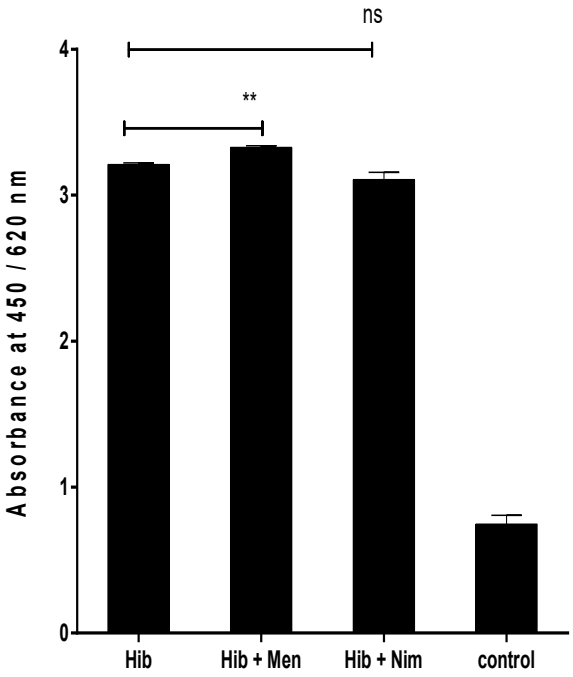

Figure 2. Evaluation of antibody response for *H. influenza* (hib) measured as absorbance values after vaccine coadministration. Balb /C mice (n=6 per group per experiment) were immunized intramuscularly with *H. influenza* type B vaccine (act-hib) at days 0, 14, 28. and the antibody titer was compared to the coadministration with *N. meningitidis* polysaccharide vaccine mencevax (Men), or N. meningitidis conjugated vaccine nimenrix (Nim). A control group was given saline at the same timing. Each bar represent means ± standard error of the mean (SEM) of three experiments. Statistical analysis was performed using unpaired student t-test for comparison between single and coadministered vaccine groups.

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4. Discussion

Several epidemiological studies on meningitis have been conducted in Egypt since 1960s reviewed in [25]. These studies showed that the highest percentages of cases (39%) with meningitis due to H. influenzae were observed 1998 – 2000. Meanwhile, during 1971–1975 period, about 56% of the cases of meningitis were due to *N. meningitidis* infection [25]. In 2004, *H. influenzae* was placed as the second cause of bacterial meningitis and *N. meningitides* as the third cause [25,26]. UNICEF and WHO/EMRO along with Egyptian ministry of health and population (MOHP) aimed to incorporate many vaccines in routine immunization schedule such as Hib-influenza, rota virus, and Penta-valant vaccines. Therefore, UNICEF has been supporting MOHP to obtain DTP and Penta-valent vaccines [27].

WHO assessed that about 92% of population in developed countries was vaccinated against *H. influenzae* while only 42% in developing countries [28]. Africa and Southeast Asia had lowest estimates using Hib vaccine. Meanwhile, vaccination against meningococcus is a must in the entry to Saudi Arabia especially for people travel to the Hajj and Umrah [12].

Conjugation of vaccine antigen with carrier protein such as tetanus toxoid, non-toxic mutant of diphtheria toxin or diphtheria toxoid alters the immune response. Converting immune responses to polysaccharide from T-cell independent to T-cell dependent thus produce long term immunity [29]. Children under one year old have the highest risk to meningitis; antibody persistence for meningococcal conjugate to tetanus toxoid carrier protein shows higher rates than any others proteins [30].

Pure polysaccharides as meningitis (men) give poor immune response as it provides short term protection [29]. It is a T-cell independent, it stimulates B cells to synthesize antibodies as IgM with IgG, they fail to induce sufficient immunological memory and considered ineffective for children less than two years [29]. Nimenrix had been approved in Europe for one-year-old children. It is a quadri-valent meningococcal conjugate ACWY. Clinical trials showed that Nim was equivalent to Men in immune response against all four serogroups after one month of vaccination with added advantage of producing immune response for 7-42 months after vaccination [31].

Our study showed no interference of Nimenrix vaccine on antibody titer of *H. influenza* after the coadministration in Act-hib group compared to Act-Hib with Nimenrix group (Figure 2). These results were confirmed in the previous studies as there was no interference in the immune response when *H. influenzae* type b vaccine is concomitantly administered with diphtheria-tetanus acellular pertussis-hepatitis B-inactivated poliovirus vaccine [32].

Vaccine coadministration is sometimes recommended for increasing efficacy and reducing number of vaccines given to individuals. A previous study on meningococcal conjugated vaccine (ACWY-TT); mencevax was compared against the

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coadministration of ACWY-TT with seasonal influenza (Fluarix) [32]. This study showed that there were no significant differences between immunized groups [32]. Several other studies showed that Nimenrix can be safely and efficiently coadministered with many vaccines such as diphtheria, tetanus, acellular pertussis, hepatitis B, inactivated polio virus and *H. influenzae* type b conjugate vaccine. These are (DTaP-IPV-HBV/Hib, Infanrix- hexa), Priorix-Tetra (measles, mumps, rubella, varicella vaccine) and 10-valent pneumococcal conjugate vaccine (Synflorix) [33,34].

5. Conclusions

We tested coadministration of the commercial available quadri-valent meningococcal ACWY vaccine either polysaccharide or conjugated with *H. influenza* vaccine. This coadministration increased the immune response for both meningococcal and *H. influenzae* vaccines. Based on our results, we recommend that *H. influenzae* type B vaccine (Act-hib) to be taken with conjugated meningococcal ACWY (Nimenrix) at 18 months. Alternatively, *H. influenzae* type B vaccine (Act-hib) can be given as a booster dose with polysaccharide meningococcal ACWY (Mencevax) at 24 months.

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Conflicts of Interest: The authors declare no conflict of interest. There were no sponsors funding this research.

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Appendix A

Materials and Methods: Assessment of the antibody responses

2.5. Analysis of specific antibody responses by ELISA

Antibody levels to H. influenza were evaluated by enzyme-linked immunosorbent assay (ELISA) in sera collected from groups 3, 4 and 5. The 6thgroup was used as negative control. ELISA plates were coated with 100 µl/well of diluted purified Polyribosyl Ribitol Phosphate (PRP)/ (NIBSC) (1µg/ml) in 1% 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide EDC and incubated at 2-8°C for overnight. Next day, plates were washed three times with PBST washing solution (PBS+ 0.05% (v/v) Tween -20) then blocked by 300µl/well of blocking solution (1%BSA in PBST) and incubated at 37°C for 2hr. At the end of incubation period, Plates were washed three times with washing solution. Sera of each group were 2 fold diluted in PBST assay diluent and dispensed as 100µl/well in their reciprocal wells then incubated at 37°C for 2hr. After that plates were washed then anti-Mouse IgG peroxidase (Sigma-Aldrich, USA) diluted 1:7000 was added as 100 µl\well to all wells and incubated at 37°C for 1.5 hour. After incubation, wash step was repeated then chromogenic substrate solution (TMB, Sigma) was added as 100 µl\well and plates were kept at room temperature in dark place for 20-30 minutes. Finally, Stopping solution (1N H2SO4) was added as 50 µl\well and absorbance was measured at 450/620 nm by ELISA reader. The test was conducted three times and results were represented as mean absorbance

Antibody levels to Meningococcal ACWY were evaluated by enzyme-linked immunosorbent assay (ELISA) in sera collected from groups 1,2,4 and 5. The 6thgroup was used as negative control.96 wells ELISA plates were activated by adding 100 µl of diluted poly L lysine (purchased from Sigma-Aldrich, USA) (2 µg/ml) in carbonate buffer at 37°C for 2 hours [9]. then plates were washed three times with distill water. Plates were coated with 100 µl/well of diluted polysaccharide (NIBSC) (100µg/ml) in PBS and incubated at 2-8°C for overnight. Next day, plates were washed three times with PBST washing solution (PBS+ 0.05% (v/v) Tween -20) then blocked by 300µl/well of blocking solution (1%BSA in PBST) and incubated at 37°C for 2hr. At the end of incubation period, Plates were washed three times with washing solution. Sera of each group were 2 fold diluted in PBST/1%BSA and dispensed as 100µl/well in their reciprocal wells then incubated at 37°C for 2hr. After that plates were washed then anti-Mouse IgG peroxidase (Sigma-Aldrich, USA) diluted 1:7000 was added as 100 µl\well to all wells and incubated at 37°C for 1.5 hour. After incubation, wash step was repeated then chromogenic substrate solution (TMB, Sigma) was added as 100 µl\well and plates were kept at room temperature in dark place for 20-30 minutes. Finally, Stopping solution (1N H2SO4) was added as 50 µl\well and absorbance was measured at 450/620 nm using ELISA reader. The test was conducted three times and results were represented as mean absorbance.

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