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Immunogenic evaluation of LptD + LtgC as a bivalent vaccine candidate against *Neisseria gonorrhoeae*

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Abstract

Background *Neisseria gonorrhoeae* is an escalating global health threat due to increasing antimicrobial resistance. The emergence of multidrug-resistant (MDR) strains necessitates alternative prevention strategies. This study focused on the development of a bivalent vaccine formulation to address this challenge. Lipopolysaccharide transport protein D (LptD) and lytic transglycosylase C (LtgC) as two promising immunogenic targets were considered in this study.

Methods The *ltgC* and *lptD* genes of *N. gonorrhoeae* ATCC 19424 were amplified, then cloned into the pET-28a (+) vector, expressed in *Escherichia coli* BL21 (DE3), and purified using Ni-NTA affinity chromatography. Antigen-specific total IgG levels in serum of patients with gonorrhea were assessed using enzyme-linked immunosorbent assay (ELISA). Proteins were formulated with monophosphoryl lipid A (MPLA) adjuvant in three groups: LptD, LtgC, and a bivalent LptD + LtgC. One additional group received LptD with liposomal MPLA, along with control groups. Vaccine formulations were administered to BALB/c mice in three doses at two-week intervals. Total IgG, IgG1, IgG2a, and IgA levels in sera and vaginal samples were measured using ELISA. Moreover, serum bactericidal (SBA) and opsonophagocytic (OPA) assays were conducted.

Results The total IgG levels against both proteins were considerably higher in the patients' sera compared to healthy individuals. All vaccine formulations significantly increased total IgG levels in animal model. The LptD + liposomal MPLA group exhibited the highest specific IgG level, whereas the bivalent formulation group exhibited the highest long-term IgG level until the day 112, which also yielded the strongest total IgG response in the whole-cell ELISA. The IgG2a/ IgG1 ratio was greater than 1 in all vaccine regimens, indicating a Th1-polarized response. The LptD + liposomal MPLA formulation elicited the highest serum IgA levels, followed by the LptD + LtgC combination. In addition, the bivalent formulation achieved the highest SBA and OPA titers.

Conclusion This study successfully developed and evaluated a recombinant bivalent vaccine against *N. gonorrhoeae*. This formulation exhibited the most potent immunogenicity, as evidenced by higher antibody levels and SBA and OPA titers than single-antigen formulations. The Th1-polarized immune response further highlights the vaccine's

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potential to elicit a protective immune profile. These findings suggest that this multi-antigen formulation can be a promising vaccine candidate against gonorrhea. However, more investigations are required to confirm the vaccine efficacy.

Keywords *Neisseria gonorrhoeae*, Lipopolysaccharide transport protein D (LptD), Lytic transglycosylase C (LtgC), Recombinant protein, Monophosphoryl lipid A (MPLA)

Introduction

Neisseria gonorrhoeae is the leading cause of gonorrhea, with approximately 87 million new cases reported annually worldwide [1]. The global challenge posed by *N. gonorrhoeae* has become increasingly complex due to the rise of antimicrobial resistance (AMR) and its spread to conventional anti-gonococcal antibiotics [2]. Although this issue is not novel, the swift surge in resistance to some of the few remaining effective mono-therapeutic options, particularly broad-spectrum third-generation cephalosporins such as ceftriaxone, has raised significant concerns worldwide. This trend is alarming as it limits the number of available treatment options [2, 3].

Multidrug-resistant (MDR) *N. gonorrhoeae*, resistant to nearly all treatment options including penicillin, tetracyclines, quinolones, macrolides, as well as last-line therapeutic cephalosporins, are colloquially referred to as "super gonorrhoea" [4]. In 2016, in response to this escalating issue, the World Health Organization (WHO) revised its recommendations for empiric treatment. In regions where local AMR surveillance data are unavailable to guide national treatment guidelines, WHO advocates for dual antibiotic therapy combining ceftriaxone and azithromycin. This strategic approach aims to address the challenges of increasing AMR and preserve effective treatment options despite a diminishing arsenal of antibiotics [5].

In December 2020, due to increasing resistance of *N. gonorrhoeae* strains to azithromycin, the Centers for Disease Control and Prevention (CDC) removed azithromycin from their treatment regimen. Instead, ceftriaxone monotherapy, a single intramuscular injection of 500 mg ceftriaxone, is the current recommended treatment for *N. gonorrhoeae*, as per the guidelines provided by the CDC [6, 7]. However, other studies have reported the emergence of ceftriaxone-resistant *N. gonorrhoeae* from different geographical regions across the world, including Argentina [8], France [9], Austria [10], and the United Kingdom [11].

Several attempts have been made to prevent and control the spread of *N. gonorrhoeae*. An efficient strategy to reduce the transmission of gonorrhea is the promotion of safe sexual practices, such as consistent and correct condom use [12]. Nevertheless, this approach relies on user compliance, which introduces potential inconsistencies [2]. Another preventive approach involves the timely identification and treatment of infected individuals, along with their partners, through regular screening programs. While this method facilitates early intervention, challenges arise from issues related to the asymptomatic nature of infection and associated stigma, potentially impeding widespread participation [13].

A more recent and promising avenue of exploration for preventing *N. gonorrhoeae* is vaccination. The development of a vaccine holds promise as a proactive and targeted approach to combat this pathogen. Several ongoing studies are investigating potential vaccine candidates, aiming to elicit protective immune responses against *N. gonorrhoeae* [2]. Vaccination not only holds the potential to confer immunity at the individual level but also contributes to community-level protection through herd immunity [14]. This approach offers distinct advantages over traditional strategies because it addresses the root cause of the infection and can provide sustained protection with minimal reliance on individual behavior. While challenges persist, such as identifying suitable antigens and navigating the complex biology of the pathogen, the pursuit of gonorrhea vaccines represents a pioneering and transformative endeavor in the realm of sexually transmitted infection prevention.

A diverse array of vaccine platforms is available, encompassing inactivated or live attenuated vaccines, subunit, recombinant, or conjugate vaccines, viral vector vaccines, and nucleic acid vaccines [15]. Recombinant protein vaccines have distinct advantages over other vaccine platforms. These vaccines offer enhanced safety with a lower risk of disease induction and greater stability compared to live vaccines, simplifying storage and distribution. Additionally, these vaccines use specific pathogen components to induce a targeted immune response [16, 17]. Their adaptability and cost-effectiveness are underscored by their capacity for rapid development and modification, which is particularly pertinent for addressing emerging pathogens. This multifaceted set of properties positions recombinant protein vaccines as a promising option among the diverse vaccination strategies [18].

Numerous investigations have sought to identify potential recombinant vaccine candidates against *N. gonorrhoeae*, yielding valuable insights. However, a significant challenge in gonorrhea vaccine research is identifying targets with high conservation and prevalence among circulating *N. gonorrhoeae* strains. This is due to substantial variations in the surface proteins of this microorganism. To fill this gap, a thorough immunoinformatic

analysis was conducted on *N. gonorrhoeae* strains from all over the world. This effort has resulted in the identification of surface-exposed antigens with desirable characteristics [19]. Consequently, the current study focused on two selected antigens for immunization against *N. gonorrhoeae*.

Lipopolysaccharide Transport Protein D (LptD, WP_003689900.1) was selected as an essential putative vaccine candidate against *N. gonorrhoeae* in this study. It serves as an essential component of the lipopolysaccharide transport pathway in Gram-negative bacteria. LptD, along with LptE, forms the LptD/E translocon complex, which plays a crucial role in transporting LPS from the periplasmic side to the outer leaflet of the outer membrane, contributing to the assembly and integrity of the bacterial outer membrane [20, 21]. Besides immunoinformatics evidence, previous proteomics investigations have demonstrated the bactericidal effects of anti-LptD antibodies against *N. gonorrhoeae* [22].

Lytic transglycosylase C (LtgC, WP_050155395.1) is the homolog of MltA in *Escherichia coli*. Lytic transglycosylase enzymes produce peptidoglycan monomers that induce the death of ciliated cells in fallopian tubes. LtgC mutants lead to a defect in cell separation, heightened autolysis, a lack of release of GlcNAc-anhydro-MurNAc disaccharides, and lower production of lytic PG monomers [23, 24]. LtgC was previously introduced as a promising vaccine candidate with desirable immunogenic properties. These two proteins are conserved among circulating *N. gonorrhoeae* strains. They exhibited high antigenicity with identifiable B- and T-cell epitopes [19].

On the other hand, the Th1 immune response is critical for protection against *N. gonorrhoeae*. Th1 response enhances the bactericidal activity of antibodies [25–27]. Moreover, the use of monophosphoryl lipid A (MPLA) as an adjuvant preferentially induces Th1 responses, leading to increased production of IgG2a/b antibodies that exhibit higher complement-dependent bactericidal activity against *N. gonorrhoeae* [28].

Thus, given the compelling evidence regarding the immunogenicity of these two proteins, we formulated a combination of LptD + LtgC and MPLA adjuvant as a potent vaccine candidate against *N. gonorrhoeae*. We hope that the results of the current study would pave the way for a new era in gonorrhea prevention approaches.

Materials and methods

Ethical statement

This research project received ethical approval from the Research Ethics Committee of the Tehran University of Medical Sciences (approval ID: IR.TUMS.SPH.REC.1403.073). The study was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki and other relevant international guidelines.

Bacterial strains

N. gonorrhoeae ATCC 19424 was used to amplify the *ltgC* and *lptD* genes. In addition, *E. coli* DH5α, and *E. coli* BL21 (DE3) strains were provided by microbial collection of the Pasteur Institute of Iran and selected as cloning and expression hosts. The pET-28a (+) (Promega Co., USA) plasmid was used to clone the genomic sequences of the targeted genes.

Cloning, expression, and purification of recombinant proteins

The genomic content of *N. gonorrhoeae* ATCC 19424 was extracted using the FAVORGEN DNA extraction kit. *Nco*I and *Xho*I restriction sites were added at the 5' end of the *ltgC* and *lptD* primers. The genomic sequences of the LtgC and LptD proteins were amplified through PCR using the AccuPower Pfu PCR PreMix enzyme. The PCR program consisted of a pre-heating step at 95˚C for 7 min, followed by 30 cycles of 30 s at 95˚C, 90 s at 58˚C, 60 s at 72˚C, and a final extension step at 72˚C for 7 min. The PCR product lengths of *ltgC* and *lptD* were 1302 and 2341 bp, respectively. The purified PCR amplicons were double-digested with *Xho*I and *Nco*I restriction enzymes (Thermo Scientific Inc., USA) and ligated to the digested pET-28a (+) expression vector using T4 DNA ligase (Thermo Fisher Scientific Inc., USA). The recombinant vectors were transformed into *E. coli* DH5α competent cells via heat shock procedure [29]. The recombinant vectors were sequenced using an ABI 3730xl DNA Analyzer (Life Technologies). Sequence similarity was assessed using the BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and ORFs were identified using the ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

The recombinant vectors were then subcloned into *E. coli* BL21 (DE3) (Novagen, WI, USA) competent cells. This strain was employed as expression host for overproduction of the recombinant proteins. An overnight culture of *E. coli* BL21 (DE3) carrying recombinant vectors was used to inoculate 1 L of LB medium supplemented with final concentration of 50 µg/ml kanamycin. After the culture reached the $OD_{600} = 0.5$, Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific Inc., USA) at a final concentration of 0.5 mM was added to induce protein overexpression. Four hours later, the bacterial cells were harvested by centrifugation at 5000 rpm for 20 min at 4 ˚C.

Cell pellets were resuspended in SDS-PAGE loading buffer and separated on 12% separating gel, followed by staining with 0.25% Coomassie Brilliant Blue (Sigma Chemical, St. Louis, MO, USA). Subsequently, the recombinant proteins were transferred onto a PVDF membrane for Western blot analysis using C-terminal specific 6xHis epitope antibody (Invitrogen Life Technologies, USA) at a dilution of 1:2000 [30].

Both proteins were purified under denaturing condition. Cell pellets were resuspended in 10 ml denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 8.0) and sonicated on ice for 1 min at 80% amplitude with cycle = 0.5, and incubated for 30 min. Then the suspension was centrifuged at 5000 rpm for 20 min. The supernatant was collected and passed through a 0.45 µl filter. The suspension was mixed with 0.5 ml Ni-NTA agarose affinity resin (Thermo Fisher Scientific, USA) and incubated on ice for 30 min. The resin and suspension mixture were then added to a column. The column was washed three times with 4 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, 0.1% Triton X-114, pH 6.3). The purified proteins were then eluted by adding 4 ml of elution buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 4.5) [31]. Finally, the recombinant proteins were dialyzed using phosphate buffered saline (PBS).

Human antibody ELISA

To evaluate the presence of specific antibodies against vaccine candidates in the blood of patients with gonorrhea, blood samples were obtained from patients diagnosed with gonorrhea and attending Tehran University Hospitals. The diagnosis of gonorrhea was confirmed through endocervical swabs collected from patients with genitourinary symptoms. The presence of *N. gonorrhoeae* in genital specimens was then determined using NAAT. Twelve patients with gonorrhea and three healthy volunteers were also included as the patient and control groups, respectively. Blood samples were collected within two weeks after the identification of *N. gonorrhoeae*, and sera were isolated by centrifugation at 2000 g for 10 min. Serum samples were aliquoted and stored at -80 °C.

Human antibody ELISA was performed by coating 100 µL/well of purified LptD and LtgC proteins with a concentration of 4 µg/mL or formalin-fixed *N. gonorrhoeae* ATCC 19424 with 0.5 McFarland concentration (OD_{600} ~ 0.08–0.13) in phosphate-buffered saline (PBS). Plates were incubated at 4 °C overnight. Following incubation, the plates were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with 1% bovine serum albumin (BSA) in PBST (100 µL/well) at room temperature for 1 h.

Subsequently, 100 µL of a 1:100 dilution of human serum was added and incubated at room temperature for 1 h. After another washing step with PBST, HRP-conjugated goat anti-human IgG antibody (Sigma-Aldrich, USA) at a dilution of 1:10,000 was added and incubated at room temperature for 1 h. The plates were washed with PBST three times before adding 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate to each well. After 15 min, 50 µL of stop solution was added. The absorbance of the wells was measured at 450 nm, with a blank control included for each antigen without serum addition. All samples were tested in duplicate route.

Preparation of liposomal MPLA adjuvant

Liposomes were developed according to the protocol previously reported [32, 33]. To create a lipid shell, a 7:2 molar ratio of L-α-phosphatidylcholine and cholesterol (totaling 20 mg) was dissolved in 70% (vol/vol) nitric acid in a round-bottomed flask. Specifically, L-α-phosphatidylcholine (87.5 µL of a 100 mg/mL stock solution) was added to the flask, followed by 125 µL of a 10 mg/mL cholesterol solution in chloroform. The total volume was then adjusted to 3 mL using chloroform. Chloroform was evaporated at 25 °C to form a dried lipid shell. A separate solution containing 50 mg of octyl-β-glucopyranoside in 10 mM HEPES buffer (pH 7.2) was prepared and incubated at room temperature for 3 h. The adjuvant mono phosphoryl lipid A (MPLA, 500 µg from *Salmonella enterica* serotype Minnesota; Sigma-Aldrich) was dissolved in chloroform and combined with the L-α-phosphatidylcholine–cholesterol mixture. The dried lipid shell was then prepared as described. Control liposomes were prepared without the addition of MPLA. The dried lipid shells were then resuspended by manual agitation in either the MLPA-octyl-β-glucopyranoside solution or the control solution, and the mixtures were incubated at room temperature for 1 h. The liposome preparations were dialyzed against PBS at 4 °C for 72 h, and the buffer was replaced twice daily to remove the detergent. Subsequently, the liposome suspensions were transferred to Bijoux tubes, and small unilamellar liposomes were produced by sonication using an MSE Soniprep 150 probe sonicator (15–20 cycles of 30 s each on ice; amplitude of 10–15 µm). The final volume of each liposome preparation was recorded, and the samples were stored at -20 °C.

Vaccine formulation and mouse immunization

A total of thirty-five 6-8-week-old BALB/c female mice were provided from the Pasteur Institute of Iran and randomly divided into seven groups of five mice. MPLA was used as adjuvant. LtgC and LptD recombinant proteins were used as vaccine candidates in three formulations: LtgC + MPLA, LptD + MPLA, and the LtgC + LptD combination with MPLA. Moreover, one group received LptD + liposomal MPLA, and three control groups were also included as follows: MPLA, liposomal MPLA, and free liposomes. A final concentration of 30 µl of each protein was used in all formulations. Immunization was performed using three injections on days 0, 14, and 28. The initial two doses were administered via subcutaneous injection, and the third dose was delivered intraperitoneally. Blood samples were obtained prior to each immunization, and sera were isolated.

Blood samples were collected prior to each immunization (on days 0, 14, and 28) and on days 42, 56, and 112 post-immunization. Vaginal wash was collected from three mice in each group on day 42 and two mice on day 112. Following euthanasia, the mice were securely held by the tail base to expose the vaginal opening, then vaginal lavage was collected by introducing 100 µl of sterile PBS with repeated aspirating. The lavage fluid was collected into a microcentrifuge tube [34]. Blood and vaginal wash specimens were collected and subsequently stored at − 80 °C until further analysis.

Whole-cell ELISA

To assess the reactivity of mouse antisera against both linear and conformational B-cell epitopes of the native LtgC and LptD proteins, a whole-cell ELISA was conducted. In this assay, *N. gonorrhoeae* ATCC 19424 was grown in GC broth medium at 37 °C. Each well of a 96-well microtiter plate was coated with 100 µl of bacterial suspension in PBS (0.5 McFarland, $OD_{600} \sim 0.08$–0.13) and incubated overnight at 4 °C. Following this, the wells were washed three times with PBST. To block non-specific binding, 100 µl of PBST containing 1% BSA was added to each well, and the plate was incubated at 37 °C for 1 h. After washing the wells three times with PBST, 100 µl of diluted mice sera (day 42, 1:100 dilution) were added to each well, and the plate was incubated at 37 °C for 1 h. The wells were then washed again, and 100 µl of HRP-conjugated anti-mouse IgG (1:10,000 dilution) was added to each well. The plate was incubated for another hour at 37 °C. After a final washing step, 100 µl of TMB substrate was added to each well, and the plate was kept in the dark at room temperature for 15 min. The reaction was terminated by adding 100 µl of 0.5 M H_2SO_4 to each well and the absorbance was read using an ELISA plate reader (Biotek Instruments, Inc.) at 450 nm [35].

Evaluation of serum and vaginal antibody levels in mice

To assess humoral immune responses, ELISA was employed to examine total IgG, IgG isotypes (IgG1 and IgG2a) in serum, and IgA levels in vaginal wash samples. The ELISA procedure involved coating microtiter plates with 100 µL/well purified protein (4 µg/mL) and incubating overnight at 4 °C. Following a wash with PBST and blocking with 1% BSA, 100 µL/well of a 1:100 diluted serum/vaginal wash was added. After a washing step, 100 µl of 1: 10,000 diluted HRP-conjugated goat anti-mouse IgG, anti-mouse IgG1, IgG2a, and IgA (Sigma-Aldrich, USA) were subsequently added and incubated. Reactions were initiated using TMB and 0.5 M H_2SO_4 was added after 15 min. Finally, the optical density at 450 nm was measured using an ELISA plate reader.

Serum bactericidal assay

The SBA and OPA titers of immunized mouse sera were investigated according to the protocol presented by Semchenko EA et al. [36], which is described in brief as follows. Whole blood samples were collected from healthy volunteers using Fartest Serum vacuum blood collection test tubes. After the blood clotted in 15 min, the tubes were centrifuged at 2000 g for 10 min. Healthy human sera were used as a source of complement. *N. gonorrhoeae* was cultured on GC agar medium and incubated at 37 °C with 5% CO_2 for 16 h. Mouse sera from each group were pooled and heat-inactivated at 56 °C for 60 min, and serial dilutions of inactivated mouse sera were developed in 96-well plates. Then, $\sim 1 \times 10^3$ CFU of *N. gonorrhoeae* was added to each dilution and incubated in 37 °C with 5% CO_2 for 15 min. Then, normal human serum with a final concentration of 10% (v/v) was added to each well to provide the complement source. The plate was incubated at 37 °C, 5% CO_2 for 1 h, and bacterial CFUs were counted by plating out serial dilutions on GC agar medium. The bactericidal titer was defined as the lowest antibody dilution that induced > 50% killing after 1 h.

Opsonophagocytic titer

Blood samples were collected from healthy volunteers in Fartest K3EDTA test tubes. PMNs were isolated as previously described [37]. The heat-inactivated mouse sera were serially diluted in a 96-well round bottom plate. Then, $\sim 1 \times 10^3$ CFU of *N. gonorrhoeae* was added to each well, and the plate was incubated in 37 °C with 5% CO_2 for 15 min. PMNs ($\sim 1 \times 10^5$ cells) and the complement source (10% v/v normal human serum) were then added, and the plate was incubated at 37 °C with 5% CO_2 for 1 h. Bacterial survival was determined after plating serial dilutions on GC agar, and survival was calculated as a percentage relative to the no-antibody control. The OPA titer is defined as the lowest antibody dilution that induces > 50% killing after 1 h.

Statistical analyses

Statistical analyses were performed to evaluate the immunogenicity of various vaccine formulations and to compare antibody responses among experimental groups. Based on descriptive statistics, the mean values and standard error of the mean (SEM) were calculated for each group. For comparative analyses, one-way and two-way ANOVA were performed. Tukey's post hoc test and Sidak' multiple comparisons tests were also used for pairwise comparisons following significant ANOVA results. Statistical significance was determined using the following thresholds: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All statistical tests were conducted using

SPSS version 27 and GraphPad Prism 9.0.2 software to ensure accurate calculations and visualizations.

Results

Expression and purification of recombinant gonococcal vaccine candidates

According to SDS-PAGE visualization, LtgC and LptD have molecular weights of 48 kDa and 85 kDa, respectively. The 6xHis-tagged proteins were purified using Ni-NTA chromatography. Subsequent verification via western blotting demonstrated that both proteins exhibited reactivity with the HRP-conjugated Anti-6xHis tag antibody when transferred onto a PVDF membrane. Furthermore, the LAL assay indicated an undetectable level of LPS, measuring < 0.1 EU/mL in both protein solutions. The concentrations of the purified LtgC and LptD proteins were determined to be 700 µg/mL and 600 µg/mL, respectively.

Recognition of vaccine candidates by sera of gonorrhea positive patients

The LptD and LtgC proteins were previously identified as potential vaccine candidates via in silico analyses. In this step, the ability of serum of patients with gonorrhea to recognize these two proteins was investigated by ELISA. This process aims to investigate whether selected proteins induce antibodies during natural infection. The results indicated that the serum levels of specific antibodies against *N. gonorrhoeae* and vaccine candidates were significantly higher in patients than those in healthy individuals (*$p < 0.05$, and ***$p < 0.001$; Fig. 1). This suggests that during gonorrhea infection, antibodies against both proteins are induced, and these antibodies recognize LptD and LtgC recombinant proteins, supporting their potential as vaccine candidates.

Humoral immune responses

Serum levels of total IgG were evaluated on days 0, 14, 28, 42, 56, and 112. The antibody levels were statistically analyzed using One-way ANOVA. All vaccine formulations including LptD + MPLA, LtgC + MLPA, LtgC + LptD + MLPA, and LptD + Liposomal MPLA induced considerable levels of total IgG compared to control groups (MPLA, Liposomal MPLA, and free Liposome) ($p < 0.001$). Detailed information is presented in Fig. 2A.

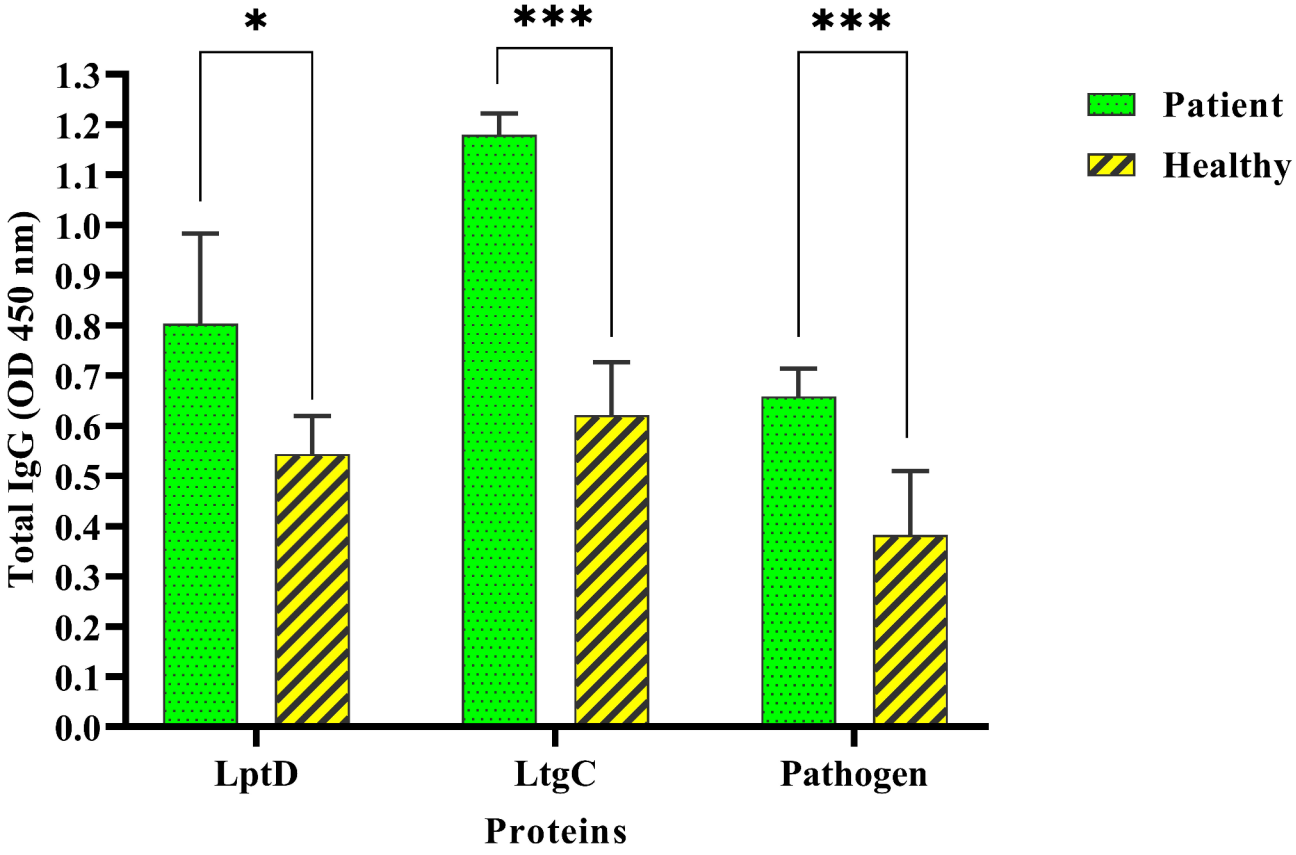

Fig. 1 Human IgG ELISA for detecting specific Antibodies against vaccine candidates. IgG levels are expressed as OD at 450 nm. Each bar represents the mean ± SEM. Patients with gonorrhea showed significantly higher IgG levels against LptD (*$p < 0.05$), LtgC (***$p < 0.001$), and the pathogen itself (***$p < 0.001$), compared with healthy controls. This suggests that both LptD and LtgC are immunogenic proteins. The elevated antibody levels in patients suggest that these antigens could be effective targets and considered as vaccine candidates

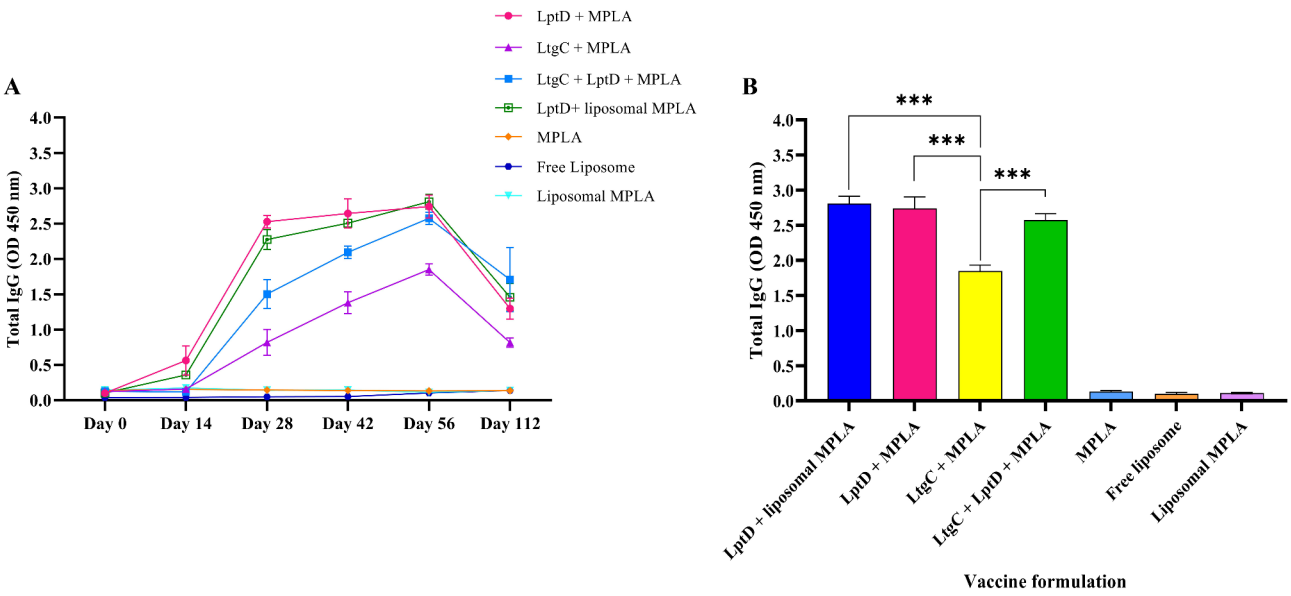

Fig. 2 Evaluation of antigen-specific total IgG. (**A**) The total IgG level of each formulation was measured on days 0, 14, 28, 42, 56, and 112. IgG levels were measured as optical density (OD) at 450 nm. Each data point represents the mean ± standard error of the mean (SEM) for each group. Compared with the other formulations, the LtgC + LptD + MPLA muti-antigen formulation showed higher total IgG levels in the long term (Day 112). (**B**) Comparative analysis of total IgG levels at Day 56 post-immunization across different formulations. The bars represent the mean $OD_{450\,nm}$ values ± SEM. Statistical significance between groups was determined using one-way ANOVA followed by Tukey's post-hoc test. Significant differences are indicated as follows: ***$p < 0.001$. The LptD + liposomal MPLA formulation showed the highest IgG level, which were significantly higher than that of the LtgC + MPLA formulation. The difference between LptD + liposomal MPLA and LptD MPLA was not significant. LtgC + LptD + MPLA combination showed higher IgG level compared to LtgC + MPLA formulation, and slightly lower level compared to LptD + MPLA which was not significant.

In all vaccine regimens, the highest serum IgG level was observed on day 56 (four weeks after the last immunization). Although all formulations induced considerable levels of specific antibodies, LptD-specific IgG was significantly higher than that of LtgC ($p < 0.001$). The LtgC + LptD combination induced a higher level of total IgG than LtgC + MPLA ($p < 0.001$), however it was slightly weaker than LptD + MPLA formulation. No significant differences were observed in total IgG levels induced by LptD + MPLA and LptD + liposomal MPLA ($p = 0.993$). To assess long-term immunity, total IgG was evaluated on day 112. LtgC + MPLA had the lowest IgG level, and the combination of LtgC + LptD + MPLA could lead to higher levels of long-term IgG on day 112 ($p < 0.001$; Fig. 2B).

Whole-cell ELISA

Whole-cell ELISA was performed to identify which formulation induces a higher level of antibody against the whole-cell *N. gonorrhoeae*. Overall, all immunization formulations induced significantly higher antibody levels compared with the control groups ($p < 0.001$). Notably, the combination of the two proteins elicited higher antibody levels than both the LptD + MPLA ($p < 0.001$) and LtgC + MPLA ($p < 0.001$) groups. Therefore, the combination of these two proteins may induce a stronger immune response. Additionally, LptD combined with the liposomal adjuvant induced higher antibody levels than LptD + MPLA ($p < 0.001$; Fig. 3).

Serum levels of IgG isotypes

Serum levels of IgG2a and IgG1 isotypes, which are the markers of Th1 and Th2 immune response, were evaluated. LptD + MPLA, LtgC + MPLA, LptD + LtgC combination, and LptD + Liposomal MPLA formulations showed higher IgG1 and IgG2a rates compared with the control groups. Moreover, all formulations produced an IgG2a/IgG1 ratio higher than 1, which suggests a Th1-mediated response. Differences of IgG1 and IgG2a levels between LptD + Liposomal MPLA and LptD + MPLA were not statistically significant. However, both of them showed significantly higher IgG2a levels compared to LtgC + MPLA ($p < 0.001$). In addition, LtgC + LptD combination led to higher IgG2a isotype compared to LtgC ($p < 0.001$), while it was slightly lower than that of LptD + MPLA. See Fig. 4.

Evaluating IgA levels in sera and vaginal lavages

IgA levels in mice sera and vaginal wash samples were measured on days 42 and 112. Among the different groups, LptD + Liposomal MPLA exhibited the highest serum IgA levels on day 42 ($p < 0.0001$). LptD + MPLA induced lower IgA levels than the liposomal formulation (Fig. 5A). This indicates that the use of liposomes

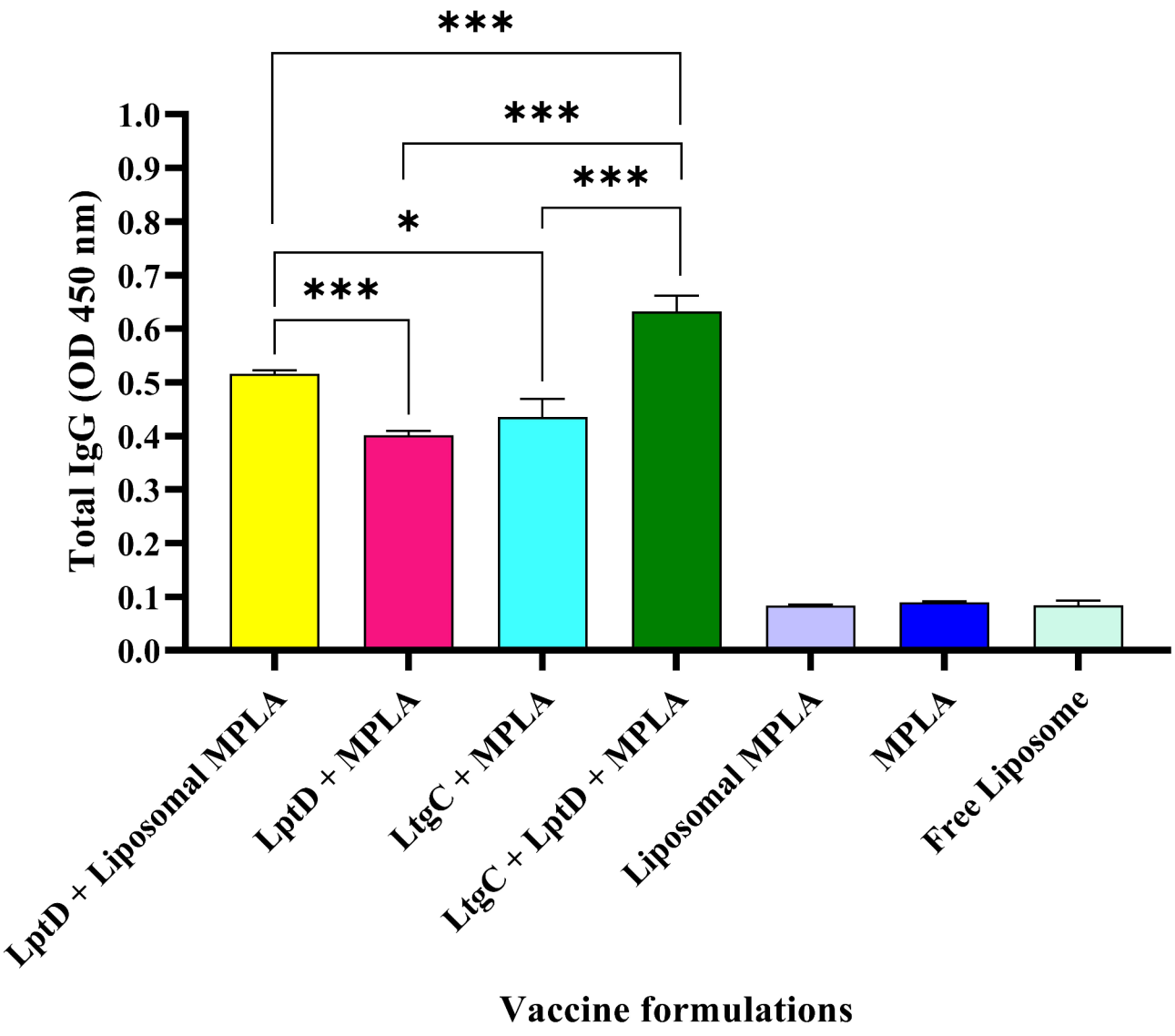

Fig. 3 Evaluating the serum level of total IgG against pathogen using Whole-Cell ELISA. Whole-cell ELISA was performed to compare the immunogenicity of bivalent formulation and the single-antigen formulations. IgG levels are expressed as OD at 450 nm. Each bar represents the mean ± standard error of the mean (SEM) for each group. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. The bivalent formulation (LtgC + LptD + MPLA) induced a higher IgG response than the single-antigen formulations LtgC + MPLA (***$p < 0.001$) and LptD + MPLA (***$p < 0.001$). Furthermore, LptD + liposomal MPLA exhibited a strong IgG response, which was significantly higher than that of LptD + MPLA (***$p < 0.001$)

as carriers significantly enhances the efficacy of the vaccine ($p < 0.001$). LtgC + MPLA produced lower IgA levels compared with LptD + MPLA, suggesting that LptD is a stronger antigen than LtgC. The combination of LtgC + LptD + MPLA also resulted in high IgA level, although lower than that in the LptD + Liposomal MPLA group. The LptD + Liposomal MPLA group exhibited higher vaginal IgA levels than the liposomal MPLA control group ($p < 0.001$). In the vaginal wash samples, apart from the LptD + Liposomal MPLA group, none of the other groups induced significant levels of vaginal IgA antibodies compared with the control groups, indicating a weaker mucosal immune response to non-liposomal MPLA adjuvant (Fig. 5A).

A gradual decrease in IgA levels was observed on day 112 compared to day 42, as it was expected that the immune response would wane over time. While significant differences were observed, the fact that IgA levels remained elevated in some groups, including LtgC + MPLA, LtgC + LptD + MPLA, and LptD + Liposomal MPLA, indicates good durability of the immune response (Fig. 5B).

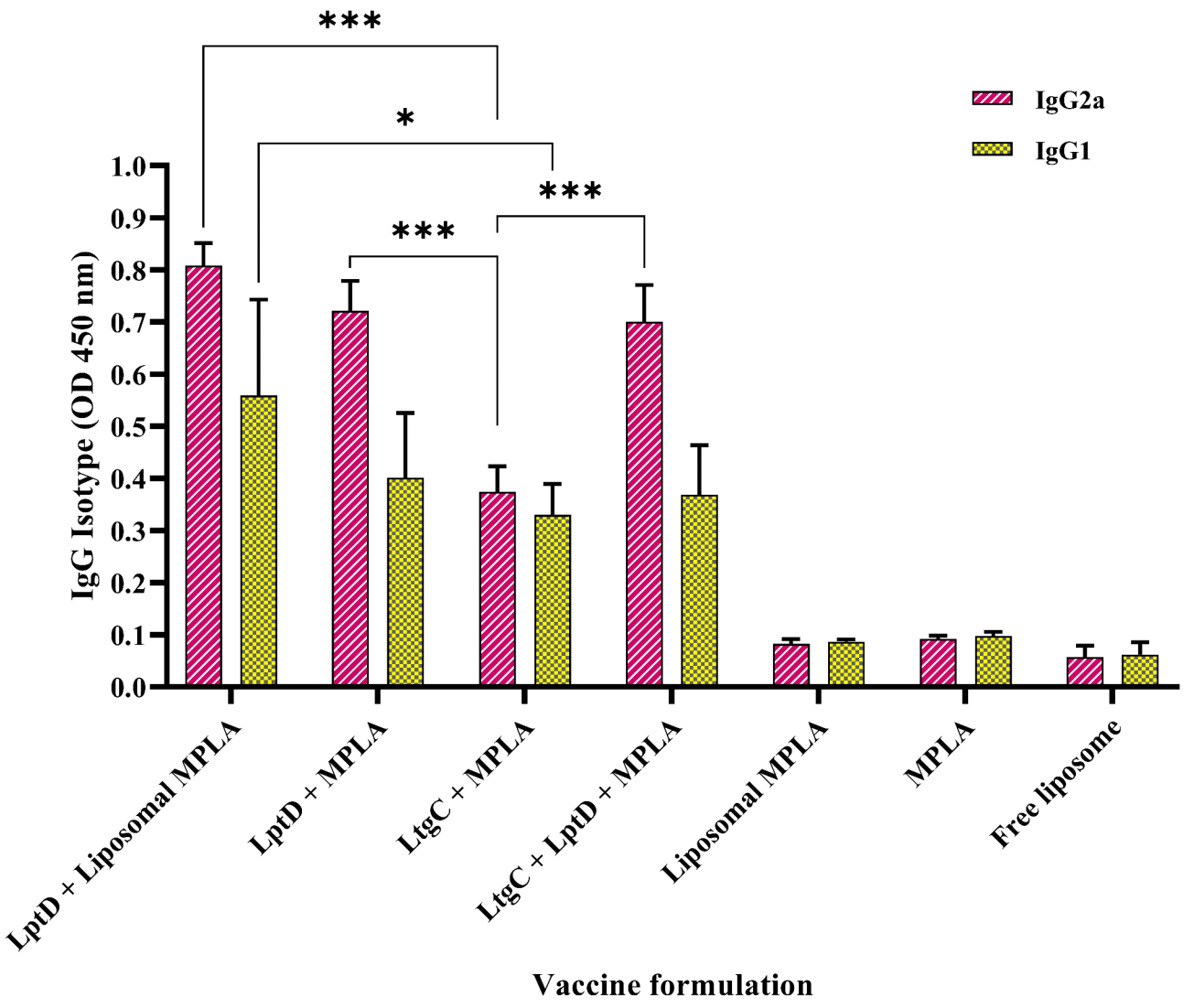

Fig. 4 Evaluation of IgG Isotype Responses Following Different Vaccine Formulations. The IgG2a/IgG1 ratio of all vaccine formulations was > 1, highlighting the Th1-polarized immune response by MPLA adjuvant. The LptD + liposomal MPLA group showed a higher IgG2a/IgG1 ratio than the LptD + MPLA group. This highlights the potential of liposomal MPLA to enhance Th1 responses, suggesting its value in vaccine formulations targeting cellular immunity. LtgC + LptD + MPLA induced a higher IgG2a level than LtgC + MPLA and a slightly lower level than LptD + MPLA. IgG levels were measured as optical density (OD) at 450 nm. Each bar represents the mean ± standard error of the mean (SEM) for each group. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. Significance is indicated as follows: $*p < 0.05$, $***p < 0.001$

Serum bactericidal and opsonophagocytic activity

The capability of antisera from each group to mediate complement-dependent lysis and opsonophagocytic killing of *N. gonorrhoeae* was evaluated by determining of SBA and OPA titers, respectively (Table 1). SBA and OPA titers represent the minimum serum dilution resulting in a 50% reduction in bacterial survival. All formulations elicited serum bactericidal and opsonophagocytic activity in a concentration-dependent manner. The LptD + Liposomal MPLA group showed SBA and OPA titers of 128 and 256, respectively, indicating that this combination not only has strong bactericidal capability but is also effective in stimulating opsonophagocytic activity. Similar results were observed in the group receiving LptD + MPLA, suggesting that the liposomal formulation of the adjuvant did not significantly affect SBA and OPA.

The LtgC + LptD + MPLA combination showed the best performance in the SBA test, with a titer of 1/256 sufficient to reduce bacterial survival by 50%. This indicates that the combination of the LtgC and LptD proteins with MPLA leads to stronger immune response. In the OPA test, this combination also maintained a high titer level (1/512), indicating that it is effective in both bacterial killing and opsonophagocytic mechanisms.

In the LtgC + MPLA group, at least an SBA titer of 1/64 was required to achieve a 50% reduction in survival. In the OPA test, a higher titer (1/128) was obtained. This suggests that LtgC and LptD alone function as weaker

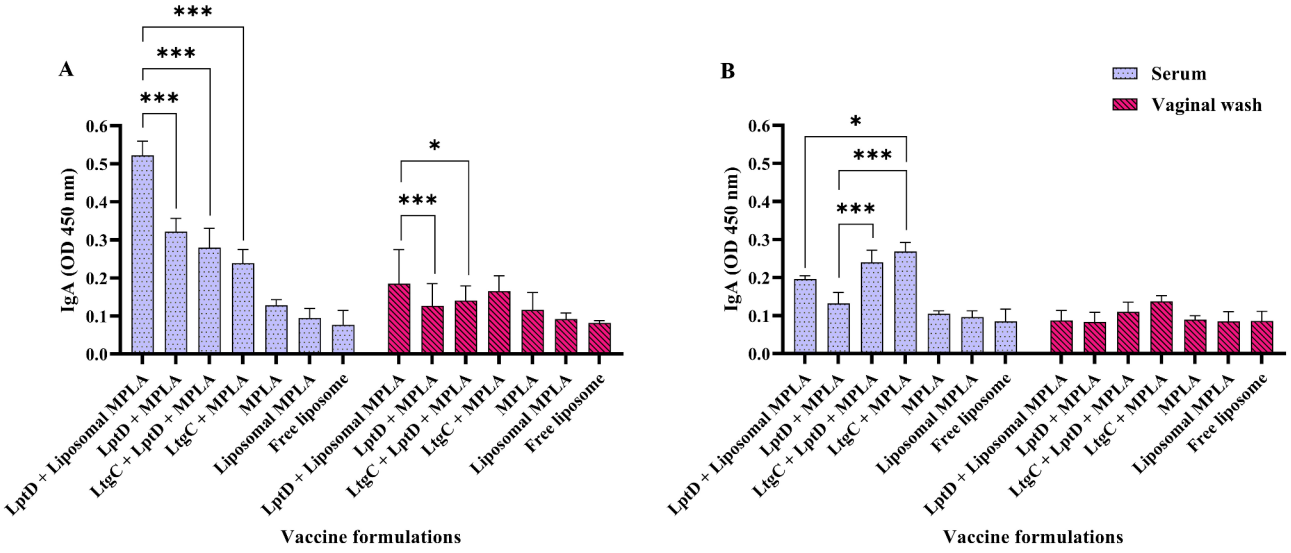

Fig. 5 IgA Levels in Serum and Vaginal Wash Following Immunization with Different Vaccine Formulations. (**A**) IgA levels in serum and vaginal wash on Day 42 post-immunization, and presented as optical density at 450 nm. Each bar represents the mean ± standard error of the mean (SEM) for each group. Generally, IgA levels were higher in serum compared to vaginal wash across all formulations. LptD + Liposomal MPLA induced the highest IgA levels in serum, which were significantly higher than in other formulations (***$p < 0.001$). In the vaginal wash, LptD + Liposomal MPLA also showed elevated IgA levels compared with LptD + MPLA (***$p < 0.001$) and LtgC + LptD + MPLA (*$p < 0.05$). (**B**) IgA levels in serum and vaginal wash on Day 112 post-immunization. By Day 112, IgA levels in serum showed a decline over time, although LptD + Liposomal MPLA maintained relatively higher levels. In the vaginal wash, IgA levels were similarly lower overall and no significant difference were observed between different groups

Table 1 Comparison of the serum bactericidal (SBA) and opsonophagocytic activity (OPA) of mice Sera immunized with different formulations against *N. gonorrhoeae*

Vaccine formulation	SBA titer*	OPA titer**
LptD + Liposomal MPLA	1/128	1/256
LptD + MPLA	1/128	1/256
LtgC + MPLA	1/64	1/128
LptD + LtgC + MPLA	1/256	1/512

* SBA titer shows the lowest mice sera dilution that results in 50% reduction of bacterial survival in presence of complement source

** OPA titer shows the lowest mice sera dilution that results in 50% reduction of bacterial survival in presence of complement source and PMNs

antigens. These findings indicate that single antigens alone are less effective in stimulating the immune system compared to combination formulation.

Discussion

The development of an effective vaccine against *N. gonorrhoeae* is imperative due to the rapid emergence of antibiotic-resistant strains [19]. Considering that untreated gonococcal infections can result in severe complications; gonorrhea is a significant public health concern. With the diminishing efficacy of current antibiotic regimens, the treatment of gonorrhea is becoming increasingly complex and challenging. Thus, vaccination seems to be the main possible core component strategy to address the global burden of gonorrhea in the long term [38].

Compared with vaccines targeting a single antigen, multi-antigen vaccines offer several benefits, including enhanced immunogenicity, which promotes more robust immunity. Various studies have confirmed the effectiveness of multi-antigen vaccines, revealing their capacity to substantially improve the magnitude, quality, and durability of the immune responses they elicit [39]. Similarly, in the case of *Neisseria meningitidis*, the 4CMenB vaccine targets several proteins to protect against a broader range of serogroup B strains and induce stronger immune responses.

The 4CMenB (Bexsero) vaccine, primarily designed to combat meningococcal disease, is currently being studied in a clinical trial involving men who have sex with men [40]. Previous studies have shown that this vaccine can provide approximately 30–40% protection against gonorrhea [41]. However, evidence highlights that among the antigens present in the 4CMenB vaccine, only NhbA shares moderate sequence identity (73%) with its gonococcal homolog and is highly conserved across *N. gonorrhoeae* strains. Additionally, NhbA is predicted to be expressed on the bacterial surface. In contrast, the gonococcal version of FHbp was predicted to lack surface expression, whereas NadA was entirely absent in all examined *N. gonorrhoeae* isolates [42]. Among the 4CMenB antigens, NhbA is the only conserved and surface-exposed protein in *N. gonorrhoeae*. It appears that anti-NhbA antibodies induced by 4CMenB offer cross-protection against gonorrhea [43]. Thus, efforts to identify protective immunogen targets are still ongoing.

Given the successful application of reverse vaccinology in developing vaccines against *N. meningitidis* serotype B,

we previously conducted a computational study leading to introduction of several potential vaccine candidates against *Neisseria gonorrhoeae* such as LptD and LtgC. These proteins, selected for their surface accessibility and minimized cross-reactivity with human proteome, demonstrated high antigenicity (>0.5) and low allergenicity (<0.2). Both exhibited numerous linear and conformational B-cell epitopes, MHC I and II binding sites, and strong conservation among *N. gonorrhoeae* strains. Docking simulations confirmed significant interactions with human TLRs, and immune simulations predicted robust IgG1/IgG2a responses and pro-inflammatory cytokine induction (e.g., IFN-γ, IL-2). Additionally, their stability, solubility, and non-toxic nature further support their potential as effective vaccine targets [19].

These findings build a strong rationale for selecting LptD and LtgC as promising vaccine targets for a bivalent vaccination strategy against *N. gonorrhoeae*. To our knowledge, this is the first comprehensive study to evaluate the immunogenicity of this combination. Antigenic variations pose substantial challenges to the induction of immunity against gonorrhea [44]. Therefore, the conserved sequences of LtgC and LptD among *N. gonorrhoeae* strains would overcome this issue [19]. Moreover, in the current study, both LtgC and LptD proteins were immunoreactive against the sera of patients with gonorrhea, which indicates that they induce antibodies following natural infection, supporting their potential as vaccine candidates.

Protection against gonococcal infection is primarily associated with Th1 immune responses in both mice and humans, which accelerates the clearance of infection [25–27]. Th1-skewed antibody subclasses are more effective at complement activation than IgG1 and enhance serum bactericidal activity against *N. gonorrhoeae* [45]. To boost Th1 responses, we employed MPLA as an adjuvant. MPLA is known for its strong ability to induce Th1-dominant immune responses [46]. Fortunately, all vaccine formulations in the current study induced a Th1-biased immune response due to higher IgG2a/IgG1 ratios, verifying the effectiveness of MLPA adjuvant in Th1 response polarization.

All formulations resulted in high serum IgG levels, with the highest level observed on day 56. The total IgG level was higher in mice that received LptD protein. Differences in antibody levels in response to different proteins may be due to several factors, including structural characteristics, stability, the presence of conserved or repetitive epitopes [47], molecular weight of protein [48], the adjuvant and antigen–adjuvant interactions [49]. Although protein-specific IgG levels of the bivalent formulation were lower than those of LptD, whole-cell ELISA showed higher levels of antibody against *N. gonorrhoeae* was induced by the bivalent formulation compared with single antigens. This indicates that the bivalent formulation induces a stronger immune response against native form of these antigens.

Liposomal adjuvants provide a multifaceted approach to vaccine development, combining enhanced antigen delivery, controlled release, sustained immune responses, and reduced adjuvant toxicity. Moreover, liposomes possess intrinsic adjuvant properties, allowing them to stimulate the immune system on their own [50]. We included a vaccine formulation composed of the LptD protein adjuvanted by liposomal MPLA. Surprisingly, LptD + liposomal adjuvant induced higher IgG and IgA levels than other formulations.

Among the different formulations, LtgC + LptD had the highest total IgG level on day 112. This indicates the effect of the bivalent approach on maintaining the antibody level in the long-term duration. However, as expected, parenteral administration of antigens did not result in significant levels of IgA at mucosal surfaces. Thus, mucosal administration routes should be considered in future studies. As studies with intranasal and intravaginal route of administration have shown strong mucosal immunity with higher vaginal IgA levels [26, 51].

Both LptD-based formulations resulted in equal SBA and OPA titers, indicating that liposomal MPLA had no greater effects on opsonophagocytic and bactericidal activities. While, the bivalent formulation resulted in higher SBA titers than the single antigen formulations. Although both single antigens could induce antibodies with opsonophagocytic activity, similar to SBA, the bivalent formulation exhibited stronger OPA activity. These findings suggest the stronger effect of bivalent formulation on survival of *N. gonorrhoeae*.

The SBA and OPA titers of different vaccine candidates have been assessed and reported previously. However, the results were not comparable due to the variations in *N. gonorrhoeae* strains and protocols. A recent protocol was presented by Semchenko et al. [36], that recent research on gonococcal vaccines has employed this approach. For example, Roe SK et al.., evaluated a trivalent vaccine containing two different adjuvants, alum and alum + MLPA. It is noteworthy that MLPA adjuvant therapy resulted in a higher SBA titer (1/160-1/320) due to activation of Th1 response [28]. Similar to our findings, this trivalent formulation showed higher SBA titers than single antigen formulations [52]. Similarly, combination of passenger and translocator fragments of adhesion and penetration protein (App) resulted in higher SBA and OPA titers (1/80) compared with the groups receiving only one fragment [51].

In addition, the combination of TbpA and TbpB proteins resulted in a higher SBA titer (1/800) against *N. gonorrhoeae* FA19 compared with single antigens (1/400 and 1/200) [53]. These findings demonstrate that

co-administration of multiple antigens such as LtgC and LptD would induce a superior bactericidal and opsonophagocytic activity in case that both antigens have no antagonistic negative effect on the bactericidal activity of each other.

While this study offers useful insights into the potential of LptD and LtgC as vaccine candidates against *N. gonorrhoeae*, we acknowledge that the lack of an in vivo animal protection experiment represents a limitation of the current study. Although an estradiol treated lower reproductive tract infection mouse model has been described previously, developing such models is complex and challenging, as highlighted by other researchers in the field [54, 55]. Nevertheless, we focused on alternative in *vitro* approaches to assess the bactericidal activity of mouse immune sera, which are well-supported in the literature. As noted, performing animal protection experiments will be a priority for our future research to further validate the vaccine candidates.

Conclusion

This study highlights the potential of a multi-antigen vaccination approach, specifically targeting the immunogenic proteins LptD and LtgC, to elicit a robust immune response capable of addressing the challenges associated with gonorrhea prevention. Our findings demonstrate that this combination not only induced higher long-term total IgG levels but also enhanced serum bactericidal activity and opsonophagocytic responses compared with single antigen formulations. The use of MPLA as an adjuvant further underscores the importance of Th1-dominant immune responses to gonorrhea. Although challenges remain, particularly in achieving sufficient mucosal immunity, the promising results of this multi-antigen strategy provide a foundation for future research and development efforts. Moreover, the formulation of a multi-antigen vaccine along with a liposomal adjuvant warrants further investigation in future studies, as it may elicit a robust and prolonged immune response. As we continue to explore and refine this approach, there is hope for significant progress in the battle against gonorrhea, ultimately leading to a reduction in its global health burden and improved public health outcomes.

Abbreviations

AMR	Antimicrobial Resistance
MDR	Multi-drug resistant
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
ELISA	Enzyme-Linked Immunosorbent Assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MPLA	Mono phosphoryl lipid A
OPA	Opsonophagocytic assay
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline with Tween 20
PMN	Polymorphonuclear cells
SEM	Standard Error of the Mean
SBA	Serum Bactericidal Assay
TMB	3,3′,5,5′-Tetramethylbenzidine
WHO	World Health Organization

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Authors' contributions

NNG and FB: methodology, conducting experiments; data interpretation, validation, writing original draft, and writing–review & editing; SMB: methodology, conducting experiments; SA: validation, visualization, and writing–review & editing; MC: methodology, conducting experiments; MSY: methodology, software, Data analysis; FB & MRP: conceptualization, funding acquisition, methodology, supervision, validation, writing–review & editing.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

This study received ethical approval from the Research Ethics Committee of the Tehran University of Medical Sciences (approval ID: IR.TUMS.SPH.REC.1403.073).

Consent for publication

All authors have agreed to publish this manuscript.

Competing interests

None to declare.

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