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Development, Qualification and Validation of Innovative High-throughput Serological Assays to Evaluate Bacterial Vaccines Immunogenicity

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This study was conducted at VisMederi-srl, and due to confidentiality, it was not possible to provide all information about the analysed samples.

ABSTRACT

The increase in antimicrobial-resistant bacterial strains has highlighted the need for a new vaccine strategy. The primary goal of a candidate vaccine is to prevent disease, by inducing a persistent immunologic memory, through the activation of pathogen-specific immune response. Antibody titer is the main parameter used to assess the immunogenicity of bacterial vaccine candidates, and it is the most widely used as a correlate of protection. On the other hand, the antibody titer alone cannot provide complete information on all the activity mediated by antibodies which can only be assessed by functional assays, like the serum bactericidal assay and the opsonophagocytosis assay. However, due to the involvement of many biological factors, these assays are difficult to standardize. Some improvements have been achieved in recent years, but further optimizations are needed to minimize inter- and intra-laboratories variability and to allow the applicability of these functional assays for the vaccine immunogenicity assessment on a larger scale.

Here I report the development, Qualification and Validation of innovative functional and non-functional assay to support the vaccine immunogenicity evaluation. In particular, in this work is described the development of the Luminescence-Based Serum Bactericidal Assay for *B. burgdorferi*, the serotyping FACS-based assay for *Shigella flexneri* 2a, the Bio-Luminescence- Serum Bactericidal Assay and the complement deposition assay for *N. meningitidis,* and the FACS-based whole cell ELISA for *N. Gonorrhoea.* Then, I also performed the qualification of the Luminescence-Based Serum Bactericidal Assay for *Shigella flexneri* 2a, *Salmonella* iNTS and also the Validation of the Classical Serum Bactericidal Assay for *Salmonella* Paratyphi A.

INTRODUCTION

After the emergency of the COVID-19 pandemic, the focus on infectious diseases is shifting to another global public health threat caused by the rise of antimicrobial-resistant (AMR) bacterial strains. At the beginning of the 20th century, bacterial diseases were the highest cause of mortality and morbidity. The 1940–1980 period was characterized by a continuous discovery of new antibiotics, the so-called "miracle drugs," which increased life expectancy and was perceived by society as a milestone achievement in microbiological research [1]. From the mid-1980s, the focus of the pharmaceutical industry shifted to other drug development, and the discovery of new antibiotics was remarkably reduced. Therefore, since the emergence of antibiotic resistance was no longer compensated by new anti-microbial drug discoveries, bacteria became again a global threat to modern health care, raising concerns of national and international public health organizations, including the World Health Organization (WHO) [1]. For this reason, during the last few years, vaccines have become a valuable alternative to prevent pandemics caused by AMR bacteria, for which currently available treatments are no longer or less effective. For example, cases of *N. gonorrhoeae* infection are now becoming more and more challenging, with the risk of becoming untreatable with current available antibiotics. Vaccines can mitigate directly and indirectly the antimicrobial resistance consequences. Vaccines can prevent diseases by the elicitation of the immune response, reducing antimicrobial drug consumption, and the development of resistance. This is particularly important considering community and healthcare-associated infections with multidrug-resistant pathogens. Additionally, the use of viral vaccines reduces the need for antibiotic treatments, that may occur in case of secondary bacterial infections [2]. However, bacteria are much more complex organisms than viruses and represent a more difficult target for vaccine development, today only a limited number of vaccines are licensed and applied against bacterial infections. Despite these difficulties, the WHO encourages the development of new therapies and vaccines targeting AMR bacteria strains [3].However, for bacterial vaccine advancement, appropriate assays for immunogenicity assessment are needed.

Progress in antigen discovery must go along with the set-up of robust and reliable methods of evaluation performed during the strict approval process.

1. Which aspects are investigated to evaluate vaccine immunogenicity?

Vaccination aims to induce an immune response able to control exposure to an infectious agent and clear or prevent the infection. Ideally, a candidate vaccine should induce the adaptive immune response, represented by both humoral and cell-mediated immunity. The antigen presentation followed by vaccine administration, induces the selection of antigen-specific B-and T-cells in separate ways (Figure 1) [4]. The B-cell receptor (BCR) allows direct interaction with the antigen, without T-cell involvement. Humoral immunity is based on the capacity of the B-cells to secrete antibodies. Antibodies have two functional domains, called the antigen-binding fragment F(ab')2 and the crystallizable fragment (Fc) which are responsible for the specificity and functionality, respectively. The basic function of the F(ab')2 fragment is the binding of antigenic epitopes via complementarity-determining regions (CDRs) [4]. The Fc domain passes through structural variations identified as five isotypes (IgM, IgD, IgG, IgA, and IgE) which are responsible for distinct innate immune effector functions [4]. Antibodies variety is generated during Ag-dependent B-cell maturation in peripheral lymphatic tissues by isotype class switching and somatic hypermutation [5]. The Fc fragment mediates several antibody functions, such as receptor-mediated phagocytosis, cytotoxicity, the release of inflammatory mediators, the transport through the mucosa, and complement activation (Figure 1) [4]. It follows that a strong antibody–antigen affinity and specificity may generate greater potency and efficacy against bacterial infections [6]. Antibody-mediated neutralization represents one of the simplest mechanisms involved in protection against bacteria. The F(ab')2 domains bind the specific pathogen antigen, inhibiting the host-cell invasion or bacterial toxin effect. Moreover, antibodies hold back pathogen pathogenesis, with the inhibition of microbial virulence factors like biofilm formation [7].

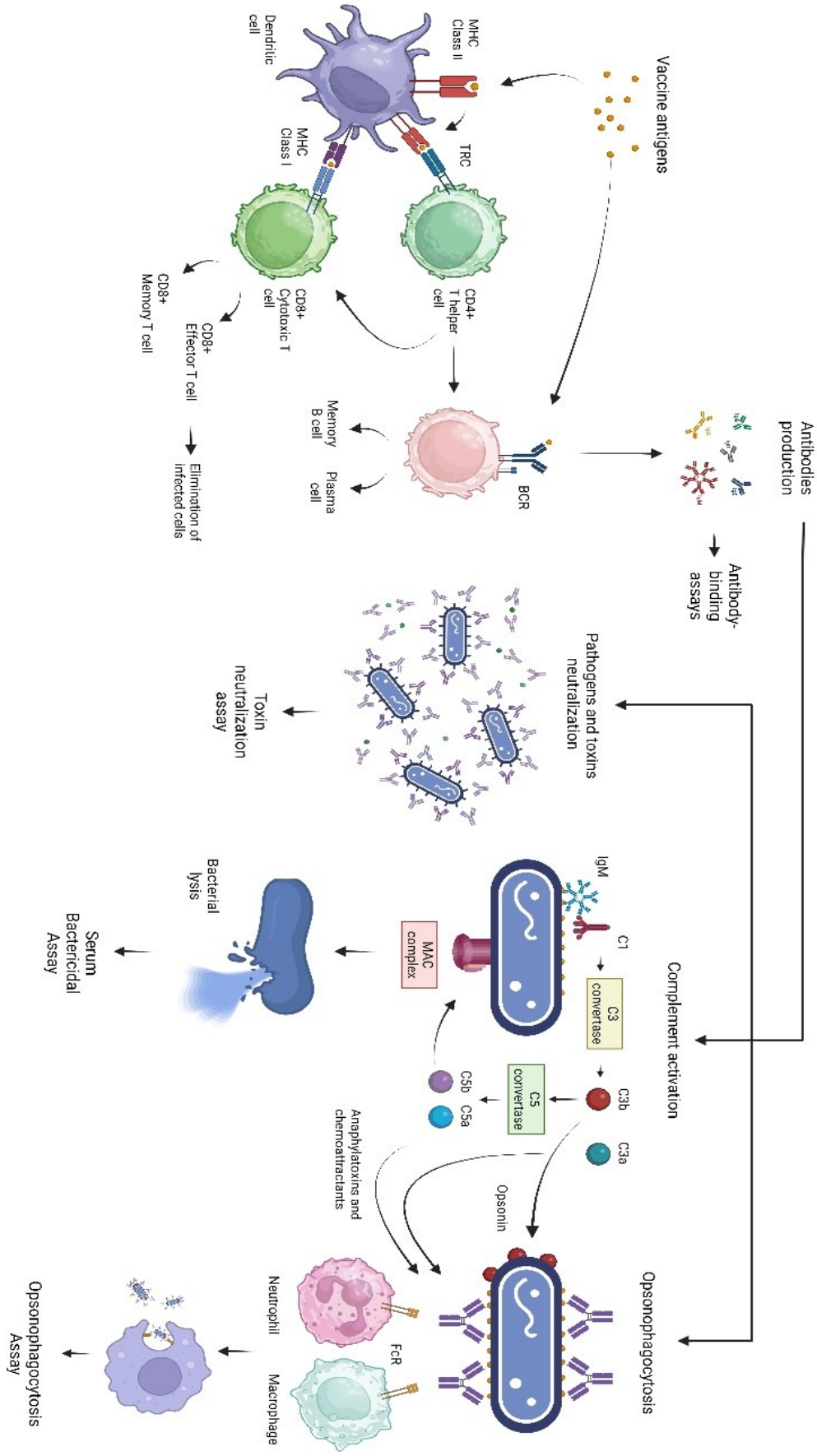

Figure 1. Graphical representation of the generation of the basic immune response induced by vaccination. The main functions mediated by antibodies against bacterial infection, and the related serological test. Antigen presentation, followed by vaccine administration, induces the selection of antigen-specific B- and T-cells. The dendritic cells (DCs) process the antigens and present them via MHC class I or MHC class II, promoting differentiation into cytotoxic T-cells (CD8+) and helper T cells (CD4+), respectively. Cytotoxic T-cells (CTLs) mediate the lysis of autologous infected cells, while helper T-cells stimulate other immune cells, like the B-cells, through cytokine secretion. B-cells can directly interact with the vaccine antigens and produce antibodies, which mediate several functions necessary to overcome bacterial infections. The main functionality of antibodies includes neutralization of pathogens and toxins, complement activation which leads to bacterial lysis through the formation of the membrane attack complex (MAC), and, finally, opsonophagocytosis mediating the elimination of bacteria by phagocytic cells. Antibody level is estimated through antibody-binding assays (ELISA), while functionality is assessed using the toxin neutralization test, the serum bactericidal assay (SBA), and the opsonophagocytosis assay (OPA). This figure was created using BioRender.com.

The complement activation represents one of the most crucial functions mediated by antibodies. The complement system consists of a complex of almost 50 plasma and surface proteins and is part of the innate immune system which cooperates with antibodies for the rapid elimination of invading bacteria. In general, the complement activation induces three different enzymatic cascades called, the classical, mannan-binding lectin (MBL) and alternative pathways. The classical complement pathway is triggered by the antibody's interaction with the bacterial surface antigens. The C1 complex interacts with the Fc region of pentameric IgM or IgG complex and leads to the formation of the C3 convertase enzyme which transforms C3 into C3a and C3b. A high concentration of C3b switches the substrate from C3 convertase to C5 convertase, which catalysis the cleavage and the release of C5a and C5b. The association of the C5b with C6, C7, and C8 allows the binding and then the polymerization of C9, generating the membrane attack complex (MAC) [8]. The MAC is a ring-structured pore that can directly kill Gram-negative bacteria without the involvement of immune cells through osmolysis [9]. The gram-positive bacteria are less susceptible to this mechanism for the presence of the peptidoglycan outer layer. In this case, the killing action is led by the strong chemoattractant C5a which recruits phagocytes in the infection site, and by the C3b opsonized particles that enhance the phagocytosis. The C3-derived products are also responsible for a long-term immune response, stimulating the adaptive immune response through the antigen presentation to B-cells and antigen-presenting cells (APC) placed in the lymphoid organs [7,8]. Then, the MBL pathway is triggered by surface-specific recognition of molecules like collectins and ficolins uniquely present in bacteria and fungi. These molecules in complex with the MBL-associated serine proteases (MASPs) lead to the

formation of C3 convertase. Lastly, in the alternative pathway, the factor B and D proteins also play a role in the amplification loop of C3b molecules. It is also considered that in the third recognition path, factors B and D can react with spontaneous hydrolysed C3 (C3H2O) to obtain C3 convertase [7,8]. In general, all three pathways lead to the formation of C3 and C5 convertase which catalyse the formation of the C3a and C5a. The C3a and C5a are anaphylatoxins and leukocyte chemoattractants, that bind their specific receptors (C3aR, C5aR1, and C5aR2) on immune cells to stimulate and modulate the inflammatory response. Although anaphylatoxins play an important role in protection against certain infections, dysregulation has been associated with several diseases related to inflammatory disorders, like allergy, autoimmunity, neurodegenerative diseases, and cancer [10]. Consequently, the C5a and C3a elicitation can be taken into consideration to evaluate the possibility of allergic reactions and hypersensitivity, during vaccine immunogenicity evaluation [11].

Concerning the ability of the immune response to face bacterial infections, cell-mediated immunity also plays an important role. The T-cells are unable to interact with antigens directly, but depend on the APC, like dendritic cells (DC) or macrophages, to process antigens and present through the MHC class I or MHC class II, enhancing the differentiation to cytotoxic T-cells (CD8+) and T helper cells (CD4+), respectively [12]. Cytotoxic T-cells (CTLs) mediate the lysis of autologous cells infected by intracellular pathogens, and T-helper cells stimulate other immune cells through the secretion of cytokines and by cell-to-cell interactions. Based on the profile of the secreted cytokines, the T-cells are classified as T helper 1 (Th1) or T helper 2 (Th2)[13]. Th1 response stimulates the cellular-based immune response, while Th2 response leads to the humoral immune response. The Th1-cells produce cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor alfa (TNF-α), which potentiate the effector function of phagocytes and increase inflammation[13]. On the other hand, Th2 cells developed under the influence of IL-4 signaling, produce another set of cytokines that support B-cells proliferation and differentiation [13]. For this reason, Th2 cells have been associated with increased humoral responses. However, the cytokines produced by Th2 cells have been linked

to IgE production; consequently, the elicitation of memory Th2 cells has become an important focus in the design of novel candidate vaccines, to evaluate and mitigate allergic reactions. Therefore, a vaccine candidate that has a balanced Th1/Th2 response can be considered optimal [12]. However, current advances in the understanding of intracellular bacterial infection revealed that the immune response is more complex than the Th1/Th2 dichotomy and recognizes the role of T-helper 17 cells (Th17) in antimicrobial host defence. The Th17 is a subset of pro-inflammatory T-helper cells defined by their production of interleukin 17 (IL-17). IL-17 exhibits proinflammatory effects and is known to mediate the immunomodulation of DC, recruitment of neutrophils, and promoting the CTL and Th1 response, which is crucial against intracellular bacteria [14]. Therefore, the Th17/IL-17 elicitation represents an important parameter to evaluate vaccines for intracellular bacteria. The Natural Killer (NK) cells are another separate lymphocyte lineage that shows both cytotoxicity and cytokine-producing effector roles. A further function of the antibodies is the enhancement of the cytotoxic destruction of the bacterial cells through the so-called antibody-dependent cellular cytotoxicity (ADCC), mediated by NK cells. The NK cells, activated by the binding with the Fc portion of the antibody, release perforin and granzyme which lead to the death of infected or tumor cells [15]. The elicitation of efficient T-cells and the generation of memory T-cells represent another challenge in vaccine development. Indeed, modern vaccines may not efficiently elicit long-term T-cell immunity due to the short persistence of the antigens. To improve this aspect, DNA-based vaccines, viral vectors, prime-boost regimes, and adjuvant combinations, have been proposed [16]. In this context, the most used techniques for the evaluation of the cell-mediated immunity response are the Enzyme-Linked ImmunoSpot assay (ELISpot) and flow cytometry applied to intracellular cytokine staining (ICS) [12]:

The last important function triggered by antibodies is the enhancement of opsonophagocytosis. These antibody-coated pathogens are labelled for phagocytosis by neutrophils and macrophages (Figure 1). In the case of antibody-dependent cellular phagocytosis, the Fc receptor recognizes the

antibodies binding the bacterial surface and starts intracellular signaling which leads to the actin cytoskeleton rearrangement and the formation of the phagosome [17]. Moreover, the Fc receptor recognition activates signaling of the immunoreceptor tyrosine-based activation motifs (ITAMs), which lead to the antigen presentation to the T-cells, and in some cases activates the immunoreceptor tyrosine-based inhibition motifs (ITIMs) for the retention of the whole pathogen antigens to be transferred to B-cells and induce also a humoral immunity [7].

2. Available bacterial vaccines

The primary goal of a candidate vaccine is to prevent disease by inducing a persistent immunologic memory through the activation of pathogen-specific immune response. Long-term protection involves memory cell activation that rapidly triggers a secondary immune response. Memory B-cells are responsible for the secretion of high-affinity functional antibodies, while memory T-cells are capable of rapid expansion and cytotoxic properties [16]. Vaccine boosters aim to increase the quality, quantity, and persistence of the immune response inducing a secondary response [18]. However, this depends on the vaccine formulation and the target pathogen. The presence of adjuvants in the formulation can induce an early activation of innate immunity which then turns into higher antibody and cellular responses to the vaccine antigens [19]. In general, adjuvants are useful for those vaccines formulated with purer components, like purified recombinant antigens, which represent a safer option but also show lower immunogenicity, in contrast to other types of vaccines [19]. In the case of the hepatitis B vaccine, the presence of adjuvant systems has been associated with positive regulation of genes associated with IFN-γ-related responses and the innate cell compartment [20]. The complexity of bacteria makes them more challenging targets for vaccine development compared to viruses [21]. However, they naturally contain various immunostimulatory components that can be exploited as in-built adjuvanticity [19].

Currently, five main groups of bacterial vaccines are available: whole-cell antigen (WCA), polysaccharide/protein conjugates; recombinant proteins including toxoids; live attenuated vaccines (LAV); and, more recently introduced, bacterial outer membrane vesicles (OMVs). Use messenger RNA (mRNA) to instruct cells to produce bacterial antigens and trigger an immune response. Primarily used for viral vaccines (SARS-CoV-2), but research is ongoing for bacterial applications. The WCA vaccine is based on the administration of inactivated bacteria (heating, irradiation, or chemical) and predominantly induces the activation of the B-cells assisted by T-helper cells for the antibodies production. For this reason, WCA immunization is efficiently used for vaccination against extracellular bacteria, like Vibrio cholerae (Dukoral and Shanchol) [22,23]. However, antibodies may lack effectiveness against intracellular pathogens, due to the difficulties in reaching the target. An efficient defence against intracellular bacteria requires the activation of cytotoxic CD8+ T-cells, mediated by the MHC class I pathway, and Th1/Th17 response. The polysaccharide conjugate vaccines are based on bacterial capsular polysaccharides conjugated with a carrier protein. Polysaccharides alone cannot be processed and displayed on MHC molecules like proteins, consequently, it activates an immune response T-cell independent. The polysaccharide conjugation with a protein carrier enhances the engagement of CD4+ T-helper cells through the interaction with the MHC class II of B-cells which leads to higher affinity and class-switched antibodies [24]. Examples of bacterial polysaccharide conjugate vaccines are vaccines against H. influenzae type b (PedvaxHIB, ActHIB, HibTITER, proHIBiT), pneumococci (Prevnar (PVC7), Pneumovax 23(PPV)), meningococci (Menactra, Menveo, MenQuadfi) [25–27]. The toxoid vaccines (DTaP) are composed of inactivated exotoxins released by bacteria like *Corynebacterium diphtheriae*, *Clostridium tetani*, *Bordetella pertussis*, induce the activation of B cells and antibody production with the cooperation of T-helper cells [21,28]). Differently, the LAVs are based on microorganisms that have lost pathogenicity but maintain the capacity for transient intracellular replication, they can activate the cytotoxic CD8+ T-cells through the interaction with MHC class I of infected cells, reaching an efficient defence against intracellular bacteria [23]. Examples of such attenuated vaccines are *Mycobacterium*

tuberculosis (BCG), *Salmonella typhi* (Vivotif), and *Vibrio cholerae* (Vaxchora) [29–31]. The OMVs carry many bacterial antigens preserving the features of the bacterial membrane, that when transferred into the cytosol of target cells have the potential to elicit T-cell responses, including cytotoxic CD8+ T-cell responses [23]. The four licensed vaccines based on OMVs are all directed against N. meningitidis serogroup B bacteria (Bexsero/4CMenB, VA- MENGOC-BC, MenBVac, MeNZB) [32].

Because of emerging AMR bacteria, the development of novel vaccine candidates against these bacterial targets is strongly encouraged by the public health authorities and a list of bacterial priority pathogens is available and updated by WHO. Vaccine strategy requires the inclusion of multiple serotypes, different adjuvants, and the continuous discovery of immunogenic epitopes. Bacteria possess a variety of antigens whose immunogenic potential is often unknown, and it is unclear which antigen can elicit a protective and long-lasting immune response [33]. There are several limitations to new bacterial vaccine development: the lack of a known correlate or surrogate of protection, the need for appropriate animal models, and the improvement in certain vaccine formulations that can increase the efficacy in high-risk groups such as infants and elders. Furthermore, intracellular bacteria represent a more challenging target for vaccine development, as the protection requires the activation of the cell-mediated immune response, besides the antibody production [34]. Vaccine development is a process that requires multiple steps and needs to take into consideration multiple aspects besides the antigen discovery. The first aspect is the epidemiological impact determined by the disease, the existence of alternatives to treat the disease, and the cost/benefit associated. Fundamental is the involvement of research groups and institutions that can be aware of the stage of the scientific knowledge of the selected disease and can identify gaps in vaccine development. Lastly, significant is the existence of laboratories capable of carrying out the process from pre-clinical to phase III clinical studies in compliance with Good Clinical Practice (GCP) and others that meet Good Manufacturing Practice (GMP) to produce the vaccines. After considering all these aspects, on average it takes 10 to 15 years to introduce a new vaccine to the market. The development of a vaccine

is pyramidal, for every success there are many failures, where most failures occur in the preclinical and phase I stages of clinical studies [35]. Traditional strategies for bacterial vaccine development aimed to induce a strong humoral response, prioritizing the use of antibody-binding assays to evaluate immunogenicity. However, binding assays, like ELISA, can only measure the level of antibodies in the serum of the patient, which may not necessarily reflect the degree of protection. Likely, the failure of some bacterial vaccines led to a broader view including the evaluation of multiple aspects of the immune response. For anti-viral vaccines, immunogenicity can be assessed through non-functional and functional assays [36]. For example, the neutralization assay has been applied to evaluate neutralizing antibodies to SARS-CoV-2. The fast approval of the COVID-19 vaccines was partly related to the reliability of these serological assays [37]. In this case, the availability of well-established functional assays, recognized by regulatory agencies, made the immunogenicity evaluation more straightforward. However, in the case of bacterial vaccines, functional assays involve critical biological components, such as live bacteria and differentiated immune cells, which are more difficult to standardize in comparison to the antibody-binding counterparts [38]. Further advancement in functional assays that evaluate the bactericidal and opsonization ability of the antibodies could help the development and approval of new candidate vaccines.

3. Immunologic correlates of vaccine-induced protection against bacteria

Immunogenicity is assessed at all clinical stages of the vaccine approval journey. Considering the complexity of the immune response, it is difficult to determine what are the specific immunological factors triggered against a specific pathogen. The correlate of protection (CoP) is defined as a laboratory marker of immune response, that correlates with the protection from infection, disease, or other defined endpoints [39]. Considering the redundancy of the immune system, more than one vaccine-induced response can be a CoP [40]. The establishment of a correlate of protection is fundamental for vaccine development strategies, as it should meet certain criteria to be accepted by regulatory agencies. The absolute CoP is a defined threshold considered for vaccine efficacy, while

the relative CoP is an indicative level where the efficacy may occasionally fail. When more than one immune function is involved in protection is defined as co-correlate [40]. In general, most vaccines induce protection through a synergic action of antibodies and cellular immunity, which may prevent the pathogen's diffusion through multiple mechanisms. The in-vitro evaluation of functional antibodies is the key to better characterize the features of the elicited humoral responses against bacterial pathogens [41]. Serological assays able to measure the antibody's functionality may be critical for vaccine licensure, however, there is the need for further optimizations and standardization to overcome the key barriers to the discovery of pathogen-specific CoP. Consequently, the principal CoP for bacterial infection is still represented by the antibodies measured by ELISA and there is not a defined threshold for protection for many diseases [42]. Usually, the toxin neutralization assay is employed to predict the protection against toxin-producing bacteria. However, in the case of anthrax bacillus and pertussis, a better correlation can occur with the assessment of specific IgG antibody levels against the toxins. Protection against pertussis also involves the cellular response elicitation [40]. CoPs for enteric vaccines include antibody responses but also undefined responses in the intestine [43]. In the case of *shigella* spp., protection involves bactericidal and opsonophagocytic response and intestinal IgG and IgA antibodies against the O antigen [43]. For pneumococcal conjugate vaccines have been established a protective cut-off value of IgG antibodies for all serotypes but protection involve also the antibodies' opsonophagocytic ability evaluated through opsonophagocytic assay (OPA) [44]. For meningococcal vaccines, clinical efficacy trials are not feasible due to the low incidence of Invasive Meningococcal Disease (IMD). In 1969, Goldschneider et al. reported that the presence of serum bactericidal antibodies is predictive of protection from the disease and today the serum bactericidal assay (SBA) is considered the surrogate of protection [45]. However, functional assays are more complex than antibody-binding assays; they require multiple biological factors that are not well or easy to standardize. It is also commonly agreed that traditional SBA and OPA assays can be labour-intensive, time-consuming, and require specialized equipment and well-trained personnel. Moreover,

the need for international standards is a limiting factor for functional test qualification and validation restricting their employment for vaccine efficacy assessment [46].

4. Current serological assays used for the evaluation of bacterial vaccine immunogenicity

The evaluation of immunological response is a critical aspect of vaccine development, and it is required by all regulatory agencies for vaccine licensure. In the case of bacterial vaccines, the immunogenicity is mainly evaluated through antibody-binding assay, SBA and OPA.

4.1. Antibody-binding assay

In the case of bacterial vaccines, the antibody titer is the major parameter evaluated for assessing the immunogenicity [44]. The Enzyme-Linked Immunosorbent assay (ELISA) represents the most widely serological assay used for the quantification and identification of specific antibody responses. Antibody concentration, evaluated by ELISA assay, is considered a reliable correlate of protection for most bacterial vaccines [40]. Similarly, the flow cytometry tool can be used to assess antibody binding to whole pathogens rather than to specific antigens. In addition, the multiplex technologies can allow the simultaneous identification and quantification of the antibody's binding to a substantial number of antigens related to several bacterial serotypes. For example, the Luminex platform takes advantage of the xMAP® microsphere technology, where beads are internally dyed with two or three spectrally distinct fluorochromes and covalently coupled with different capture molecules [47]. These specific combinations allow distinct analysis of multiplex data and the simultaneous assessment of the antibody responses to multiple antigens. This is particularly useful in the case of polyvalent vaccines like the Pneumococcal Conjugate Vaccine (PCV), which has broad coverage and individual serological immunoassay would be extremely time-consuming and laborious [47]. However, concerning PVC vaccines, the ELISA remains the gold standard for the detection of capsule-specific IgG antibodies [48]. Although, antibodies against pneumococcal polysaccharide are the primary mediators of bacterial opsonization and killing, other antibodies lacking functional activity are present in the

serum. Indeed, first-generation ELISA tended to overestimate antibody levels due to the presence of non-functional antibodies. At the same time, for third-generation ELISA the specificity has been improved via pre-absorption with both pneumococcal cell wall and 22F polysaccharide to remove non-protective antibodies [48,49]. Despite the improvements, it occurred that IgG antibodies did not reflect the level of protective immunity and OPA data confirmed that these antibodies were non-functional. For this reason, toxin neutralization assay, SBA and OPA, can provide information on the functional activity of the antibodies against the bacteria (Figure 1) [38].

4.2. The Serum Bactericidal Assay

The Serum Bactericidal Assay (SBA) aims to measure the capability of pathogen-specific antibodies present in the serum to activate the complement classical pathway, trigger the MAC formation, and consequently kill the target organism [38]. In general, the assay involves live bacteria, usually grown until the mid-exponential phase, incubated with serially diluted sera, and an exogenous source of complement (Figure 2) [38].

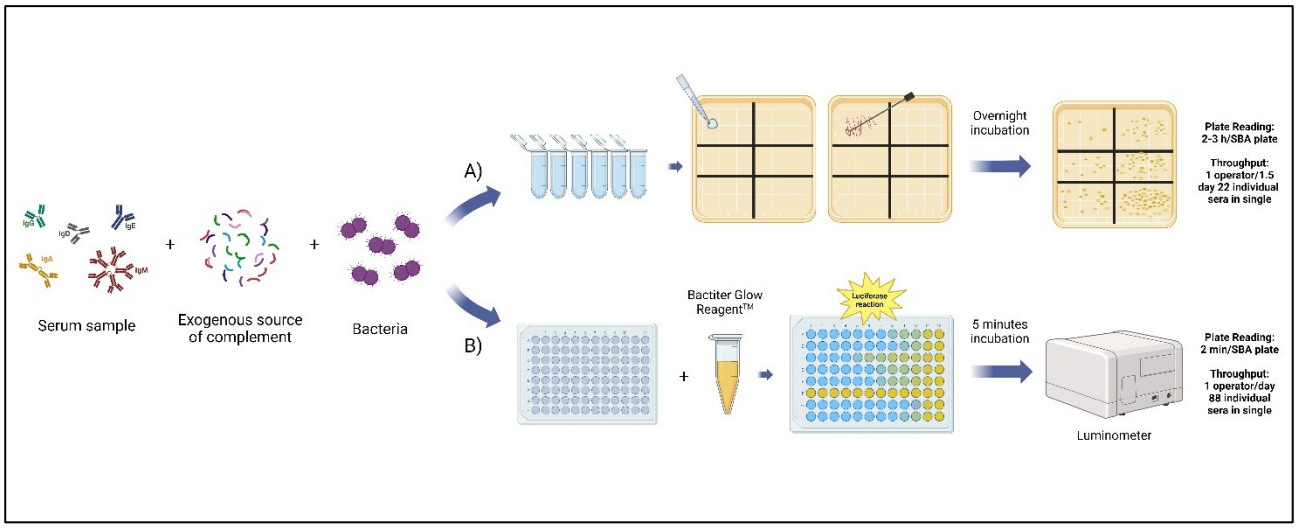

Figure 2. Graphical representation of the main steps for the Conventional SBA (A) and Luminescence-based SBA (B). The assay reaction mix is the same for the two versions of the SBA assay, composed of the serially diluted serum samples from the immunized patients, an exogenous source of complement, and bacteria. After the incubation, in the case of the C-SBA (A), the reaction mix of each well is plated on agar and incubated overnight. The day after the colony counting of each plate is performed. The readout is the colony forming unit (CFU) value for each of the sera dilution points, which is proportional to the number of surviving bacteria. In the case of L-SBA (B), the BacTiter Glow™ reagent is added to the assay plate, after the incubation, and the luminescence value is recorded within 5 min using a luminometer. The read-out is the luminescence value for each sera dilution point, which is proportional to the number of survival bacteria. The L-

SBA is a high throughput method that allows the test of 88 sera samples in single per operator in 1 day, in contrast to the C-SBA with only 22 sera samples in single per operator in 1,5 days [50]. This figure was created using BioRender.com.

Complement, bacteria, and their interaction, are the main biological factors difficult to standardize. On the other hand, the SBA makes it possible to evaluate the antibody's capacity to effectively neutralize the bacteria present in the reaction. This is a significant advantage compared to the use of binding-assays which can only estimate the specific antibodies concentration but not their functional capacity. In the classical or conventional version of the SBA (C-SBA), after the incubation time, the reaction mix is plated in solid agar plates and incubated overnight (Figure 2A). In this case, bacterial survival is evaluated through the count of the Colony Forming Units (CFU). The readout is expressed as the reciprocal titer of the serum which kills ≥50% of the bacteria when compared to the negative control [51]. Bacteria can be naturally susceptible or resistant to complement-mediated killing, even in the absence of antibodies. If the bacteria are too sensitive or resistant to complement action, the SBA assay is not applicable [46]. Theoretically, the complement source of choice should be from the same host species, as the assay should mimic the real immune response during the infection [38]. For this reason, the SBA assay used to evaluate the bactericidal activity against human host pathogens should involve human serum as a complement source. However, in the case of *Neisseria meningitidis*, the human complement from healthy donors can have intrinsic bactericidal activity against meningococci and the procurement of large amounts of those lots with normal hemolytic activity represents a problem [51,52]. Consequently, the human complement source must be validated in advance for each isolate to avoid interference with the assay result. The potential solution is the use of a complement source different from the host species. The rabbit complement is the most widely used as it was found to be a reliable and suitable source of complement for SBA (rSBA). However, while the rSBA has been used for the evaluation of MenACWY vaccines, in the case of the MenB vaccine only human complement (hSBA) is appropriate due to the higher SBA titer obtained with rabbit complement [39]. In the case of encapsulated bacteria, like *Haemophilus influenzae* type b (Hib) and *N. meningitidis* type A and C (men A, C), the SBA is considered the co-CoP together with the ELISA assay (Hib:

ELISA Long term: ≥ 1.0 g/mL, Short term: ≥ 0.15 g/mL, SBA ≥ 4 titer; men A, C: rSBA ≥8 titer or hSBA ≥4 titer)[39]. On the other hand, the assay standardization has been successfully carried out only for Men A and C serogroups, using baby rabbit sera as a complement source. No standardization for Men B, W135, and Y has been established up to now. However, Borrow et al. evaluated and optimized parameters for interlaboratory reproducibility of the assay [53]. For MenB vaccine evaluation the hSBA titer ≥1:4 is considered the threshold for protective immunity against IMD [51]. No formal method has been established due to discrepant results between laboratories, which have been reconducted to the different epidemiology of prevalent strains, making the choice of a "universal" reference strain difficult. This is particularly true in the case of protein-based vaccines, where the antigens included in the vaccine formulation may not be expressed by all the different wild-type strains. For example, for MenB vaccines only strain-specific protein-based vaccines were developed to avoid the possibility of autoimmunity. As the protein antigens sequence and expression can vary among the same serotype, the vaccine coverage against circulating strains is difficult to establish [54]. The bactericidal activity of the immunized serum samples, evaluated through SBA, increases with the number of antigens expressed by the strains and their affinity to vaccine antigens [54,55]. Consequently, the choice of the strains to be tested should consider this aspect for the immunogenicity assessment, especially to avoid very large and pointless efficacy trials [54]. To evaluate the strain coverage of the multicomponent protein-based vaccine against MenB (4CMenB (Bexsero)) the Meningococcal Antigen Typing System (MATS) has been developed. The MATS is a sandwich ELISA that uses polyclonal rabbit antibodies raised against the three antigens included in the vaccine (factor-H-binding protein, neisserial heparin-binding antigen, and neisserial adhesin A) applied to bacterial lysates [55]. The authors find out that MATS relative potency could be used as a valid parameter to predict the killing in a large panel of strains [54,55]. For the same purpose, the MEASURE assay has been developed to predict the bactericidal response induced by the Bivalent rLP2086 (Trumenba) vaccine, composed of two variants of the factor H binding protein (fHbp) [56]. This assay, based on flow cytometry, can be used to evaluate the surface expression of a large collection of MenB isolates, using an anti-fHbp

cross-reactive antibody. In this way, it becomes possible to predict the vaccine-induced strain susceptibility in SBA [56]. The complications in standardizing SBA for meningococcus Groups B, W135, and Y are the same as those encountered for other bacterial species. On the other hand, the evidence that vaccine-elicited functional antibodies can represent a valid serological CoP encouraged the development of SBA assay for multiple bacterial strains [57]. The enumeration of the CFU as readout of the C-SBA influences several assay parameters, including the assay volume, the number of bacteria, the number of replicates, and the duration of the experiment. Consequently, the conventional SBA is considered labour-intensive, with a high inter-operator and inter-laboratories variability [58]. Even if the introduction of automated colony counting represented an improvement, the conventional assay still is not applicable to test a large number of serum samples [59,60]. The need for an alternative high throughput SBA is derived from the awareness that antibody functionality is an important parameter to consider during vaccine efficacy assessment.

The Luminescence-SBA (L-SBA) applies to the same principles of the C-SBA but evaluates the antibody bactericidal activity by quantifying the ATP level as a correlate of bacterial survival (Figure 2B) [59]. In L-SBA the level of luminescence detected is directly proportional to the number of bacteria present in the assay wells, which is inversely proportional to the level of functional antibodies present in the serum [61]. Therefore, serum bactericidal titer obtained by the luminescence readout method strongly correlates with the data obtained by the conventional agar plate-based assay [59]. The L-SBA allows us to avoid the major disadvantages of the conventional SBA and can be used as a high throughput functional assay for vaccines' immunogenicity evaluation. Shimanovich and co-authors have demonstrated that the SBA is a potential CoP for Shigella since it can evaluate the bactericidal activity elicited by vaccination, associated with a reduction of shigellosis in humans [62]. The equivalence of the results obtained with the L-SBA compared to the CFU-based method have been demonstrated for several pathogens, including *Citrobacter freundii, Salmonella* serovars Typhimurium and Enteritidis*, Shigella flexneri* serotypes *2a* and *3a, Shigella sonnei, Neisseria*

meningitidis [50]. The same team has also demonstrated the applicability of a High-Throughput L-SBA for 9 bacterial species: *S. sonnei*, *S. flexneri 1b*, *S. flexneri 2a*, *S. flexneri 3a*, *S. typhimurium*, *S. enteritidis, S. Paratyphi A*, and *C. freundi* along with a further optimization using 384-wells-plate format instead the 96-wells one [50]. Another version of the conventional SBA is represented by the use the Resazurin (R-SBA), a metabolic indicator that gives a fluorescence readout when is irreversibly reduced to resorufin by living bacteria. Stazzoni and co-authors developed this method for screening monoclonal antibodies against Neisseria gonorrhoea and compared it to L-SBA and C-SBA [63]. The same fluorescent readout has been applied to develop a high-throughput version of SBA for meningococcal strains which maintains a good agreement with the conventional assay results [64].

5. *Borrelia burgdorferi*

Borrelia burgdorferi is a Gram-negative spirochete transmitted to humans through *Ixodes* ticks, that is the causative agent of Lyme disease (LD) [65]. *B. burgdorferi* in the ticks is mainly located in the midgut lumen, where the spirochaetes is able to avoid the tick innate immune responses [66]. *B. burgdorferi* is part of the Lyme Borrelia group which contains 19 species, however only 4 are able to cause human infection (*Borrelia burgdorferi sensu stricto*, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia bavariensis*)[65]. The path of LD is divided into different stages: early localized, early disseminate and late LD phase. The early localized infection typically presents a painless skin rash which can be treated with antibiotics. Usually, an early treatment with antibiotics efficiently kills the pathogen and cures most LD cases [66]. In the 10-20% of cases even after an extended period of antibiotic therapy can occur the persistent disease which is associated to symptoms such as headache, fatigue, and joint pain. *B. burgdorferi* present a high number of specific virulence factors such as outer-surface protein OspC, OspA and VlsE [66]. When *B. burgdorferi* is inoculated in immunocompetent inbred mice, antigen-specific antibodies are generated, like strong IgM and IgG antibody responses in particular to OspA and OspB but also to flagellin [67].

Currently no approved vaccines against LD for human use is available, however *B. burgdorferi* express several surface-exposed proteins such as BBA52, BB0405, BBI39, DbpA, BBK32, OspC which were shown to be promising vaccine candidates. Multivalent OspA-based formulations are currently common targets in vaccine development [66].

The only approved vaccine to prevent LD was LYMERix, that was based on the recombinant surface protein called outer-surface protein A (OspA). However, due to the numerous reports of severe side effects like arthritis associated with vaccination, the vaccine was pulled from the US market [68].

Currently, the Valneva-Pfizer "VLA15" is the only LD vaccine candidate in advanced clinical development (phase 3). It is a multivalent recombinant protein vaccine targeting six serotypes of the most common borrelia strains found in the United States and Europe (Lyme Disease - VLA15 - Valneva). Results of Phase 2 showed that VLA15 is generally safe, well tolerated, and elicited functional antibody responses to all six OspA vaccine serotypes. Functional antibody titers , evaluated using the SBA assay, raised at 1 month after the third vaccination, and significantly correlated with the ELISA titres [69].

6. *Shigella flexneri* 2a

Shigella is a gram-negative non-spore forming and non-motile that belong to *Enterobacteriaceae* family and it is invasive pathogen and etiologic agent of human shigellosis [70]. *Shigella* species comprises *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Shigella boydii* serogroups, also known as *Shigella* subgroups A, B, C, and D, respectively [71]. These four species are distinguished based on different biochemical reactions and O antigen lipopolysaccharide [72]. Most used method for the identification of *Shigella* subgroups and serotypes is the agglutination method by using commercial antisera [70]. However, this method is expensive, time-consuming and can led to improper interpretation related to the limit of the visual evaluation of the agglutination reactions [70]. More effective alternative can be the multiplex PCR assay targeting the gene involved in O-antigen synthesis and the FACS-based serotyping described in this work.

Nowadays, the antibiotic treatment plays a key role in reducing prevalence and death rates of shigellosis. However, antibiotic incorrect use or overuse has increases antibiotic resistance in *Shigella* spp. [73]. *Shigella* has been listed by the WHO among those pathogens for which the development of new effective strategies is a priority [74]. Since *Shigella* is a human-restricted pathogen, animal infection does not mirror human infections well, testing candidate *Shigella* vaccines in humans seem to be the most valuable way to assess their efficacy. For this purpose, *Shigella* CHIMs trials have been established, however since they are conducted in adults in high-income countries, the appropriateness of their use to assess vaccine efficacy in infants in LMICs is controversial [75]. Moreover, the necessity for multivalency vaccines increases complexity to the development of new vaccines.

Currently, only two *Shigella* vaccines are on the market, however these are limited to Russia (Shigellvak, solution of LPS extracted from *S. sonnei* bacteria) and China (FS, a live attenuated, oral bivalent *S. flexneri* 2a and *S. sonnei* vaccine developed at the Lanzhou Institute of Biological Products). Further candidate vaccines based on different technologies are in different clinic phases [75]. LimmaTech Biologics developed a recombinant glycoconjugates produced in genetically engineered *E. coli*, with a bioconjugate against *S. flexneri* 2a (phase 1 clinical trial) [76]. A well-defined synthetic glycoconjugate vaccine made of synthetically produced *S. flexneri* 2a oligosaccharides chemically linked to tetanus toxoid (TT) carrier protein have been proposed by the Institute Pasteur (phase 2 clinical trial and CHIM studies)[77].

The traditional glycoconjugate approach has been used to develop a bivalent vaccine (ZF0901) by Beijing Zhifei Lvzhu Biopharmaceuticals, composed by *S. sonnei* and *S. flexneri* 2a OAg conjugated to TT (phase 3 study) [78].

GSK Vaccines Institute for Global health (GVGH) developed a *S. sonnei* antigen (Generalized Modules for Membrane Antigens) combined with antigen from *S. flexneri* 1b, 2a, and 3a in a four-component formulation (altSonflex1-2-3) (phase 1–2 clinical trials)[79].

7. Invasive Non-typhoidal *Salmonella (*iNTS*)* and *Salmonella* Enterica Paratyphi A

All *Salmonellae* species are members of the family *Enterobacterales* and a Gram-negative non-lactose fermenting, motile intracellular bacterium [80]. The genus *Salmonella* is divided taxonomically into two species: *S. enterica* and *S. bongori*. The *S. enterica* is divided into six distinct subspecies (S. *enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, S. *enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and S. *enterica* subsp. *Indica*), however around 99 % of all human *Salmonella* infections are caused by *S. enterica* subsp. *enterica* serovars. Among the *S. enterica* subsp. *enterica* the most relevant serovars are the typhoidal *Salmonella* (TS) serovars (*S.* Typhi and *S.* Paratyphi) and the non-typhoidal *Salmonella* (NTS) (S. Typhimurium and S. Enteritidis) [81]. In humans, the TS are the cause of the typhoid fever, a systemic febrile illness, while the NTS results in diarrheal disease but also severe, extra-intestinal, invasive bacteraemia, referred to as invasive NTS (iNTS) disease [82]. The high mortality and morbidity related to TS and iNTS, as well as the increasing prevalence of multidrug resistant strains have encouraged the development of new vaccines candidates [83].

Bharat Biotech International, Ltd (Hyderabad, India) has licensed the live oral *S.* Typhi vaccine strain, (CVD 909) and live attenuated vaccine for *S.* Paratyphi strains (CVD 1902) [84]. Two live-attenuated iNTS vaccines candidate (*S.* Typhimurium) have been conducted in humans. Both studies resulted safe and generally well tolerated, however neither achieved strong or uniform immunogenicity [85,86].

GSK Vaccines Institute for Global Health (GVGH) developed a bivalent formulation of *S.* Typhimurium and S. Enteritidis antigen-based vaccine (iNTS-antigen) and a trivalent combination of iNTS antigen with the glycoconjugate Vi-CRM_{197} (iNTS-TCV). Both vaccines resulted to mediate complement-mediated killing also against a broad panel of epidemiologically relevant heterologous *Salmonella* species [87].

More recently, their attention has shifted to the antigen platform, which demonstrated comparable immunogenicity to O-antigen conjugates vaccine with manufacturing and dose-sparing advantages.

8. *Neisseria Meningitidis*

N. meningitidis is a Gram-negative capsulated diplococcus that can cause septicemia and meningitis in susceptible individuals, mainly in infants under 1 year of age, teenagers, and young adults [88]. There are twelve meningococcal serogroups each with a chemically distinct capsular polysaccharide; of these, six serogroups, A, B, C, W, X, and Y, cause disease [88]. *N. meningitidis* is a causative agent of invasive meningococcal disease (IMD) characterized by severe illnesses such as meningitis and septicemia. Vaccination can effectively control and prevent invasive meningococcal disease (IMD) [89]. Among the 12 known serogroups of *N. meningitidis* that are defined by the antigenicity of the meningococcal capsule, six—A, B, C, W, X, and Y—are responsible for the vast majority of IMD cases around the globe [90]. Moreover, meningococcal isolates can be classified according to their genotypes into sequence types (ST), which can be clustered into clonal complexes (CC). The sequence data that allow this genotyping can now be extracted from whole genome sequences (WGSs) using molecular tools available on the PUBMLST platform (https://pubmlst.org/ accessed on 18 November 2024). Several CC are considered hyperinvasive and are responsible for most IMD cases, including CC11, CC32, CC41/44, and CC269 [91]. Nevertheless, the distribution of these serogroups is not static; it varies geographically and fluctuates over time due to shifts in population immunity, the introduction of vaccination programs, and localized outbreaks.

However, beyond these six common serogroups, the less frequent or "rare serogroups" of *N. meningitidis*, including E and Z, non-groupable (NG) isolates, and serogroup X in Europe, have also been sporadically implicated in IMD cases. Such isolations constitute a marginal proportion of overall IMD cases in the general population. The incidence and pathogenicity of these rare serogroups are described less often, given their minimal impact on public health in comparison to the more prevalent serogroups. As a result, literature on their epidemiology remains limited [91]. Despite this, some studies suggest that patients with terminal complement pathway deficiencies exhibit higher susceptibility to both rare meningococcal serogroups (like serogroup E) and NG strains, being inherently defective in forming the terminal C5–9 membrane attack complex (MAC) on the bacterial surface and therefore

complement-mediated bacterial clearance [92]. Additionally, recent advances in monoclonal antibody therapies targeting complement factor C5, such as eculizumab and, more recently, C3, have revolutionized the management of diseases caused by the dysregulation of the complement system like paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). However, these treatments also induce acquired complement deficiencies by specifically inhibiting the terminal complement pathway, leaving treated patients vulnerable to infections from encapsulated bacterial pathogens, including rare meningococcal serogroups [93].

While current vaccines targeting serogroup B (4CMenB and fHbp bivalent vaccines) are also effective against other highly prevalent serogroups such as C, W, and Y [94], there is limited data regarding their efficacy against rare serogroups, such as serogroup E. Moreover, complement deficiencies (acquired and hereditary) impair bacterial lysis despite vaccination [95,96]. Given this uncertainty, further research is required to assess whether existing vaccination strategies provide adequate protection against these less common isolates.

9. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae (also known as the gonococcus) is the etiologic agent of a sexually transmitted infection (STI) called gonorrhea [97]. Together with *N. meningitidis*, *N. gonorrhoeae* represent the pathogenic species of the genus *Neisseria* [97]. Between the two species there is 80–90% genetic homology, however there are several differences in disease manifestation [98]. *N. gonorrhoeae* is an obligate human pathogen which causes urethritis in men and cervicitis in women. Since the past 70 to 80 years Gonorrhea has been treated successfully by use of antimicrobials. However, nowadays an international high prevalence of *N. gonorrhoeae* strains with resistance to several antimicrobials have been recorded [99]. In 2024 World Health Organization (WHO) surveillance of clinical strains of *N. gonorrhoeae* published a list of strains that are resistant to most available antibiotics, highlighting the imminent risk of widespread untreatable gonorrhea infections [99]. The pathogenesis of the gonococcus is based on the capacity of the bacteria to adherence to the mucosa epithelium, which is

mediated through different bacterial surface structures that include Type IV pili, opacity (Opa) proteins, the LOS, and the major porin, also referred to as PorB. The major feature of host adaptation of *N. gonorrhoea* is the ability to evade recognition and attack from the human complement system. Indeed, *N. gonorrhoeae* shows susceptibility to animal complement systems to but not to human complement [97,100]. Gonococcus is able to escapes complement-mediated killing by inhibiting complement cascade components (preventing MAC formation), expressing host molecules on the bacterial surface, and binding to complement regulatory proteins [97].

Vaccine development for *N. gonorrhoeae* has been difficult due to unpredictable nature of this bacteria, the lack of knowledge on which aspect of the immune response need to be target to achieve protective immunity, and the absence of robust animal models that accurately mimic the disease [101]. The establishment of a correlates of protection to gonococcal infection and disease is because natural infection doesn't generally protect against reinfection [102]. Until now no vaccine for gonorrhea are licensed, and all vaccine candidates tested in efficacy studies in humans have failed [102]. However, surveillance data suggest that outer membrane vesicle (OMV) meningococcal group B vaccines affect the incidence of *gonorrhoea* [98].

MATERIALS AND METHODS

10. Development of Luminescence-Based Serum Bactericidal Assay for *Borrelia burgdorferi* B31

10.1. Bacterial stocks for *Borrelia burgdorferi* B31

B. burgdorferi B31 (ATCC 35210) was grown in 12 ml of Complete BSK-H medium (BioConcept) (with 6% of Rabbit Serum (Bio&Sell)) for 72 hours at 33°C. After 3 days, the bacterial concentration was adjusted to 5 x 10^7 spirochetes/ml in complete BSK-H medium containing with 20% of glycerol (Sigma-Aldrich) and stored at -80°C for long-term storage.

10.2. Correlation between Luminescence value and Bacterial concentration

The luminescence signal is directly correlated to the bacterial ATP content and therefore measures bacterial viability. Specifically, the luminescence signal is obtained by adding the BacTiter-Glo Reagent (Promega) in the reaction mix. BacTiter-Glo contains a luciferase that oxidizes luciferin and consequently generates light in the presence of ATP Bacterial viability activity was tested measuring the bacterial ATP content [59].

B. burgdorferi B31 was grown in Complete BSK-H medium for 72 hours at 33°C (5% CO_2) and adjusted at the concentrations of 5 x 10^7 spirochetes/ml. The bacterial solution was diluted 2-fold and 3-fold in Complete BSK-H medium. In a plastic round-bottom 96-well plate, 200 µl/well of bacterial suspension was added in the row B and C of column 1, and 150 µl/well of bacterial suspension was added in the row D and E of column 1. 100 µl/well were added from B2 to E12 wells. To obtain serial 1:2 dilutions, 50µL were transferred starting from row B-C of column 1 to column 2 and then again from column 2 to column 3 and so on until column 12 was reached. The last 50µL were discarded. To obtain serial 1:3 dilutions, 50µL were transferred starting from row D-E of column 2 to column 3 and then again from column 3 to column 4 and so on until column 12 was reached. The last 50µL were discarded. The plate was centrifuged at 3220 g for 15 minutes, and the pellet was resuspended in 100 µl of PBS1X (Sigma-Aldrich) and 50 µl was transferred into a flat Costar White Opaque 96-

well. The luminescence signal was obtained by adding 50 µl of Promega BacTiter-Glo reagent and by reading luminescence using a multimode reader (Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

10.3. *B. burgdorferi* B31sensitivity to Guinea Pig Complement

B. burgdorferi B31 was grown in Complete BSK-H medium for 72 hours at 33°C (5% CO_2) and adjusted at the concentrations of 1 x 10^6 spirochetes/ml. Two solutions containing the active and heat inactivated (30 minutes at 56°C) Guinea Pig Complement (GCP and HI-GPC) diluted at 8% (2% final dilution in the assay) and 4% (1% final dilution in the assay) in BSK-H medium were prepared. 100 µl/well of bacterial suspension were added in all wells from A4 to B12 and wells F10-F12. 50 µl/well of active guinea pig complement at 8 % were added in wells A4-A9; 50 µl/well of active guinea pig complement at 4% in wells B4-B9. Add 50 µl/well of HI guinea pig complement at 8 % were added in wells A10-A12 and 50 µl/well HI guinea pig complement at 4 % were added in wells B10- B12. 50 µl/well of heat inactivated positive mouse serum diluted 1/125 (1/500 final dilution in the assay) was added in wells A4-A6 and B4-B6. The plate was sealed with a sealing tape, covered with the lid, and incubated for 72 hours at 33°C (5% CO_2). After 3 days, the plate was centrifuged at 3220 g for 15 minutes, and the pellet was resuspended in 100 µl of PBS1X (Sigma-Aldrich) and 50 µl was transferred into a flat Costar White Opaque 96-well. The luminescence signal was obtained by adding 50 µl of Promega BacTiter-Glo reagent and by reading luminescence using a multimode reader (Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

10.4. Comparison of different sources of GPC

B. burgdorferi B31 was grown in Complete BSK-H medium for 72 hours at 33°C (5% CO2) and adjusted at the concentrations of 1 x 10^6 spirochetes/ml. Six different solutions containing the active and heat inactivated (30 minutes at 56°C) Guinea Pig Complement (GCP and HI-GPC) at 8% (2% final dilution in the assay) in BSK-H medium were prepared. Following the plate layout reported in

Figure 3, 100 µl/well of bacterial suspension were added in all wells from A4 to C12 and wells F10-F12. 50 µl/well of active guinea pig complement at 8 % were added in row A, B and C from column 4 to 9 for the 3 diverse sources of GPC. Add 50 µl/well of HI GPC solutions in row A, B and C from column 10 to 12 for the 3 diverse sources of GPC. 50 µl/well of heat inactivated positive mouse serum diluted 1/125 (1/500 final dilution in the assay) was added in wells A4 to C6. The plate was sealed with a sealing tape, covered with the lid, and incubated for 72 hours at 33°C (5% CO2). After 3 days, the plate was centrifuged at 3220 g for 15 minutes, and the pellet was resuspended in 100 µl of PBS1X and 50 µl was transferred into a flat Costar White Opaque 96-well. The luminescence signal was obtained by adding 50 µl of Promega BacTiter-Glo reagent and by reading luminescence using a multimode reader (Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

10.5. Luminescence-Based Serum Bactericidal Assay for *B. burgdorferi* B31

B. burgdorferi B31 was grown in Complete BSK-H medium for 72 hours at 33°C (5% CO_2) and adjusted at the concentrations of 1 x 10^6 spirochetes/ml. Solutions containing the active and heat inactivated (30 minutes at 56°C) GCP (HIGPC) diluted at 8% (2% final dilution in the assay) in BSK-H medium were prepared. The sera sample was serially with 1:2 dilutions, 50µL were transferred starting from wells A1- A7 with a multichannel pipette from row A to row B, pipette 3-5 times to mix, and continued the dilution series up to row H and discarded the leftover volume. 50 µl/well of Active GPC solution were added form column 1 to 8 and 50 µl/well of HI GPC were added in column 9. Then, 100 µl/wells of bacterial suspension were added in each assay well. Finally, 50 µl/wells of BSK-H were added in each assay well. The plate was sealed with a sealing tape, covered with the lid, and incubated for 72 hours at 33°C (5% CO_2). After 3 days, the plate was centrifuged at 3220 g for 15 minutes, and the pellet was resuspended in 100 µl of PBS1X (Sigma-Aldrich) and 50 µl was transferred into a flat Costar White Opaque 96-well. The luminescence signal was obtained by adding 50 µl of Promega BacTiter-Glo reagent and by reading luminescence using a multimode reader

(Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

10.6. Precision assessment of the L-SBA for *B. burgdorferi* B31

In order to assess the precision of the assay, the methods described in section 10.5 was performed by three analysts over two different days. The precision of the method expresses the ability of a measurement to be consistently reproduced. It is considered at two levels: repeatability and intermediate precision. The positive mouse serum (starting dilution 1:128) have been tested in duplicate as positive control sera, while a pre-immune mouse serum (starting dilution 1:128) was tested in single as a negative control serum. The assay controls in the plate were: the active GPC, the HI-GPC, the negative control (bacteria only) and mAb (positive control) at the final concentration of 0,8 μg/mL).

The percentage coefficient of variation (%CV) has been calculated based on the base-10 logarithmic transformation of the IC50 results. For repeatability, the %CV will be determined for each operator on each day, resulting in a total of six individual %CV values. The intermediate precision will be calculated per day, considering the IC50 results from both operators, while also analysing the results separately for each operator based on the data collected over the three days. The calculated CV% should not exceed 15%.

11. Qualification of a Luminescence-Based Serum Bactericidal Assay for *Shigella flexneri 2a.*

11.1. Working stocks preparation for *Shigella flexneri 2a*.

*Shigella flexneri 2a s*train 2457T (ATCC-700930) was grown in 12 ml of LB medium (Gibco) at 37°C, overnight with gentle shaking. The day after, the bacterial concentration was adjusted OD_{600} of 0,5 LB medium and grown until OD_{600} equal of 2. The bacterial culture was centrifuged at 3220g for 5 minutes and resuspended in equal volume of a solution containing LB with 20% of glycerol (Sigma-Aldrich) and stored at -80°C for long-term storage.

11.2. FACS-based Serotyping experiment for *Shigella flexneri 2a*

11.2.1. Titration Experiments of primary and secondary antibody

Plastic round-bottom 96-well plates were coated with *Shigella flexneri 2a* cultures (50µL/well) grown to exponential phase (OD_{600} = 0,18-0,25), the plate was centrifuged for 7 minutes at 3220g and the pellets were resuspended in 150µl of Polyvalent and/or Monovalent sera against *Shigella flexneri* 2a (Denka Seiken, Biogenetics) diluted at 1:50, 1:100, 1:200 and 1:400 (diluted in PBS with 1% of BSA (Sigma-Aldrich)) following the plate layout reported in Figure 4. The plate was incubated for 1h at RT before being centrifuged for 7 minutes at 3220g. Each pellet was resuspended in 150µl of PBS1X-BSA1% containing secondary antibody (BV421 Goat Anti-Rabbit IgG, BD Horizon™) diluted 1:250, 1:500, 1:1000 and1:2000 (diluted in PBS-BSA1%) following the plate layout reported in Figure 3, and the plate was incubated for 30 minutes at RT in the darkness. The samples washed twice in 100µL of PBS-BSA1%, and were fixed with 100µL/well of 1% paraformaldehyde (Sigma-Aldrich) prepared in PBS1X for 30 minutes at RT. Then the plate was centrifuged for 7 minutes at 3220 x g and washed in 100µL of PBS1X and again centrifuged for 7 minutes at 3220 x g. Finally, each pellet was resuspended in 100µl of PBS1X-BSA1%, from which 50µL were transferred to a mirror 96-well round-bottom plate where 100µl of PBS1X-BSA1% were added to each well. The plate was stored at 4°C, overnight in the dark. The plate was acquired by the Cytometer Cytoflex LX (Beckman) according to the manufacturer's recommendations and analyse and plot the data by using FlowJo.

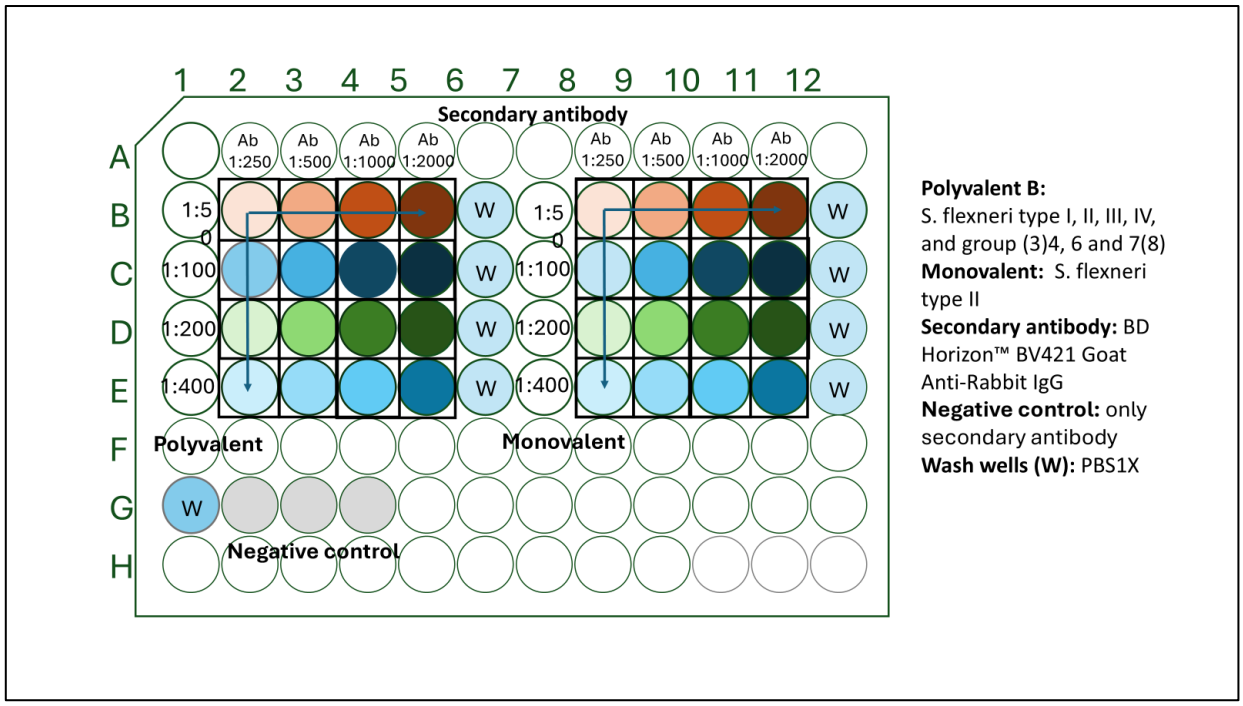

Figure 3. Plate layout used for the titration experiment of Shigella flexneri 2a.

11.2.2. Serotyping for *Shigella flexneri* 2a

Plastic round-bottom 96-well plates were coated with *Shigella flexneri 2a* cultures (50µL/well) grown to exponential phase (OD_{600}= 0,18-0,25), the plate was centrifuged for 7 minutes at 3220 x g and the pellets were resuspended in 150 µl of Polyvalent and/or Monovalent sera against *Shigella Flexneri 2a* diluted in PBS with 1% of BSA at 1:400 and 1:200, respectively. The plate was incubated for 1h at RT before being centrifuged for 7 minutes at 3220 x g. Each pellet was resuspended in 150µl of PBS1X-BSA1% containing secondary antibody (BV421 Goat Anti-Rabbit IgG, BD Horizon™) diluted in PBS with 1% of BSA at 1:250, and the plate was incubated for 30 minutes at RT in the darkness. The samples washed twice in 100µL of PBS-BSA1%, and were fixed with 100µL/well of 1% paraformaldehyde prepared in PBS1X for 30 minutes at RT. Then the plate was centrifuged for 7 minutes at 3220 x g and washed in 100µL of PBS1X and again centrifuged for 7 minutes at 3220 x g. Finally, each pellet was resuspended in 100µl of PBS1X-BSA1%, from which 50µL were transferred to a mirror 96-well round-bottom plate where 100µl of PBS1X-BSA1% were added to each well. The plate was stored at 4°C, overnight in the dark. The plate was acquired by the Cytometer

Cytoflex LX (Beckman) according to the manufacturer's recommendations and analyse and plot the data by using FlowJo.

12. Luminescence Based Serum Bactericidal Assay for *Shigella flexneri 2a*

Bactericidal activity on *Shigella flexneri 2a* strains was tested using Luminescence-based Serum Bactericidal Assay (L-SBA). The L-SBA assay takes advantages of bacterial ATP content as a correlate of bacterial viability [59]. *Shigella flexneri 2a* cultures was grown to exponential phase (OD_{600}= 0.18-0,25), diluted 1:100 in PBS1X and mixed with Baby Rabbit Complement (BRC) with a final dilution of 7,5%. The sera sample (previously heat inactivate for 30 minutes at 56°C) and the mAbs pool were serially diluted (2-fold) in PBS1X in a final volume of 25µL until column 11. 75 µL/wells of the solution containing BRC and diluted bacteria were added in the assay wells from column 1 to 12. The plate was sealed with a sealing tape, covered with the lid, and incubated for 3 hours at 37°C (5% CO_2). After 3 hours, the plate was centrifuged at 3220 g for 10 minutes, and the pellet was resuspended in 100 µl of PBS1X and 50 µl was transferred into a flat Costar White Opaque 96-well. The luminescence signal was obtained by adding 50 µl of Promega BacTiter-Glo reagent and by reading luminescence using a multimode reader (Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

12.1. Qualification experiments of the Luminescence-Serum Bactericidal Assay for *Shigella flexneri 2a*

The qualification of the Luminescence-Based Serum Bactericidal Assay for *Shigella flexneri* 2a aim to demonstrate that the methods is appropriate for serological detection of anti-*S. flexneri* 2a functional antibody titers in human serum samples. The purpose of the L-SBA is to detect and measure serum-neutralizing antibodies in subjects belonging to epidemiology studies, preclinical studies or vaccine clinical trials. The assay parameters assessed were Precision (distinguished between Repeatability – i.e., Intra-assay - Precision, and Intermediate - i.e., Inter-assay – Precision),

Dilutional Linearity, Specificity and Lower Limit of Quantification (LLoQ)/Limit of Detection (LOD).

- Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions. Precision has been considered at two levels: repeatability and intermediate precision:

Repeatability is the variability observed within a single run of a specific sample. It measures the precision of the assay when conducted under consistent conditions, such as the same day, operator, and run. In contrast, intermediate precision accounts for the variability across multiple runs and replicates of the same sample. This means it assesses the assay's precision over different days, with various operators, and across multiple running conditions.

For both parameters have been used the serum samples which cover the analytical range of the assay:

- Low titer sera sample tested in 8 replicates with starting dilution 1:40;
- Medium titer sera sample tested in 8 replicates with starting dilution 1:100;
- High titer sera sample tested in 16 replicates with starting dilution 1:500;

Serum samples were serially diluted in 2-fold steps. Each operator ran 4 plates per day for 3 days. For each sample, the IC50 values were determined through six runs conducted over three days by two operators. These results were analysed to evaluate the precision of the assay in terms of percentage coefficient of variation (%CV). The performance target for both intermediate precision and repeatability was a %CV not exceeding 50%.

- Dilutional Linearity

The linearity of dilution experiment provides information about the precision of assay results for samples tested at different levels of dilution in the chosen sample diluent. The linearity has been

assessed by pre-diluting the high titer sera sample in a 3-fold step dilution scheme, starting from the neat serum to the highest dilution expected to yield an SBA titre below the expected lower limit of quantitation of the assay. Each pre-diluted serum sample has been run as an independent sample, serially diluted in 2-fold step and tested in duplicates, performing two identical microplates (with each replicate in a different microplate), by 2 different operators, on 3 different days.

Dilutional linearity has been evaluated utilizing dilutions whose coefficient of variation (CV) did not exceed 25%. A linear regression analysis has been performed on the base-3 logarithmic transformation of the geometric mean of the estimated IC50 values, in relation to the base-3 logarithmic transformation of the dilution levels. The performance target for the regression analysis included a coefficient of determination (R-squared) of at least 0,95 and a slope that falls within the range of 0,8 to 1,2.

- Specificity

In order to evaluate the ability of the assay to exclusively detect the target analyte, the high titer sera sample has been diluted 1:1 in PBS only or spiked with different amounts of the purified homologous LPS (500, 250, 100, 50, 10, 5, 1 µg/mL) and compared with the High QC control serum spiked with PBS only (non-depleted). The lowest concentration of the homologous LPS Ag able to inhibit the titer of ≥ 70% compared to the non-depleted sample, has been used for LPS antigens of the heterologous STs and of the unrelated species (*Porphyromonas gingivalis*), the inhibition of the IC50 compared to the control serum (spiked with PBS only) must be < 30%. Each spiked serum sample has been tested in two replicates and serially diluted in 2-fold step.

The inhibition rate was expressed as % reduction compared to the control serum was calculated as follows:

*Reduction %= 1-(IC50 of spiked serum/IC50 of control serum)] *100*

- Limit of Detection (LOD) and Lower Limit of Quantification (LLoQ)

The limit of Detection (*LoD*) and the limit of Quantitation (*LoQ*) are the lowest SBA titer that can be detected under the assay conditions and the lowest SBA titer that can be quantified with suitable precision, respectively. One sample of commercial IgG-depleted human serum has been used to calculate the limits of detection for all Shigella STs. One run with 12 replicates of the same serum has been performed by one operator in one day (2 plates). Each replicate has been tested neat and serially diluted in 2-fold step.

Calculations of LoD and LoQ were performed according to the ICH guideline Q2(R2)[103] by using the standard deviation (SD) of log-transformed SBA titers obtained for the samples and the lowest serum concentration evaluated in the assay according to the following formulas:

$$LoD = 10\text{^}(\ 3.3 * SD)\ * X$$

$$LoQ = 10\text{^}(10 * SD) * X$$

13. Luminescence-Based Serum Bactericidal Assay for Salmonella iNTS

Bactericidal activity on Salmonella iNTs strains (*Salmonella* Typhimurium (STm) and *Salmonella* Enteritidis (Sen)) were tested using Luminescence-based Serum Bactericidal Assay (L-SBA). The L-SBA assay takes advantages of bacterial ATP content as a correlate of bacterial viability[59]. The bacteria cultures were grown to exponential phase (OD_{600}= 0.18-0,25), diluted 1:500 in PBS1X and mixed with Baby Rabbit Complement (BRC) with a final dilution of 50%. The sera sample (previously heat inactivate for 30 minutes at 56°C) were serially diluted (2-fold) in PBS1X in a final volume of 25µL until column 11. 75 µL/wells of the solution containing BRC and diluted bacteria were added in the assay wells from column 1 to 12. The plate was sealed with a sealing tape, covered with the lid, and incubated for 3 hours at 37°C. After 3 hours, the plate was centrifuged at 3220 g for 10 minutes, and the pellet was resuspended in 100 µl of PBS1X and 50 µl was transferred into a flat Costar White Opaque 96-well. The luminescence signal was obtained by adding 50 µl of Promega

BacTiter-Glo reagent and by reading luminescence using a multimode reader (Molecular Device). The plate was incubated for 4 minutes at RT in orbital shaker, and the luminescence read within 5 minutes.

13.1. Qualification of the Luminescence-Based Serum Bactericidal Assay for *Salmonella* iNTS

The scope of this qualification is to demonstrate and document that the L-SBA assay for invasive Nontyphoidal *Salmonella* (iNTs) spp. is suitable for the detection and quantitation of bactericidal activity of antibodies in clinical samples. The purpose of the L-SBA is to detect and measure serum-neutralizing antibodies in subjects belonging to epidemiology studies, preclinical studies or vaccine clinical trials. The assay parameters assessed were Precision (distinguished between Repeatability – i.e., Intra-assay - Precision, and Intermediate - i.e., Inter-assay – Precision), Dilutional Linearity, Specificity and Lower Limit of Quantification (LLoQ)/Limit of Detection (LOD).

- Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions. Precision has been considered at two levels: repeatability and intermediate precision. Repeatability expresses the precision under the same operating conditions and is also named intra-assay precision. Intermediate precision (occasionally called within-lab reproducibility) is expressed within-laboratory variations.

Two different High titer sera sample were tested in 16 replicates (2 plate) with starting dilution 1:100. The samples, serially diluted in 3-fold steps, were tested in 8 identical replicates, on three different days, by two operators, independently (a total of 24 replicates for each sample, 8 per operator each day, per sample). Each operator ran 2 plates each day, for a total of 3 days (with 48 samples in total, 24 per operator).

The coefficient of variation (CV%) calculated considering the day and the operator as random and no fixed factors, was used to estimate the repeatability (defined as the variability under the same operating conditions over a short interval of time) to estimate the intermediate precision (defined as the variability among different days and different operators), and to evaluate the contributions of the operator and the day of analysis to the variability.

- Dilutional Linearity

A linearity of dilution experiment provides information about the precision of assay results for samples evaluated at various levels of dilution in the chosen sample diluent. The linearity was assessed by evaluating a positive serum neat and pre-diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in PBS prior to performing the assay. Each serum sample was run as an independent sample, serially diluted in 3-fold step, by 1 operator in 1 day.

Dilutional linearity was evaluated utilizing dilutions whose coefficient of variation (CV) does not exceed 25%. A linear regression analysis will be performed on the base-2 logarithmic transformation of the geometric mean of the estimated IC50 values in relation to the base-2 logarithmic transformation of the dilution levels. The performance target for the regression analysis includes a coefficient of determination (R-squared) of at least 0.95 and a slope that falls within the range of 0.75 to 1.25

- Specificity

The specificity of the assay is the ability of an analytical procedure to determine solely the concentration of the analyte that it intends to measure.

Two sets of samples were prepared to assess the homologous and heterologous specificity of the assay using the positive serum diluted at 1:1 (v:v) in PBS alone and PBS supplemented with different quantities of homologous or heterologous purified antigens. The positive serum sample was diluted 1:1 in PBS with different amounts of homologous antigens for STm and SEn respectively (100, 50,

10, 5 and 1 µg/mL) and compared with the serum diluted 1:1 in PBS only (non-depleted). The lowest homologous antigen concentration (among the ones tested) resulting in an IC50 reduction ≥ 70% will be used for the heterologous antigen and polysaccharide from different species. The inhibition of the titer compared to the control serum (spiked with PBS only) must be <30% when a heterologous competitor is used. The inhibition rate was expressed as % reduction compared to the control serum was calculated as follows:

*Reduction %= 1-(IC50 of spiked serum/IC50 of control serum)] *100*

- Limit of Detection (LOD) and Lower Limit of Quantification (LLoQ)

The limit of Detection (LoD) and the limit of Quantitation (LoQ) are the lowest SBA titer that can be detected under the assay conditions and the lowest SBA titer that can be quantified with suitable precision, respectively. The HI positive serum was pre-diluted in PBS to generate a sample with low but detectable SBA titer, defining the limits of detection and quantification.

Calculations of LoD and LoQ were performed according to the ICH guideline Q2(R2)[103] by using the standard deviation (SD) of log-transformed SBA titers obtained for the samples and the lowest serum concentration evaluated in the assay according to the following formulas:

$$LoD = 10\text{^}(\ 3.3 * SD)\ * X$$

$$LoQ = 10\text{^}(10 * SD) * X$$

14. Classical Serum Bactericidal Assay for *Salmonella* Enterica Paratyphi A

Bactericidal activity on *Salmonella* Enterica Paratyphi A was tested using the classical Serum Bactericidal Assay (L-SBA). *Salmonella* Enterica Paratyphi A culture was diluted 1:4000 in assay buffer (HBSS+1%FBS). 20 µL of assay dilution buffer to each well of a flat-bottom 96-well plate. The sera sample (previously heat inactivate for 30 minutes at 56°C were serially diluted (2-fold) in assay buffer in a final volume of 20 µL until column 9. 10 µL/wells of the solution containing Baby

Rabbit Complement (BRC) (12,5% final concentration) were added from column 1 to 10. While in column 11 were added 10 µL of heat inactivated BRC. Then, 10 µL/wells of diluted bacteria were added in from column 1 to 11. The plate was incubated at 37°C, 5%CO_2 for 60 minutes. After 1 hour, 10 µL of each condition was plated on LB agar plate and grown overnight at 37°C, 5%CO_2. The day after was performed the colony count an automatic colony counter (ProtoCOL3).

15. Validation of the Classical Serum Bactericidal Assay for *Salmonella* Enterica Paratyphi A

- Precision

Precision is defined as the extent to which the same result is measured when multiple analyses of the same homogeneous sample are performed under the normal assay conditions by multiple analysts constituting both intra and inter assay precision.

For assessing the precision, minimum seven operators will test 2 high, 2 medium, 2 low and 1 negative serum sample (IgG depleted serum) selected from the precision panel in Six replicates, in 3 days.

Each analyst will thus generate 18 determinations for each sample, for a total of 126 determinations considering all six analysts. Median titre and agreement will be calculated for each sample. Agreement is defined as the number of titres obtained within ± 1 SBA titre of the median titre.

The global precision must have ≥ 85% of the samples within the median titre ± 1 SBA titre. Samples having >50% valid results should be considered for calculating the global precision.

Determinations of analyst 1 will be considered for calculation of median titres, results for remaining analyst should be compared to analyst 1 median titres.

- Dilutional Linearity

The linearity is defined as the ability of an analytical process within a given range to provide a result directly proportional to the titre of the analyte being estimated in the sample. The Accuracy is defined as the closeness of agreement between the value which is accepted either as a conventional true value

or an accepted reference value and the value found. The linearity will be assessed by two analysts, by testing 2 high, 2 medium and 1 low sample, tested in duplicate pre-diluting the sample. Each prediluted sample will be run as an independent sample, serially diluted at 2-fold step. The results of linearity should be adjusted by their dilution factor. The titre for each fractional/initial dilution (median of the titres) of the pooled results are calculated. The accuracy will be also evaluated by calculating the difference between the reference titre (median of the titres) and observed titre (median of the adjusted titres) for each sample. The linearity of each sample will be assessed using a linear regression graph comparing the log 10_2 transformed values for dilution used and the log 102 transformed observed SBA titres against each of the dilution.

The linearity is demonstrated if >80% samples (determinations) titres obtained (using dilution factor) are with predefined acceptance limits of [+ 1 SBA titre] to the median titre and R^2 value for linear regression must be > 0.9.

For the accuracy, the criterion for acceptable assay accuracy is that the observed SBA titre of the spiked serum specimen be within + 1 SBA titre (i.e. agreement) for ≥ 80% of the samples tested.

- Robustness

The robustness is defined as a measure of the capacity of the analytical procedure to remain unaffected by small but deliberate changes in the test conditions. A set of 2 high, 2 medium and 2 low serum samples selected from the robustness panel, will be tested in duplicate by two analysts at different incubation time intervals (30, 60 and 90 mins).

For each condition, ≥ 80% of the samples must be within ± 1 SBA titre of the corresponding 60 mins/reference value.

- Specificity

The specificity is defined as the ability of an analytical procedure to solely determine/detect the analyte that it intends to measure. Specificity is assessed by testing these sera after pre-treatment for

1h at 37°C with an equal volume of homologous competitor polysaccharide at a final concentration of 100 µg/mL. Specificity will be assessed by testing 2 high and 2 medium sera samples selected from the specificity panel by two analysts in duplicate, spiked with homologous, heterologous, unrelated species competitor and non-spiked serum as control.

The specificity is evaluated by calculating the difference between the reference titre (untreated sample) and observed titre (treated sample) for each sample. The criterion is met, if ≥ 80% of the samples are inhibited with the homologous competitor by 2 SBA titre and for heterologous polysaccharide, the criterion is met if ≥ 80% of the samples are not inhibited with the heterologous competitors by more than 1 SBA titre

- Ruggedness

The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Sera samples may need to be re-tested and as such, may undergo many freeze/thaw cycles to perform those additional tests. Therefore, the impact of multiple freeze/thaw cycles must be assessed accordingly. Ruggedness will be assessed by testing 2 high, 1 medium and 2 low sera sample with concentrations covering the analytical range. Each freeze/thaw cycle should mimic clinical testing conditions. Each cycle began with the removal of samples from -70°C, which were thawed at ambient temperature (15°C to 30°C) for at least 1 hour aliquoted and then inactivated for use in the assay. The remaining part is kept back at storage condition (-70°C). The cycle is repeated to obtain SBA titres for different freeze thaw cycles.

For each condition, ≥ 85% of the samples must be within ± 1 SBA titre of the corresponding Cycle 0 reference value.

- LLOQ Verification

The LLOQ is defined as the lowest value of a sample that can be quantitively determined with suitable precision and linearity. The lowest dilution of sera achievable for the SBA is 4. The LLOQ is verified by the analysis of the results obtained by testing 4 low and 4 negative titre serum samples, which fall within the range between half the LLOQ and 4X LLOQ (titre corresponding to 16). Each sample should be tested in duplicate by 3 analysts to generate 6 determinations per sample.

Acceptance criteria: the LLOQ of 4 (reciprocal dilution) is considered verified if:

Positive agreement is ≥ 66%

Negative agreement is ≥ 66%

That will be calculated using the following formulas:

Positive agreement (%): [A/(A+C)] x 100

Negative agreement (%): [D/(B+D)] x 100

Where:

A = number of results for double positive results (expected and determined);
B = number of expected results negative with a positive result determined;
C = number of expected results positive with a negative result determined;
D = number of results for double negative results (expected and determined);

- Matrices effect

The matrices effect will be evaluated by using a spike/recovery approach to explore whether the interference components from the serum matrices may affect the performance of the assay. Four sera from positive samples (2 medium, 1 low and 1 high sera sample selected trough other validation panels) will be spiked with: Hemolysate (final concentration 10.5 mg/mL, Sun Diagnostics), Triglyceride (final concentration 3.0 mg/mL, Sun Diagnostics) or Bilirubin (final concentration 0.5 mg/mL, Sun Diagnostics). All samples will be tested un-spiked (baseline) and spiked with the selected interferents in duplicate.

The criterion is met if ≥ 70% of the samples are not inhibited with the interferents by more than 1 SBA titre.

16. Development of a Bioluminescence-Serum Bactericidal Assay for *N. meningitidis*

16.1. Working stocks preparation for *N. meningitidis* serogroup B and sera samples

The *N. meningitidis* serogroup B (MC58) was transformed with the previously described recombinant plasmid pDG34 in which the P*porB-luxCDABE-aph3'* was flanked by the meningococcal *pilE* gene and 120 bp downstream *pilE* gene to facilitate the recombination and allelic replacement in the parental strain MC58 chromosome. The resulting bioluminescent strain MC58lux expresses the luciferase constitutively. Bacteria were cultured on GC medium (Difco, ThermoFisher Scientific, Illkirch, France), supplemented with Kellog supplements. Bacteria were stored in 20% glycerol-containing GC liquid medium at −80 °C until use. A fresh culture of bacteria (16 h at 37 °C, 5% CO_2) was prepared from frozen stocks by streaking onto GC agar plate.

The previously described collection of 48 serum samples from children 1–5 years of age vaccinated in 2008 with the MenBvac® vaccine using a 2+1+1 schedule (at D0 and W6 then at M8 and M36) was used to develop the BioLux SBA and for the complement deposition assay. The sera from this collection were sampled 6 weeks after the last dose, i.e., in 2011, at a time Bexsero® was not available in the area [104]. Ethical approval for use of serum samples for research studies was provided by the regional ethics committee (Comité de Protection des Personnes Nord-Ouest-1) and written informed consent was obtained from parents or legal guardians of every participant.

16.2. Complement source validation experiments

Normal human sera (NHS) from healthy unvaccinated human donors were used as a source of complement for SBA assays. From overnight GC plate-grown culture, the bioluminescent strain MC58lux was 10-fold serially diluted in a 96-well plate (from the concentration of 10^6 CFU/ml to 10^2 CFU/ml) and incubated with 25% of different sources of complement in Hanks balanced salt

solution supplemented with calcium chloride and magnesium chloride ($HBSS^{++}$) (Gibco, Thermo Fisher Scientific Illkirch, France) for one hour at 37°C, 5% CO_2. The reaction mix in the absence of the complement was used as a reference control. After the incubation time, 10µl from each well were plated on a GC agar plate which was then incubated overnight at 37°C, 5% CO_2 for enumeration of CFU. A validated complement serum does not show higher than 15% of killing when compared to complement-free control.

16.3. Conventional Serum Bactericidal Assay for *N. meningitidis*

The bioluminescence *N. meningitidis* serogroup B was grown overnight in a GC agar plate with supplements and adjusted to 10^4 CFU/ml concentration in $HBSS^{++}$. The human sera were decomplemented by heating for 30 minutes at 56°C and then two-fold serially diluted in different tubes, starting from a dilution of 1/4 until 1/128 in a final volume of 50 µl. A mixture containing the bacterial suspension, and the complement was prepared in a proportion 1:1, and 50 µl were added to each well containing the diluted serum. The final volume in each well was 100 µl. The reaction mix in the absence of the sera represents the negative control. The reaction was incubated at 37°C, 5% CO_2, for 1 hour. After one hour of incubation, the entire reaction mix was plated on the GC agar plate with Kellogg supplements. The plates were incubated overnight at 37°C, 5% CO_2. The day after, the colony counting was performed, and the titer was assigned based on the highest serum dilution that caused 50% of the killing with respect the negative control. The serum bactericidal activity titers corresponded to the inverse of the final serum dilution causing 50% killing of the inoculum after 1 hour of incubation. When indicated, luminescence emitted from the bacterial GC agar plate were acquired using ChemiDoc Imaging System (Biorad, France) and Image J 1.51K software (National Institutes of Health https://imagej.net/) was used to quantitate the signals.

16.4. Bioluminescence Serum Bactericidal Assay (BioLux-SBA)

The bioluminescent *N. meningitidis* serogroup B was grown overnight in a GC agar plate with supplements and adjusted to the concentration of $8x10^6$ CFU/ml in $HBSS^{++}$. The human sera were decomplemented by heating for 30 minutes at 56°C and then were two-fold serially diluted in a 96-well white-wall flat plate, starting from a dilution of 1/4 until 1/128 in a volume of 25 µl, then 12.5 µl of a previously validated human complement source has been added to the reaction with a final concentration of 25%. Lastly, the 12.5 µl of the bacterial suspension was added to the reaction. The final assay volume was 50 µl. The reaction mixes in the absence of the sera represented reference and the negative control. The plate was incubated at 37° C, 5% CO_2 for 5 hours. After one hour of incubation 10 µl of each reaction were plated on the GC agar plate as an additional control of the sera-killing action. After 5 hours of incubation, the luminescence of the assay plate was recorded using the Centro XS LB 960 microplate reader (Berthold Technologies), and data were expressed as a ratio to the reference condition without serum (BioLux-SBA titer).

16.5. Terminal Complement Complex Deposition assay

The bioluminescent *N. meningitidis* serogroup B was grown overnight in GC agar plate with supplements. A suspension containing $2x10^6$ CFU diluted in 50µl of $HBSS^{++}$ was mixed with 50µl of sera in a 96-well plate. The final assay volume is 100 µl. The reaction was incubated for 30 min at 37° C, 5% CO_2. After the incubation, the bacteria were washed twice (centrifugation at 4000 rpm for 10 min) using $HBSS^{++}$ with 1%BSA and resuspended in the same volume with a solution containing the FITC-anti-human C5b-C9 antibody (clone aE11 FITC conjugated antibody (HycultBiotech)) diluted 1:50 and incubated for 30 min at room temperature (RT) in the dark. The bacteria unstained with fluorescence represents the negative control. After the incubation, the bacteria were washed twice in $HBSS^{++}$ with 1%BSA, resuspended with formalin solution, and incubated for 30 min at RT in the dark. After the incubation, the plate was centrifuged at 4000 rpm for 10 min and resuspended in 100 µl $HBSS^{++}$ with 1%BSA. The plate was acquired using the CytoFlex S (Beckman Culture) and the data were analyzed using FlowJo.

16.6. Measure of complement deposit on different serogroups of N. *meningitidis*

Isolates of each serogroup of *N. meningitidis* were grown overnight in a GC agar plate with supplements (Gibco, Thermo Fisher Scientific, Illkirch, France). A suspension containing 2 × 10^6 CFU diluted in 50 µL of Hanks balanced salt solution supplemented with 0.15 mM calcium chloride and 0.5 mM magnesium chloride (HBSS++) (Gibco, Thermo Fisher Scientific, Illkirch, France) (HBSS++) with 1%BSA were mixed with 50 µL of normal sera from consent anonymous donors in a 96-wells plate and incubated for 30 min at 37 °C, 5% CO2. After the incubation, the bacteria were washed in HBSS++ with 1%BSA and then resuspended in a solution containing the FITC-conjugated anti-human C5b-C9 antibody (clone aE11 FITC conjugated antibody (HycultBiotech, Uden, The Netherlands)) diluted to 1:50 and incubated for 30 min in the dark at room temperature. Unstained bacteria served as negative control. After incubation, the bacteria were washed twice in HBSS++ with 1%BSA, resuspended with 2% paraformaldehyde (PFA) solution, and incubated for 30 min at room temperature in the dark. After incubation, the plate was centrifuged at 4000 rpm for 10 min and resuspended in 100 µL HBSS++ with 1%BSA. The plate was acquired using the CytoFlex S (Beckman Coulter, Villepinte, France), and the data were processed using FlowJo version 10.4.1 (BD Biosciences, East Rutherford, NJ, USA).

17. FACS-Based Whole cell ELISA for *N. Gonorrhoea*

With a sterile loop the *N. Gonorrhoea* strains were streaked onto an agar, Chocolate PolyViteX (BioMérieux), and incubated ON at 37°C (5% CO_2), and 95% humidity. The next morning, about 10 colonies were collected from the agar plate with a sterile loop and inoculated in a 50 mL conical tube with 6 mL of GC medium (Sigma-Aldrich) (complemented with 1% Isovitalex (Fisher Scientific) and 10% Fetal Bovin Serum (FBS) (Gibco)). The bacterial culture grew at 37°C (with 5% CO_2), for 3-4 hours, with shaking at 200 rpm until an OD_{600} of 0,5 ± 0,1 is reached.

Plastic round-bottom 96-well plates were coated with *N. Gonorrhoea* culture (50µL/well) and centrifuged for 7 minutes at 3220 x g. The pellets were resuspended in 100 µl of Rabbit monoclonal antibody 2C7 ((Rb2C7) Genescript, tested at the final concentration of 10 µg/mL) or sera samples (previously heat inactivated for 30 minutes at 56°C) diluted in DPBS with 1% of BSA at 1:400 and 1:200, respectively. The plate was incubated for 1h at RT before being centrifuged for 7 minutes at 3220 x g. Each pellet was resuspended in 150µl of DPBS-BSA1% containing secondary antibody (Secondary antibody: Alexa Fluor™ 488 goat anti-rabbit IgG (H+L) (Life Technologies Corporation)) diluted in PBS with 1% of BSA at 1:250 following the plate layout reported in Figure X, and the plate was incubated for 30 minutes at RT in the darkness. The samples washed twice in 100µL of DPBS-BSA1%, and were fixed with 100µL/well of 1% paraformaldehyde (Sigma Aldrich) prepared in PBS1X for 30 minutes at RT. Then the plate was centrifuged for 7 minutes at 3220 x g and washed in 100µL of DPBS-BSA1%, and again centrifuged for 7 minutes at 3220 x g. Finally, each pellet was resuspended in 100µl of PBS1X-BSA1%. The plate was stored at 4°C until acquired. The plate was acquired by the Cytometer Cytoflex LX (Beckman) according to the manufacturer's recommendations and analyse and plot the data by using FlowJo.

AIM OF THE WORK

This work aims to develop and qualify innovative functional and non-functional assays to support the vaccine effectiveness evaluation, for those candidates targeting the major antimicrobial-resistant bacterial pathogens (AMR), recognized as one of the most pressing threats to global health. These developed assays include both non-functional and functional assay to broadly evaluate vaccine immunogenicity by measuring not only antibody binding antigen-specific, but also their bactericidal activity. While binding assays are well established and, commonly used as correlates of protection in bacterial vaccinology, functional assays are not yet fully utilized due to limited regulatory standardization and acceptance criteria established. By qualifying these functional assays to be used in preclinical models, clinical trials or epidemiological studies, this work will help to reduce the methodological gap and enable more accurate assessment of vaccine immunogenicity. The availability of robust and validated functional serological assays can significantly strengthen the global vaccine development pipeline against AMR bacteria, accelerating progress toward effective immunization strategies.

RESULTS AND DISCUSSION

18. Correlation between Luminescence value and concentration of *B. burgdorferi*

In the L-SBA assay, the luminescence signal is obtained by adding the BacTiter-Glo Reagent in the reaction mix which correlates to the bacterial ATP content. BacTiter-Glo contains a luciferase that oxidizes luciferin and consequently generates light in the presence of ATP Bacterial viability activity was tested measuring the bacterial ATP content [59]. The Figure 4 shows that the concentration of *B. burgdorferi* B31 diluted 2-fold and 3-fold correlates with the luminescence value. The simple linear regression analysis shows a significant R squared (p value< 0,0001) equals to 0,99 for both dilutions type. Investigate the correlation between the bacterial concentration and the luminescence emitted is a required step previous development of any Luminescence based SBA, and it is particular important in the case of *B. burgdorferi* for its elongated spiral shape. In fact, the length and degree of elongation of *B. burgdorferi* can vary significantly from one bacterium to another depending on the phase of its growth cycle, with cells in different stages (e.g., early exponential vs. late stationary phase) exhibiting different filament lengths. Since ATP content is directly linked to cellular volume and metabolic activity, these morphological variations can lead to substantial differences in the amount of ATP present in individual organisms. As a result, luminescence signals may not correlate linearly with cell number unless these shape-dependent variations are properly characterized and accounted for.

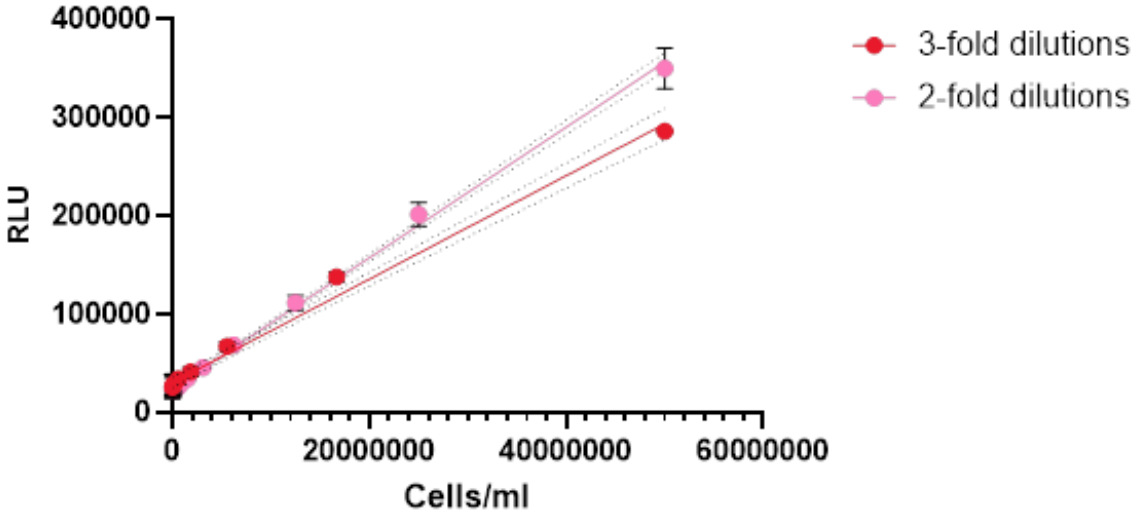

Figure 4 shows the linear correlation between the bacterial concentration (serially diluted 2- and 3-fold) and the luminescence emitted.

18.1. *B. burgdorferi* B31 sensitivity test to Guinea Pig Complement

Testing bacterial sensitivity to complement is required before assessing sera sample potency in complement-dependent killing, or serum bactericidal assays. In fact, the antibodies present in the sera samples exert their bactericidal activity by recruiting the complement cascade upon binding to the bacterial antigens. This concept is at the basis of the serum bactericidal assay (SBA), which evaluates bacterial viability in the presence of antibodies and of an exogenous complement source. By exposing the bacterium to different dilutions of Guinea Pig Complement (GPC) it is possible to understand which the suitable percentage is to be used in SBA. Ideally, the right concentration of GPC should be the one that has little or no effect on the bacteria viability in the active complement control but promotes bacterial killing in the presence of the sera. The assay was carried out by measuring luminescence generated by the reaction. Figure 5 shows the results of the complement sensitivity test. Comparison between GPC and HIGPC tested at 2% indicates that the bacteria may be sensible to the complement showing a decrease in the bacterial viability between the HI GPC and GPC control. On the other hand, the GPC tested at 1% showed no complement sensitivity and at the same time enhance the antibodies-dependent killing in the presence of the positive serum sample diluted 1:500.

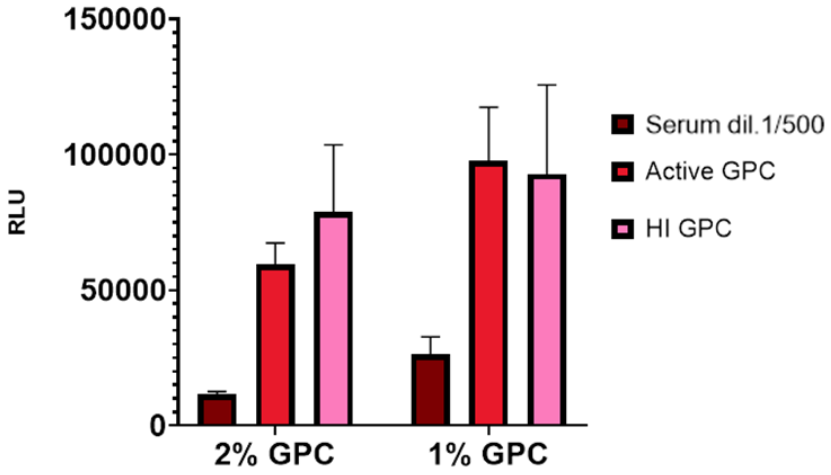

Figure 5 shows Borrelia Burgdorferi B31sensitivity to Guinea Pig Complement. The histogram shows the bacterial viability when tested with different concentration of GPC (red), HI GPC (Pink) and with serum sample (dark red). The percentage of the GPC is reported on the X axis and the Relative Light Units (RLU) on the Y axis.

Once the 1% concentration of the GPC was confirmed to be used in the L-SBA for *B. burgdorferi,* different sources of GPC were also tested. Figure 6 shows the bacterial viability in the presence of different sources of GPC alone, Heat Inactivated and with the serum sample diluted at 1:500. No differences were found in the different source of GPC. Indeed, all the three different sources were validated to be used in the L-SBA.

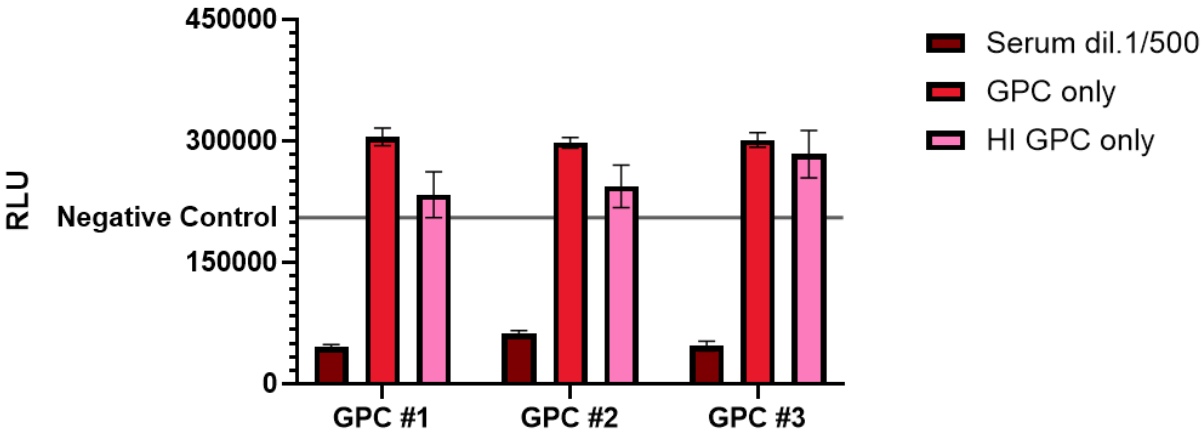

Figure 6 shows the comparison of different source of GPC. The different GPC sources are reported on the X axis and the Relative Light Units (RLU) on the Y axis. The negative control (bacteria only) is represented as continued line on the Y axis.

18.2. Precision assessment of the Luminescence-Based Serum Bactericidal for *B. burgdorferi* B31

The precision test evaluates the closeness of agreement between a series of measurements obtained from multiple sampling of a sample under different conditions such as different operators and performing day. Precision experiment has been performed by three operators on 2 different days using one high positive serum sample against *B. burgdorferi* B31. Figure 7 shows the curve obtained by the three operators over the two days. The calculated CV% are reported in Table 1.

Table 1 Precision Experiments results obtained by three Analysts over 2 days.

Inter-Assay Precision among operators in the single day	DAY	1	2
		14%	12%
Inter-Assay Precision of the single OP between the 3 days	OP1	8%	
	OP2	11%	
	OP3	5%	

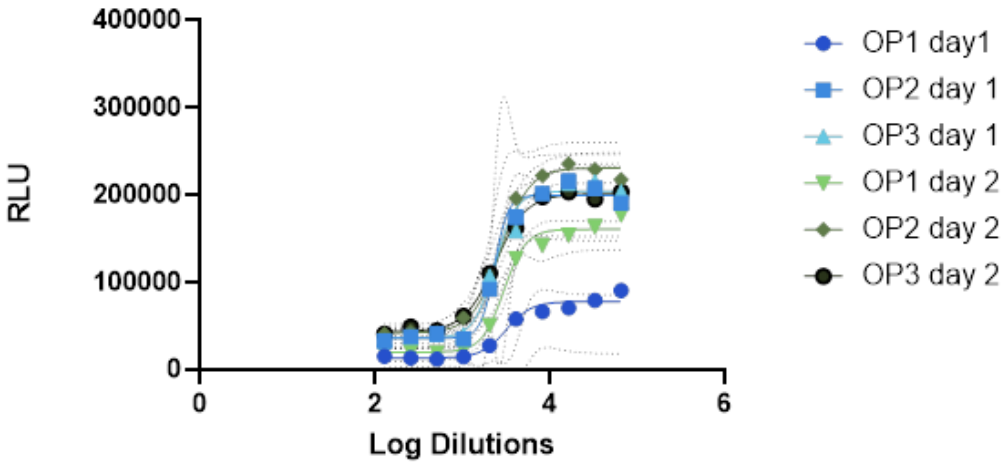

Figure 7 shows the results obtained by the precision experiments performed by three operators in two days. The logarithmic transformed sera concentrations are reported on the X axis and the luminescence values expressed as Relative Light units (RLU) are reported on the Y axis.

19. FACS-based Serotyping for *S. flexneri* 2a: the titration experiments

Before performing the FACS-based serotyping experiment, it was necessary to perform a titration experiment to choose the best dilution to be used for the Polyvalent/Monovalent antisera and the secondary antibody. To perform the titration experiments were tested the Polyvalent B serum S.

flexneri type I, II, III, IV, and group (3)4, 6 and 7(8) and the Monovalent serum *S. flexneri* type II were diluted at 1:50, 1:100, 1:200, 1:400. The secondary antibody (BD Horizon™ BV421 Goat Anti-Rabbit IgG) was tested at the following dilutions of 1:250, 1:500, 1:1000, 1:2000 and the negative control was represented by the absence of the secondary antibody in the reaction mix. Figure 9 shows the histograms created using FlowJo software at the different dilutions of the antisera and the secondary antibody. For the Polyvalent antisera the intensity of the fluorescence signal remains stable at all the different dilutions, while for the Monovalent antiserum an increasing of the fluorescence signal proportional to the dilution of the serum can be observed. To obtain the best fluorescence signals, 1:250 dilution was selected for the secondary antibody and 1:200 and 1:400 was selected for the polyvalent and monovalent antisera, respectively.

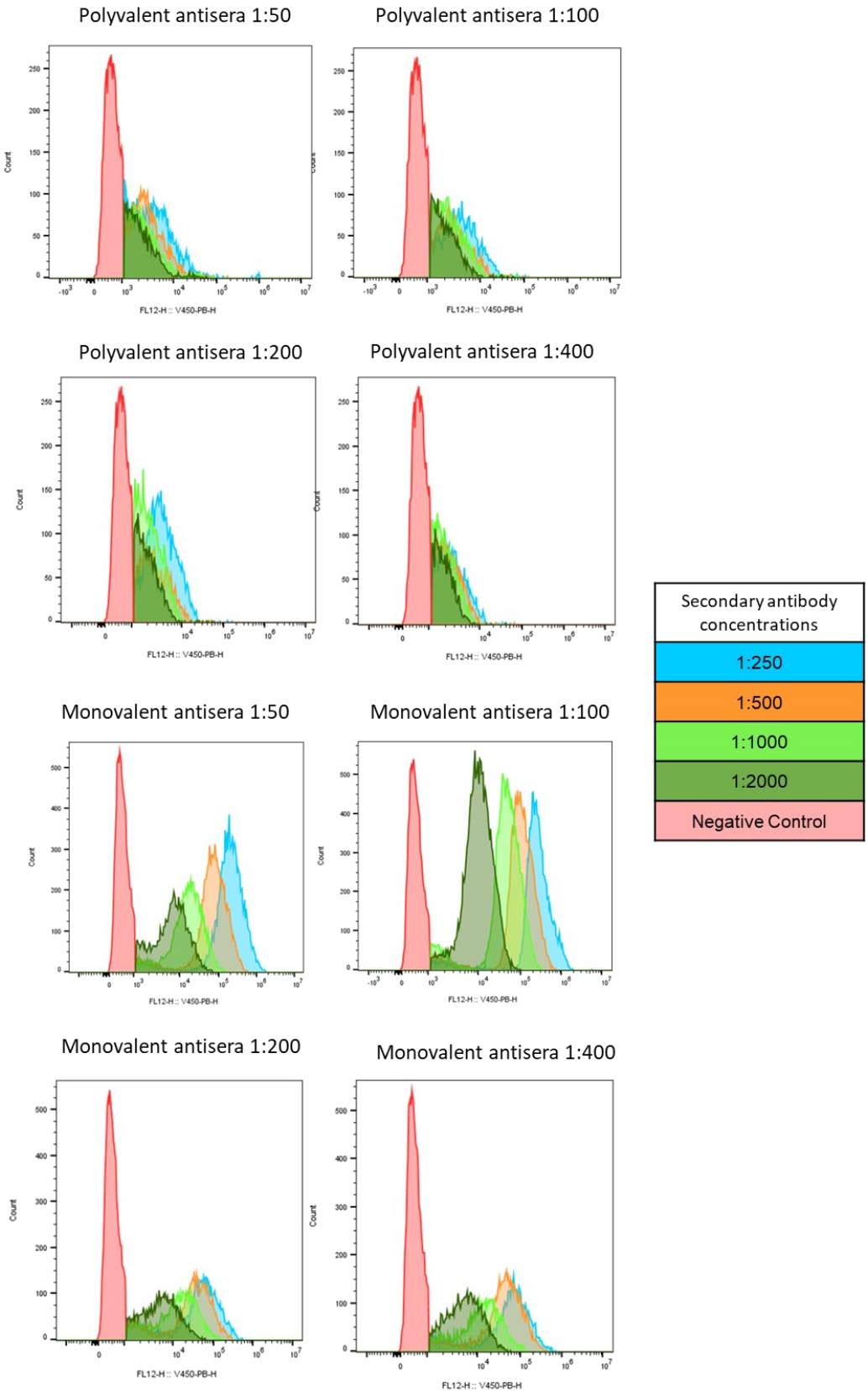

Figure 8 shows the results obtained for the titration experiment of the polyvalent (1:50, 1:100, 1:200, 1:400) and monovalent (1:250, 1:500, 1:1000, 1.2000) antisera and the secondary antibody concentration (blue (1:250), orange (1:500), light green (1:1000), dark green (1:2000) and pink (absence of the secondary antibody)). On the Y axis are reported the number events and on the X axis is reported the intensity of the fluorescence signal for the BV421 Goat Anti-Rabbit IgG.

19.1. FACS-based Serotyping experiment for *Shigella Flexneri 2a*

The serotyping experiment was performed using prepared antisera that bind to a set of known antigens to evaluate and confirm the serotype of *S. flexneri* 2a. The binding between a surface antigen and antiserum can be observed experimentally in many forms. The FACS-based serotyping evaluates the antisera binding on the bacterial surface using the fluorescence signal generated by the secondary antibody BV421- conjugated Goat Anti-Rabbit IgG to evaluate the serotype of the bacteria. Table 2 shows the results of the serotyping experiment performed to confirm the S. *flexneri* 2a serotype, reporting the percentage of positive events for the fluorescence signal. The percentage of positive fluorescence signals confirmed the serotype of *S. flexneri* 2a, even if the results show also a positivity to the *S. flexneri* group 3(4) which is known for sharing a common epitope founded in serotypes la, 2a, 3b, 4a, 5a, and Y [105]. Figure 9 shows the specific histograms of the negative control (A), the Polyvalent B: *S. flexneri* type I, II, III, IV, and group (3)4, 6 and 7(8) (B) and Monovalent *S. flexneri* type II (C).

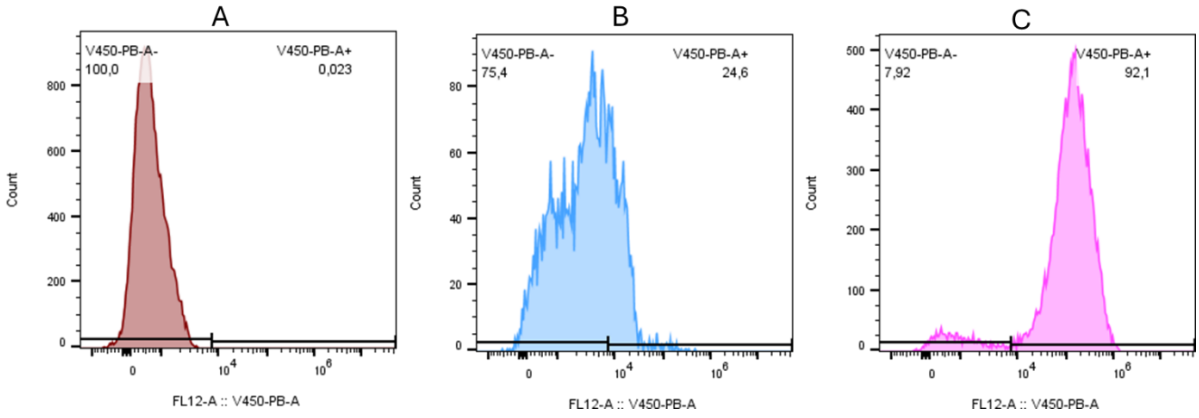

Figure 9 shows the histograms created using the FlowJo analysis software for the negative control (A), Polyvalent B: S. flexneri type I, II, III, IV, and group (3)4, 6 and 7(8) (B) and Monovalent S. flexneri type II (C). On the Y axis are reported the number of events and on the X axis is reported the intensity of the fluorescence signal for the BV421 Goat Anti-Rabbit IgG.

Table 2 shows the FlowJo analysis results for each antisera used, the total bacteria acquired (Count total), the % of the positive events for V450-PB.

Antisera	Count total	morfology/Single Cells/V450-PB-A+ | Freq. of Single Cells
Polyvalent A: S. dysenteriae type 1, 2, 3, 4, 5, 6 and 7	36705	0,29 %
Polyvalent A1: S. dysenteriae type 8, 9, 10, 11 and 12	40490	0,086 %
Polyvalent B: S. flexneri type I, II, III, IV, and group (3)4, 6 and 7(8)	6561	24,6 %
Polyvalent C: S. boydii type 1, 2, 3, 4, 5, 6 and 7	37854	0,17 %
Polyvalent C1: S. boydii type 8, 9, 10 and 11	34672	0,076 %
Polyvalent C2: S. boydii type 12, 13, 14 and 15	36672	0,15 %
Polyvalent C3: S. boydii type 16, 17 and 18	29489	0,18 %
Polyvalent D: S. sonnei phantiserume I and II	28902	0,34 %
Monovalent S. flexneri type I	31848	0,17 %
Monovalent S. flexneri type II	22010	92,1 %
Monovalent S. flexneri type III	29657	0,18 %
Monovalent S. flexneri type IV	27417	0,30 %
Monovalent S. flexneri type V	29477	0,16 %
Monovalent S. flexneri type VI	30633	0,058 %
Monovalent S. flexneri group (3)4	19074	94,1 %
Monovalent S. flexneri group 6	28788	0,16 %
Monovalent S. flexneri 7(8)	28074	14,3 %
S. sonnei phantiserume I	30535	0,21 %
S. sonnei phantiserume II	25559	0,12 %
Negative Control S. Flexneri 2a	26670	0,032 %
Negative Control S. Flexneri 2a	27988	0,023 %
Negative Control S. Flexneri 2a	28578	0,076 %

20. Qualification of the Luminescence-Based Serum Bactericidal Assay for *Shigella flexneri 2a*

- Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions. Precision has been considered at two levels: repeatability and intermediate precision. Repeatability is the variability observed within a single run of a specific sample. It measures the precision of the assay when conducted under consistent conditions, such as the same day, operator, and run. In contrast, intermediate precision accounts for the variability across multiple runs and replicates of the same sample. This means it assesses the assay's precision over different days, with various operators, and across multiple running conditions.

For both parameters three different sera samples covering the broad range of the assay (high, medium and low titer sera) have been used. Serum samples were serially diluted in 2-fold steps. Each operator ran 4 plates per day for 3 different days.

For each sample, the mean values of IC50s were determined through six runs conducted over three days by two operators (Figure 10). These results were analysed to evaluate the precision of the assay in terms of percentage coefficient of variation (%CV). The performance target for both intermediate precision and repeatability was a %CV not exceeding 50%. The %CVs calculated for the intermediate precision and repeatability resulted below 50% for each serum and are reported in Table 3.

Table 3. Precision Experiments results obtained by two Analysts over 3 days.

		LOW			MEDIUM			HIGH		
Inter-Assay Precision among two Analysts in the single day	DAY	1	2	3	1	2	3	1	2	3
		3%	13%	5%	2%	4%	4%	3%	3%	3%
Inter-Assay Precision of the single OP between the 3 days	OP1	10%			3%			4%		
	OP2	2%			2%			2%		

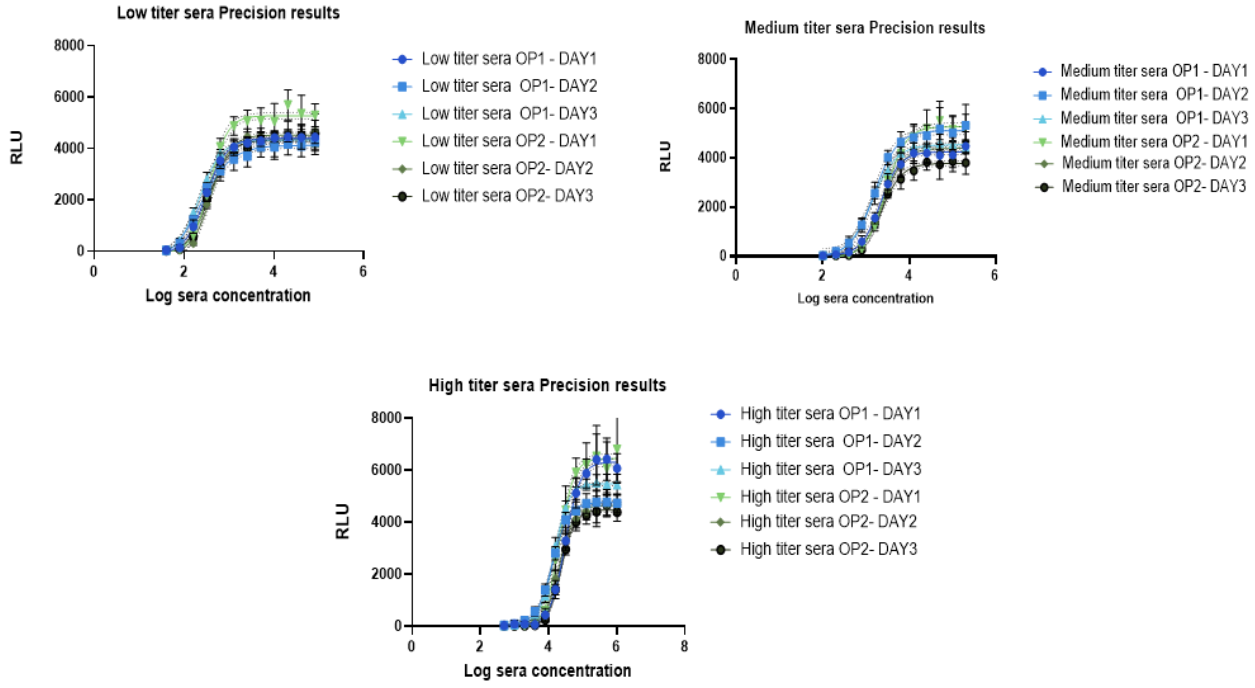

Figure 10 shows the Precision experiments low, medium and high titer sera results of the L-SBA for S. Flexneri 2a. The logarithmic transformed sera concentrations are reported on the X axis and the luminescence values expressed as Relative Light units (RLU) are reported on the Y axis.

- Dilutional Linearity

A linearity of dilution experiment provides information about the precision of assay results for samples tested at different levels of dilution in the chosen sample diluent. The linearity has been assessed by pre-diluting the High titer serum in a 3-fold step dilution scheme, starting from the neat serum to the highest dilution expected to yield an SBA titre below the expected lower limit of quantitation of the assay. Each pre-diluted serum sample was run as an independent sample, serially diluted in 2-fold step and tested in duplicates, performing two identical microplates (with each replicate in a different microplate), by 2 different operators, on 3 different days.

Dilutional linearity has been evaluated utilizing dilutions whose coefficient of variation (CV) did not exceed 25%. A linear regression analysis has been performed on the base-3 logarithmic transformation of the geometric mean of the estimated IC50 values, in relation to the base-3 logarithmic transformation of the dilution levels. The performance target for the regression analysis included a coefficient of determination (R-squared) of at least 0,95 and a slope that falls within the range of 0,8 to 1,2 was satisfied (Figure 11).

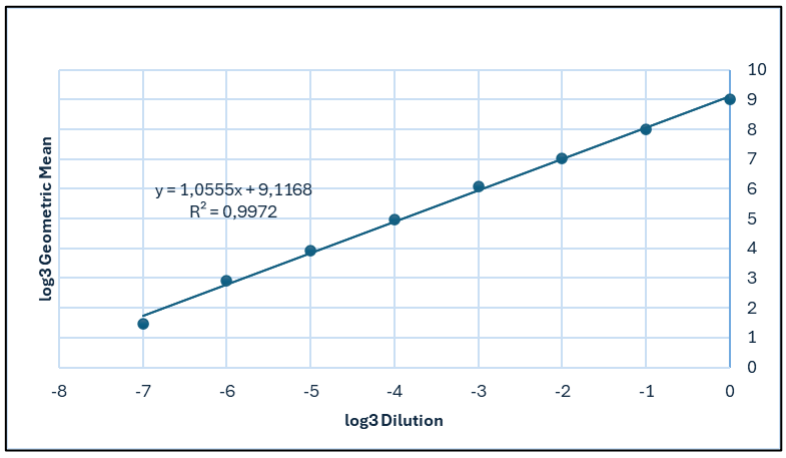

Figure 11 shows the linear regression plots of the L-SBA for Shigella Flexneri 2a. The logarithmic transformed sera dilutions are reported on the X axis and the logarithmic transformed Geometric mean of the IC50s obtained are reported on the Y axis.

- Specificity

To evaluate the ability of the assay to exclusively detect the target analyte, the high positive sample high titer sera was tested. The serum sample has been diluted 1:1 in PBS only or spiked with

different amounts of the purified homologous LPS for each ST (500, 250, 100, 50, 10, 5, 1 µg/mL) and compared with the control serum spiked with PBS only. The lowest concentration of the homologous LPS Ag able to inhibit the IC50 of ≥ 70% compared to the non-depleted sample, has been used for LPS antigens of the heterologous STs and of the unrelated species (Porphyromonas gingivalis), the inhibition of the IC50 compared to the control serum (spiked with PBS only) must be < 30%. Each spiked serum sample has been tested in two replicates and serially diluted in 2-fold step.

The inhibition rate was expressed as % reduction compared to the control serum. For *Shigella flexneri 2a* strain, not statistically significant difference cross reactivity was observed, however a % reduction close to the threshold (37%) was obtained with the *S. sonnei* LPS, serum spiked with *Shigella flexneri* 3a and 1b LPS showed inhibition rates of 86% and 72% respectively (Figure 12).

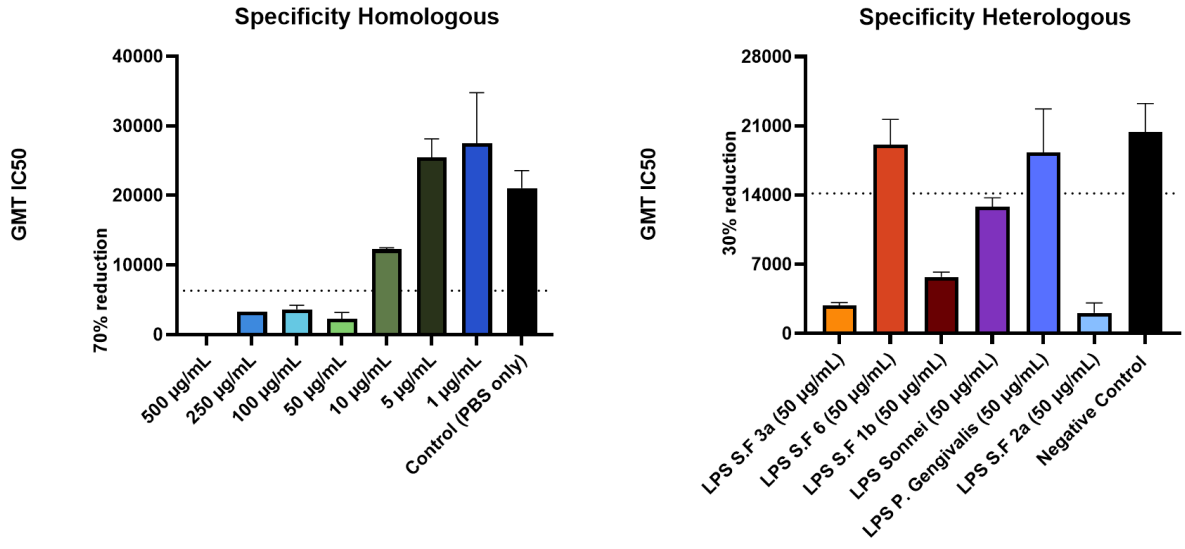

Figure 12 shows the results obtained by the Specificity homologous (graph on the left) and heterologous experiments (graph on the right) of the L-SBA for Shigella flexneri 2a. The different antigen concentrations are reported on the X axis and the IC50s obtained are reported on the Y axis. The dotted line represents the 70% (graph on the left) and 30% (graph on the right) reduction threshold respect the control.

- Limit of Detection (LOD) and Lower Limit of Quantification (LLoQ)

The limit of Detection (LoD) and the limit of Quantitation (LoQ) are the lowest SBA titer that can be detected under the assay conditions and the lowest SBA titer that can be quantified with

suitable precision, respectively. One run with 12 replicates of the same serum was performed by one operator on one day. Calculations of LoD and LoQ were performed according to the ICH guideline Q2(R2) by using the standard deviation (SD) of log-transformed SBA titers obtained for the samples and the lowest serum concentration tested in the assay. The results showed a LoD of 19,95 and a LoQ of 521,16.

21. Qualification of the Luminescence-Based Serum Bactericidal Assay for *Salmonella* iNTS

- Precision

The precision of the method expresses the ability of a measurement to be consistently reproduced. It is considered at two levels: repeatability and intermediate precision. Two high titer samples, serially diluted in 3-fold steps, were tested in 8 identical replicates, on three different days, by two operators, independently (a total of 24 replicates for each sample, 8 per operator each day, per sample). Each operator ran 2 plates each day, for a total of 3 days (with 48 samples in total, 24 per operator). The coefficient of variation (CV%) calculated considering the day and the operator as random and no fixed factors, was used to estimate the repeatability (defined as the variability under the same operating conditions over a short interval of time), and to estimate the intermediate precision (defined as the variability among different days and different operators), in order to evaluate the contributions of the operator and the day of analysis to the variability. The CV% calculated to estimate the repeatability and intermediate precision did not exceed 15%. The graphical representation of the single curve obtained by the two operators in 3 days are reported in Figure 15.

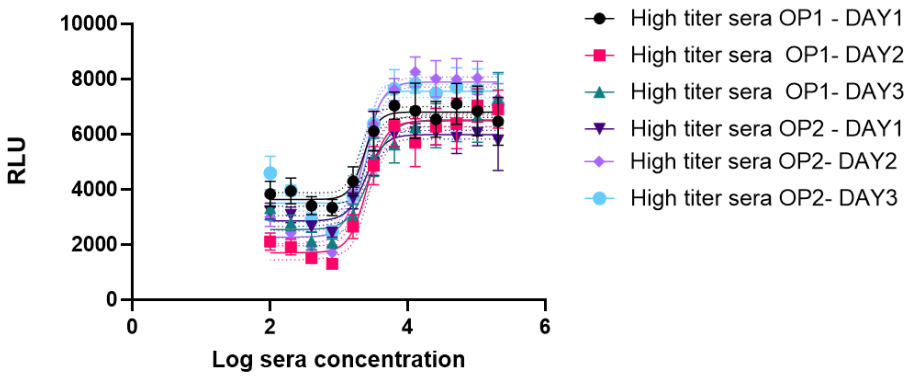

Figure 13 shows the curve obtained from the precision experiment performed by two operators (OP) over 3 different days. The logarithmic transformed sera concentrations are reported on the X axis and the luminescence values expressed as Relative Light units (RLU) are reported on the Y axis.

- Dilutional Linearity

A linearity of dilution experiment provides information about the precision of assay results for samples tested at different levels of dilution in the chosen sample diluent. The linearity was assessed by testing high titer serum neat and pre-diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in PBS prior to performing the assay. Each serum sample was run as an independent sample, serially diluted in 3-fold step, by 1 operator in 1 day.

The evaluation of dilutional linearity was conducted utilizing dilutions with a coefficient of variation (CV) not exceeding 25%. A linear regression analysis was performed on the base-3 logarithmic transformation of the geometric mean of the estimated IC50 values in relation to the base-3 logarithmic transformation of the dilution levels. The results met the desirable criteria, demonstrating a coefficient of determination (R-squared) of at least 0.95 and a slope that falls within the range of 0.75 to 1.25 for both strains. For *S.* Enteritidis (SEm), the R-squared value was 0.965, indicating that 96.5% of the variability in the IC50 results is explained by the linear regression model. The slope of the regression line was 0.995, demonstrating a nearly perfect linear relationship (Figure 14). Similarly, for S. Typhimurium (STm), the R-squared value was 0.994, suggesting that 99.4% of the variability is accounted for by the model. The slope of the regression line was 0.967, indicating a strong linear relationship (Figure 16). These results confirm the consistency and reliability of the assay in measuring the IC50 values for both Salmonella strains.

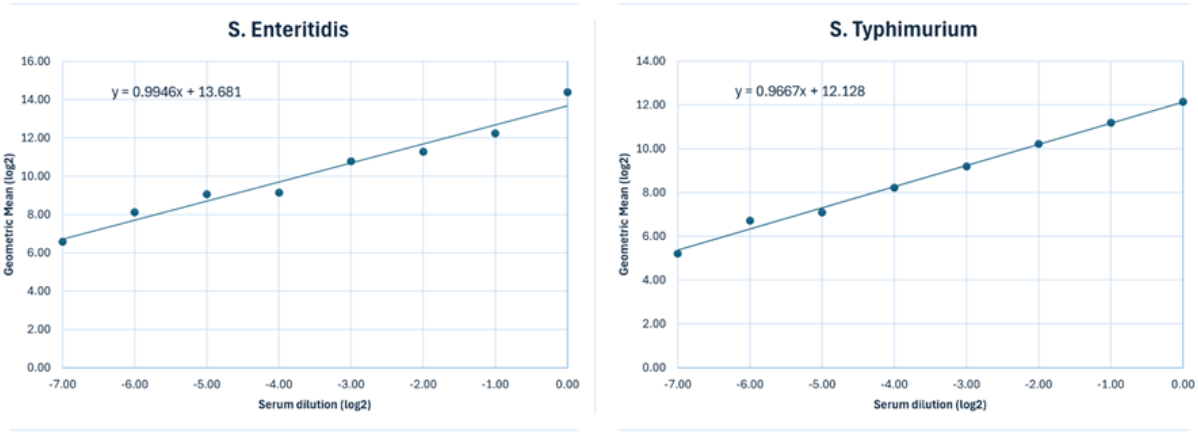

Figure 14 shows linear regression plots of the L-SBA for S. Enteritidis and S. Typhimurium. The logarithmic transformed sera dilutions are reported on the X axis and the logarithmic transformed Geometric mean of the IC50s obtained are reported on the Y axis.

- Lower Limit of Detection (Lod) And Lower Limit of Quantitation (LoQ)

The limit of Detection (LoD) and the limit of Quantitation (LoQ) are the lowest SBA titer that can be detected under the assay conditions and the lowest SBA titer that can be quantified with suitable precision, respectively. The high titer serum was pre-diluted in PBS to generate a sample with low but detectable SBA titer, defining the limits of detection and quantification.

One run with 16 replicates of the same serum was performed by one operator (2 plates) on one day. Calculations of LoD and LoQ were performed according to the ICH guideline Q2(R2) by using the standard deviation (SD) of log-transformed SBA titers obtained for the samples and the lowest serum concentration tested in the assay. The results showed a LoD of 5.56 and a LoQ of 10.83 for SEm and LoD 5.61 of and a LoQ of 11.15 for STm.

- Specificity

The specificity of the assay is the ability of an analytical procedure to determine solely the concentration of the analyte that it intends to measure. Two sets of samples were prepared to assess the homologous and heterologous specificity of the assay using a high titer Serum diluted at 1:1 (*v: v*) in PBS alone and PBS supplemented with different quantities of homologous or heterologous purified antigen. The high titer serum was diluted 1:1 in PBS with different amounts of homologous

antigen for STm and SEn respectively (100, 50, 10, 5 and 1 µg/mL) and compared with the serum diluted 1:1 in PBS only (non-depleted). The lowest homologous antigen concentration (among the ones tested) obtaining in an IC50 reduction ≥ 70% resulted to be 5 µg/mL for both strains as shown in Figure 15. This concentration was selected for the heterologous antigen and polysaccharide from the different species for both strains. The inhibition of the IC50 compared to the control serum (spiked with PBS only) resulted < 30% when a heterologous competitor was used as shown in Figure 15-16. In the case of heterologous competitors S. paratyphi antigen (5 µg/mL) against S. Enteritidis was observed an inhibition >30%.

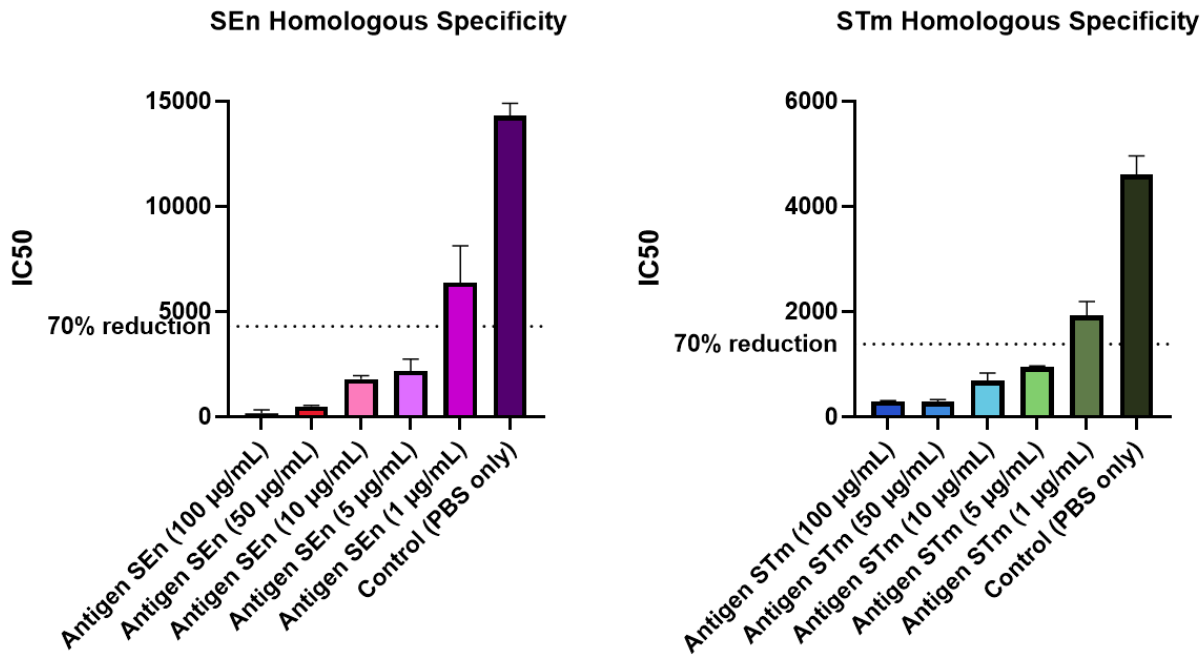

Figure 15 shows the results (IC50) obtained for the Homologous Specificity experiment of the L-SBA for SEn (graph on the left) and for STm (graph on the right). The different antigen concentrations are reported on the X axis and the IC50s obtained are reported on the Y axis. The dotted line represents the 70% reduction threshold respect the control.

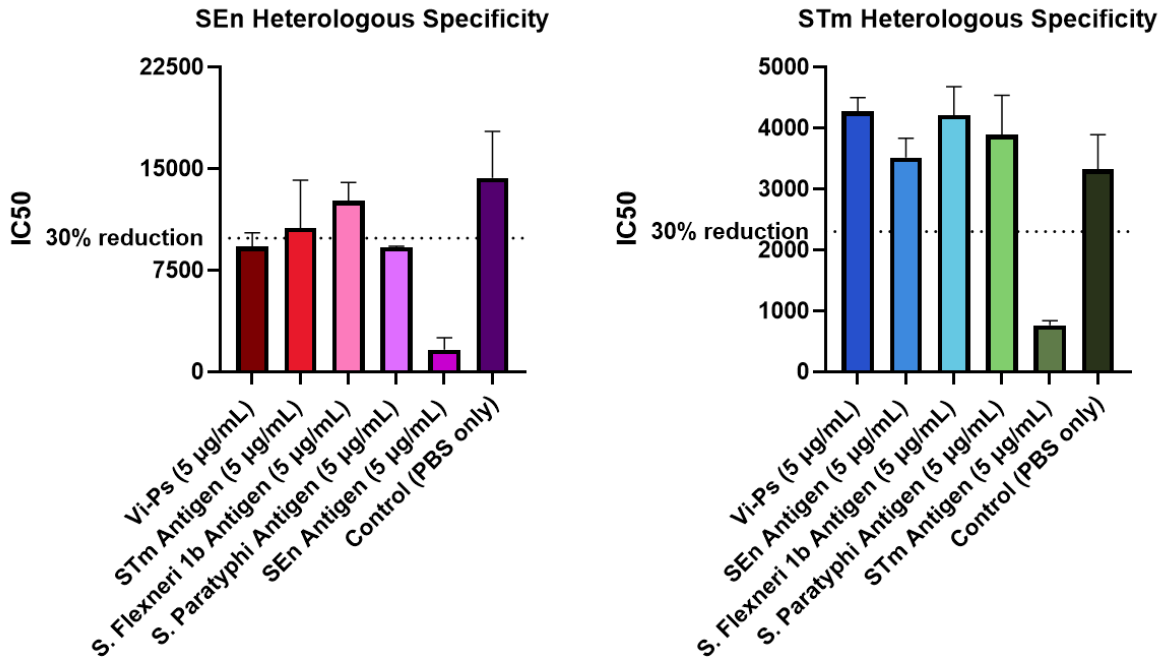

Figure 16 shows the results (IC50) obtained for the Heterologous Specificity experiment of the L-SBA for SEn (graph on the left) and for STm (graph on the right). The different antigen concentrations are reported on the X axis and the IC50s obtained are reported on the Y axis. The dotted line represents the 30% reduction threshold respect the control.

22. Validation of a Classical Serum Bactericidal Assay for *Salmonella* Enterica Paratyphi A

- Precision

For assessing the precision, seven operators have tested 2 high, 2 medium, 2 low (tested at 1:8 final dilutions) and 1 negative serum sample (IgG depleted serum) selected from the precision panel in Six replicates each, in 3 days. Each analyst has thus generated 18 determinations for each sample, for a total of 126 determinations considering all seven analysts. Median titre and agreement to Analyst 1 (reference titer) have been calculated for each sample.

The global precision has ≥ 85% of the samples within the median titer ± 1 SBA titer. Samples having >50% valid results have been considered for calculating the global precision. Combined analysis done for global precision for all 7 Analysts has been reported in Table 4.

Conclusion for Precision parameter: the intra- and inter- assay precision resulted to have an agreement ≥ 85% for all the samples and Analysts.

Table 4 Agreement calculated for the global precision.

Overall % Agreement 7 OPs - 3 DAYs	
Intra-Assay Analysis	**% Valid results inside the range**
DAY 1	95%
DAY 2	95%
DAY 3	98%
Analyst 1	99%
Analyst 2	97%
Analyst 3	97%
Analyst 4	99%
Analyst 5	97%
Analyst 6	97%
Analyst 7	100%

- Dilutional Linearity

The linearity has been assessed by two analysts, by testing 2 high, 1 medium and 2 low sample, selected from the linearity panel, tested in duplicate pre-diluting the sample. Each prediluted sample have been run as an independent sample, serially diluted at 2-fold step.

The accuracy has been also evaluated by calculating the difference between the reference titre (median of the titres) and observed titre (median of the adjusted titres) for each sample.

The linearity of each sample has been assessed using a linear regression graph comparing the log 10_2 transformed values for dilution used and the log 10_2 transformed observed SBA titres against each of the dilution. In Figure 17 and 18 are reported the Linearity results of Analyst 1 and 2, respectively. Log2 transformed dilutions are plotted on the X axis and corresponding Log2 transformed SBA titres are plotted on the Y axis. Equations and R^2 values for each of the results are displayed on each individual graph.

The SBA titres for HS, MS, and LS sera have demonstrated strong linearity when analysed by linear regression, consistently yielding R^2 values greater than 0.9 for both Analysts (Figure 17 and 18). The criterion for assay accuracy is also met; the observed SBA titre of the spiked serum specimen be within + 1 SBA titre for ≥ 80% of the samples tested.

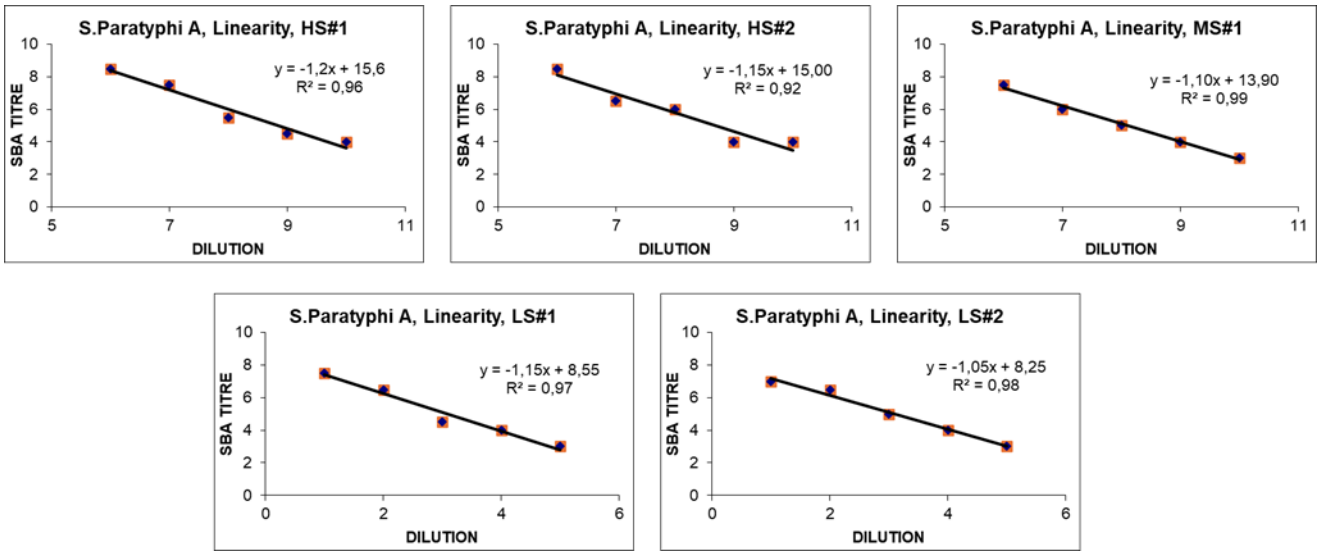

Figure 17 shows Linearity experiments result of Analyst 1.

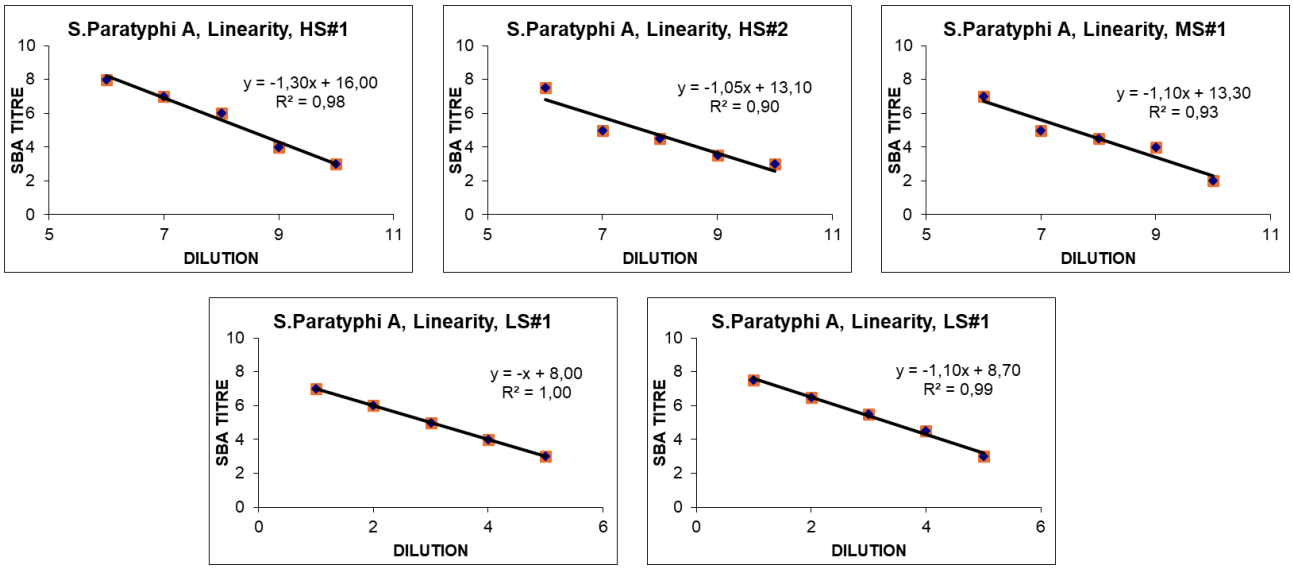

Figure 18 shows Linearity experiments result of Analyst 2.

- Robustness

The robustness is defined as a measure of the capacity of the analytical procedure to remain unaffected by small but deliberate changes in the test conditions. A set of 2 high, 1 medium and 2

low serum samples selected from the robustness panel, have been tested in duplicate by two analysts at different incubation time intervals (30, 60 and 90 mins). In figure 19 are reported the. SBA titres obtained for all five sera sample tested (2 HS, MS and 2 LS), measured at 60 and 90 minutes of incubation agreed within ± 1-Fold. While in the case of 30-minute incubation times, consistently yielded SBA titres lower than 1-fold compared to the 60-minute median titres. This suggests that incubation interval of 30 minutes is insufficient for complete reaction (i.e- complement activation and killing of bacterial cells). Hence assay results are robust only at 60- and 90-time intervals.

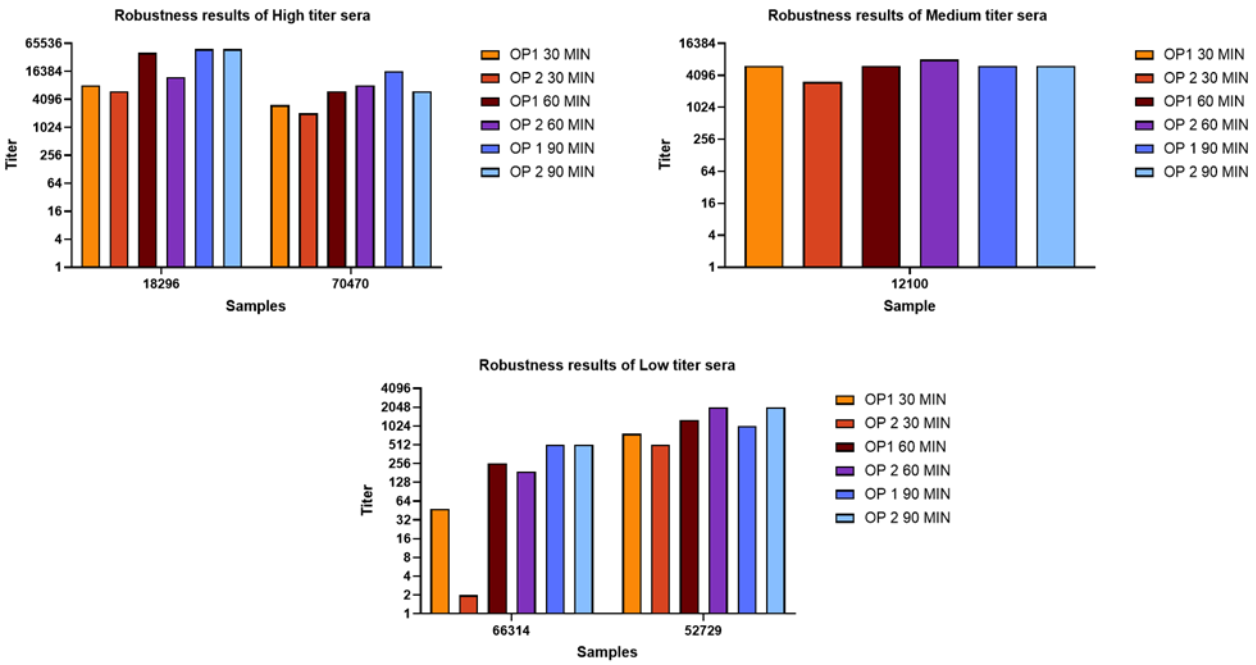

Figure 19 shows SBA titres obtained during the robustness experiments for the HS, MS and LS of analyst 1and 2, plotted on the Y axis and the different sera samples on the X axis.

- Specificity

The specificity is defined as the ability of an analytical procedure to solely determine/detect the analyte that it intends to measure. Specificity is assessed by testing these sera after pre-treatment for 1h at 37°C with an equal volume of homologous competitor polysaccharide at a final concentration of 100 µg/mL. Specificity have been assessed by testing 2 high and 2 medium sera samples selected from the specificity panel by two analysts in duplicate, spiked with homologous, heterologous,

unrelated species competitor and non-spiked serum as control. The specificity is evaluated by calculating the difference between the reference titre (untreated sample) and observed titre (treated sample) for each sample.

SBA titre obtained for Homologous PS treatment showed reduction in titre (> 4-fold) as compared to the titre for the non-spiked/no PS control (Figure 20). Whereas no inhibition (> 4-fold) of SBA titre is seen for any of the other 2 treatment groups (Heterologous PS and Unrelated PS), thus the specificity of the assay is proved.

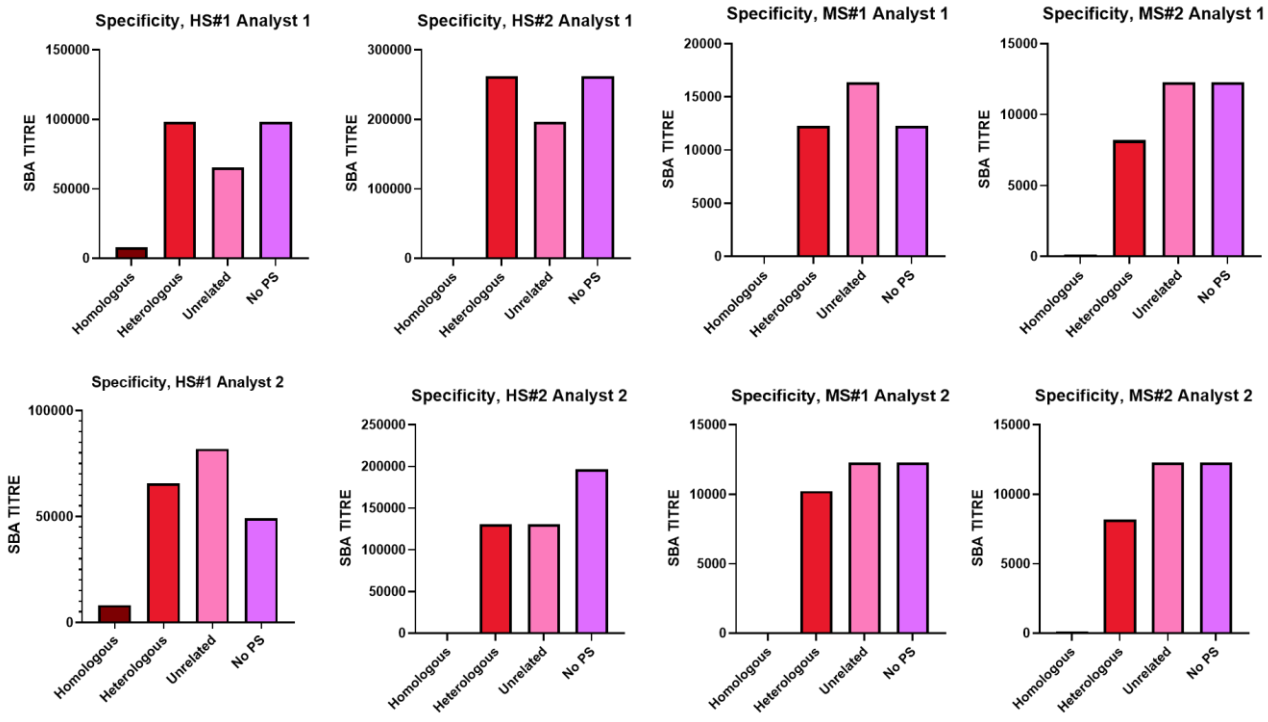

Figure 20 shows SBA titres obtained in the specificity experiment plotted on Y axis and the different PS (Homologous, Heterologous, unrelated PS and no PS) used to spike the MS and HS on X axis.

- Ruggedness

The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Sera samples may need to be re-tested and as such, may undergo many freeze/thaw cycles to perform those additional tests. Therefore, the impact of multiple freeze/thaw cycles must be assessed accordingly. Ruggedness have been assessed by testing 2 high, 1 medium

and 2 low sera sample with concentrations covering the analytical range following the Figure 8 and 9. Each freeze/thaw cycle should mimic clinical testing conditions. Each cycle began with the removal of samples from -70°C, which were thawed at ambient temperature (15°C to 30°C) for at least 1 hour aliquoted and then inactivated for use in the assay. The remaining part is kept back at storage condition (-70°C). The cycle is repeated to obtain SBA titres for different freeze thaw cycles. SBA titer obtained for all the 5 cycles were within ± 1 SBA titre of the corresponding Cycle 0 reference value, thus the sera sample results remain unaffected by 5 freeze/thaw cycles.

- LLOQ Verification

The LLOQ is defined as the lowest value of a sample that can be quantitively determined with suitable precision and linearity. The lowest dilution of sera achievable for the SBA is 4. The LLOQ was verified by 3 analysts by testing 4 low and 4 negative titre serum samples, which fall within the range between half the LLOQ and 4X LLOQ (titre corresponding to 16).

The LLOQ of 4 (reciprocal dilution) is considered verified if:

- Positive agreement is ≥ 66%
- Negative agreement is ≥ 66%

The positive and negative agreement met the acceptance criteria (≥ 66%) resulting 97% and 100%, respectively.

- Matrices Effect

The matrices effect has been evaluated by using a spike/recovery approach to explore whether the interference components from the serum matrices may affect the performance of the assay. Four sera from positive samples (2 medium, 1 low and 1 high sera sample selected trough other validation panels) have been spiked with: Hemolysate (final concentration 10.5 mg/mL, Sun Diagnostics), Triglyceride (final concentration 3.0 mg/mL, Sun Diagnostics) and Bilirubin (final concentration 0.5 mg/mL, Sun Diagnostics). SBA titer obtained for the ≥ 70% sample spiked with the different

interferents were within ± 1 SBA titre of the corresponding reference value (non-treated), thus the sera sample remain unaffected in presence of interference components from the serum matrices.

23. Setup of a Bioluminescence-Serum Bactericidal Assay for *N. meningitidis*

23.1. Complement source validation experiments

Selection of a suitable source of complement represented a crucial step in setting up an SBA. To select the appropriate source of complement for measuring SBA responses, we first tested several human complements from normal human sera for their intrinsic bactericidal activity against the strain MC58lux. Bacteria incubated without complement were used as control. The comparison between the presence and absence of 25% complement source in the colony counting spots at different bacterial concentrations allowed the estimation of the complement-killing action. We assumed that a complement source was not bactericidal at the dilution used in the SBA (25%), when the killing did not exceed 15% the number of target bacteria incubated without complement source. We identified one serum out of 6 tested sera as a suitable complement source for the assay. Indeed, similar colony growth was observed in the presence and absence of this complement source by direct counting. Similarly, luminescence activity emitted from bacterial spots was closely comparable for both conditions (Figure 21). This complement source was selected to carry out the subsequent tests.

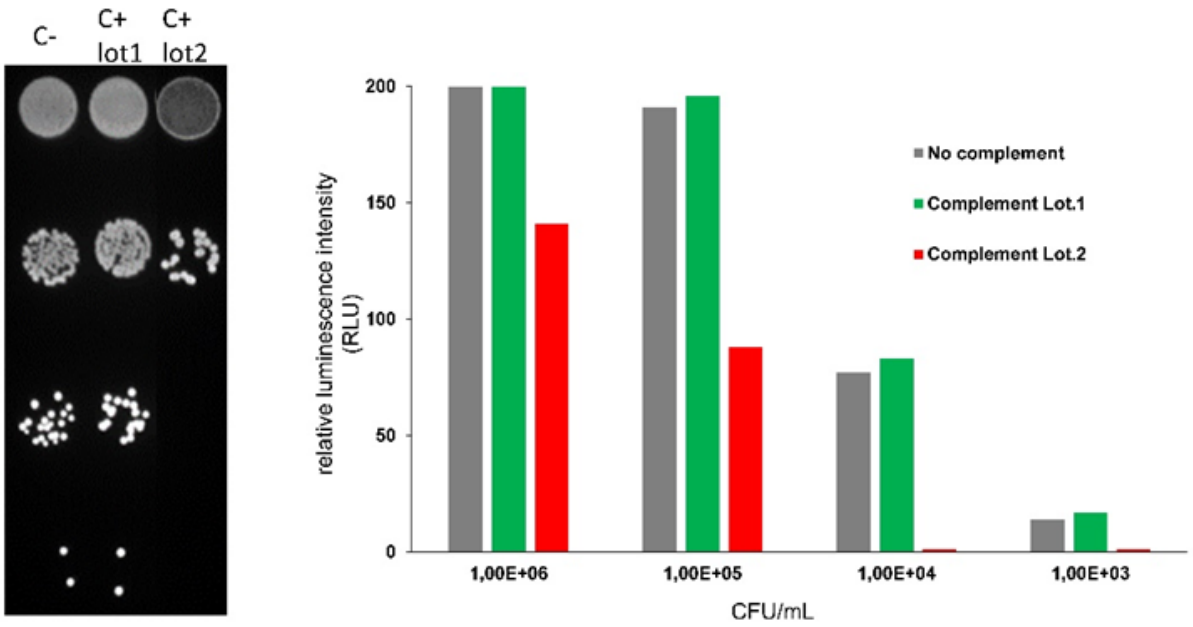

Figure 21 Complement source validation. Different concentrations of MC58Lux strain were assessed for survival at nonimmune human serum as a source of complement (C+) at a concentration of 25% for 60 min. Bacteria without complement (C-) were used as negative control. Luminescence emitted by the different bacterial concentrations was acquired after plating using the ChemiDoc Imaging system (Biorad) (left) and the intensity of the luminescence values of the related bacterial spots was quantitated using Image J software. The results expressed as the relative luminescence intensity (RLU) are shown as histograms (right). Two complements are shown, one validated (green) and another non-validated (red).

23.2. Conventional Serum Bactericidal Assay for *N. meningitidis*

In the conventional SBA, the incubation time of the reaction mix before being plated is one hour. However, bacterial lysis may not immediately impact the bioluminescence signal as the luciferase enzyme, and its substrate may still be available mixture to provide bioluminescence. In fact, after 1 hour the curve obtained was flat while the spot in the agar plate showed bactericidal activity. Therefore, we performed a time incubation setup using 12 human sera previously immunized with the MenBvac® vaccine, and the bioluminescence was recorded every hour for six hours. The IC50 was compared to the CFU results of the agar plate. The results showed that the reliability of the curve (Figure 22), the IC50, and the related R2 increased with time until reaching a plateau at 5 hours, which was selected as the optimal time point to score bioluminescence.

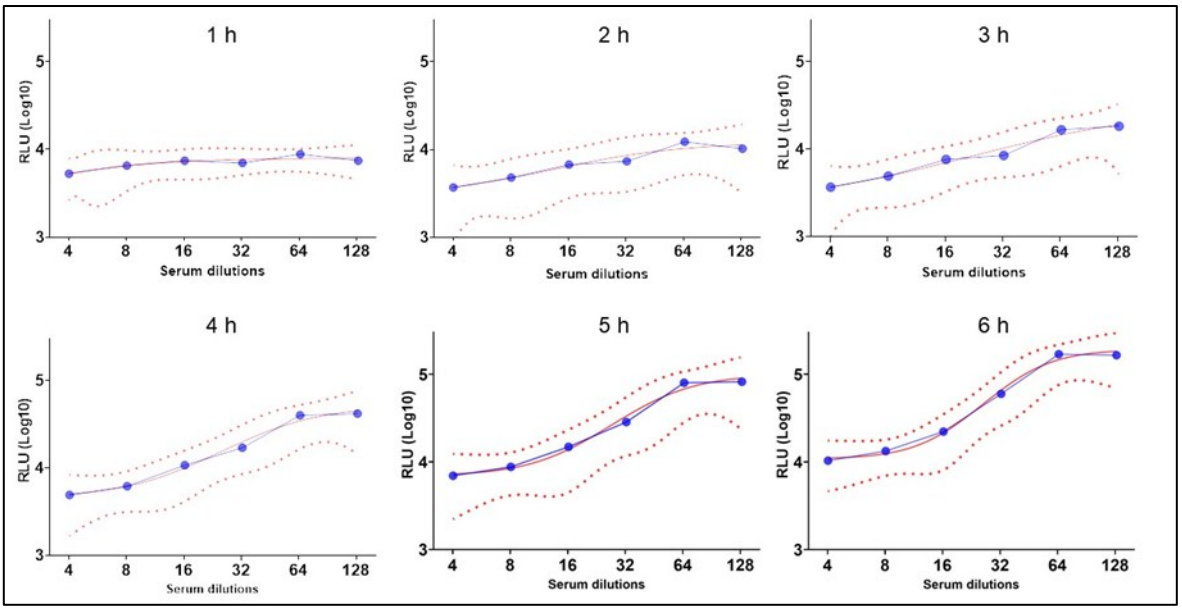

Figure 22 shows six hours timeline of a serum sample tested in BioLux-SBA where the luminescence is recorded every hour. The assay has been performed on 12 sera samples. RLU (Y-axis) stands for Relative Luminometer Units

23.3. Repeatability and intermediate precision of the BioLux-SBA

The repeatability and the intermediate precision of the BioLux-SBA were evaluated to demonstrate the assay precision were evaluated as described in the Methods section. To evaluate the intra- and inter-day precision the standard error (SE%) was calculated using the IC50. The repeatability showed limited variation with SE% of 2% for the first day, 7% for the second day, and 3% for the third day (Figure 23). For the intermediate precision, the SE% was calculated using the average of IC50 from each day, with a result of 17%. These results suggest that variance attributed to both, repeatability and intermediate precision are not significant.

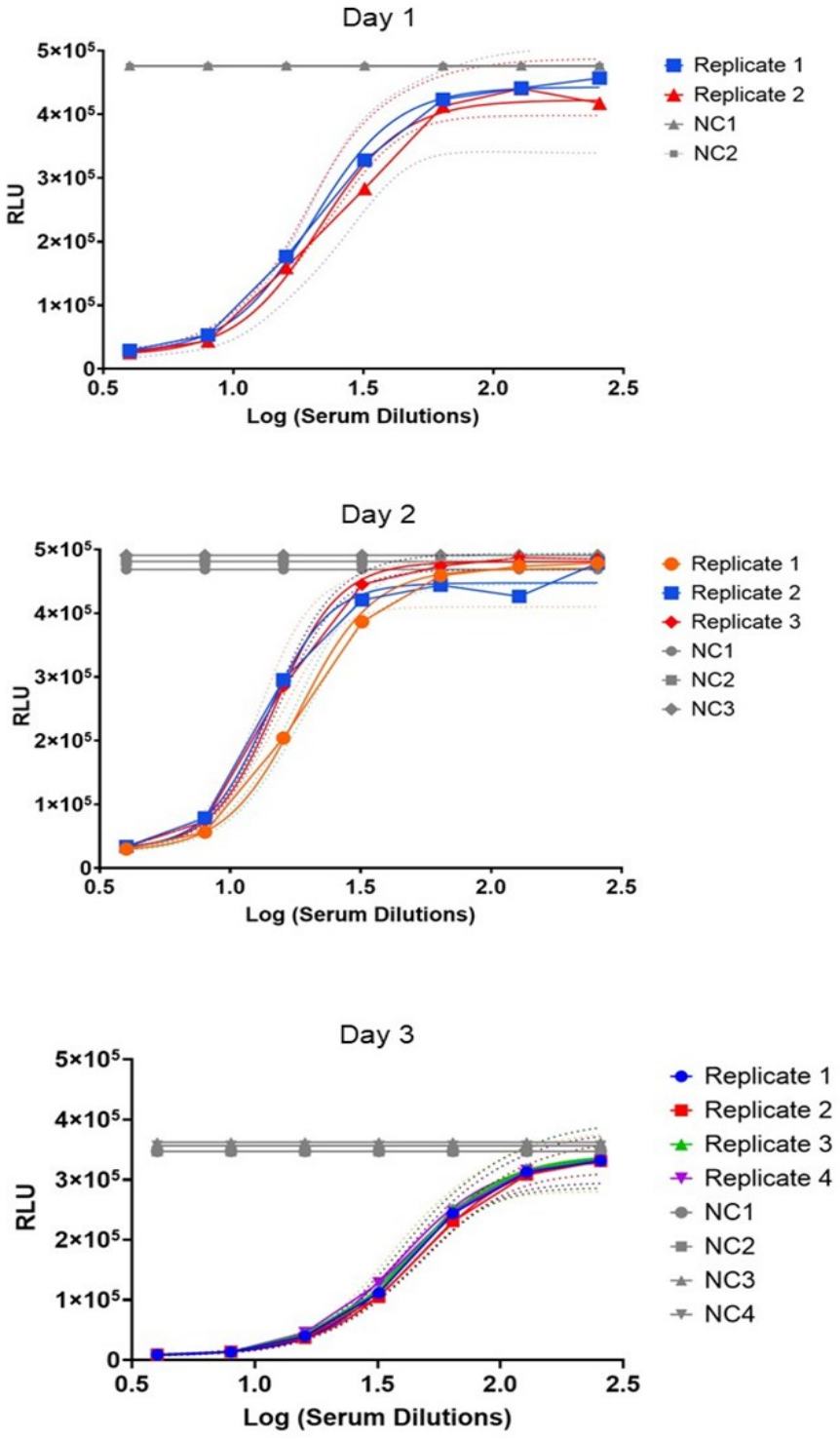

Figure 23 The same serum sample as in Figure 3 was tested in BioLuxSBA in duplicate on day 1, in triplicate on day 2, and in four replicates on day 3 for the evaluation of repeatability and intermediate precision. In the graph in grey (NC1, NC2, NC3 or NC4), the negative control value of each replicate which is the reaction mix (bacteria + complement) is reported in the absence of sera. Replicates are represented with symbols. Colored solid lines without symbols represent the curve fitting by nonlinear regression for each replicate. Dashed lines represent the 95% confidence interval (CI) of each replicate. RLU (Y-axis) stands for Relative Luminometer Units.

23.3.1. Correlation between C-SBA and BioLux-SBA

The C-SBA and the BioLux-SBA were performed in parallel for 10 sera samples to calculate the correlation between the results obtained. The results of 10 sera samples evaluated showed a good agreement between the two assays as demonstrated by the Pearson r of 0.99 with a significant P-value <0.0001 and a Spearman r of 0.96 with a P-value <0.0001. The simple linear regression calculated showed an R^2 of 0.98 with a P-value <0.0001 (Figure 24). These results suggest a linear correlation between the two assays.

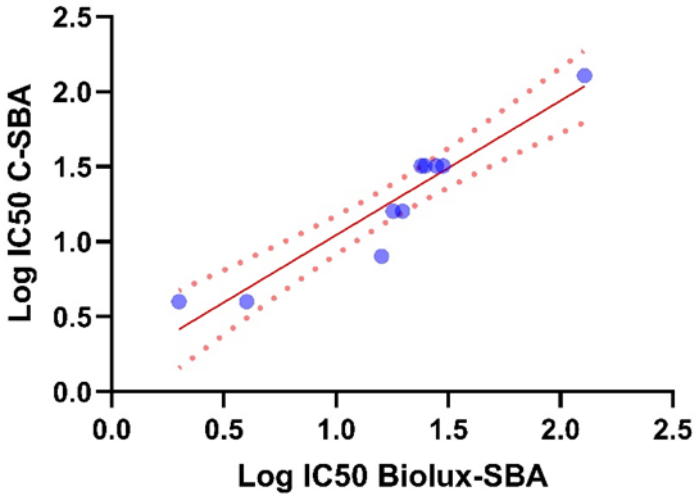

Figure 24 Log IC50 C-SBA (Y axis) vs Log IC50 BioLux SBA (X axis) with the red line representing the linear regression trendline, and red dot line the 95% CI (confidence interval).

23.4. Defining the threshold of BioLux-SBA

The BioLux-SBA was used for the screening of bactericidal activities in 48 human sera samples of vaccinated patients that were previously described[104]. Compared to the C-SBA established threshold, 13 of the sera (27%) showed BioLux titers lower than 4, while 35 sera (73%) showed titers equal to or higher than 4 with a complete correlation between C-SBA data and BioLux-SBA. Indeed, Figure 25 shows the positive sera (C-SBA of 4 or higher in blue circles) and negative sera (C-SBA lower than 4 in red circles). These data suggest that the titer of 4 in BioLux-hSBA can be also used as a threshold for protective titers as in the case of C-SBA.

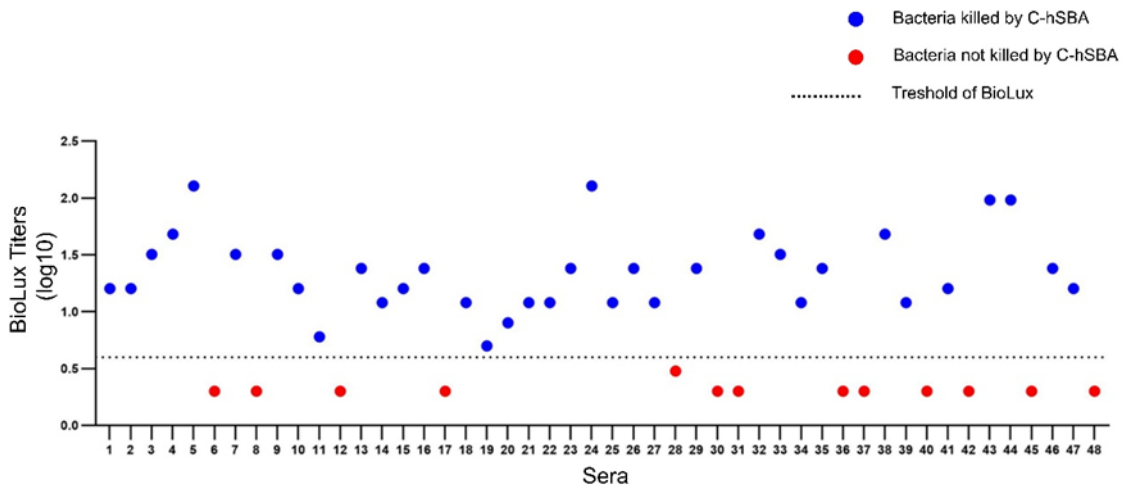

Figure 25 BioLux-SBA titer of 48 serum samples expressed in logarithmic scale (Y axis). using the human complement. Red circles correspond to sera with titers of <4 (bacteria not killed) and blue circles stand for the sera with titers of = or ≥4 (killed bacteria) as determined by C-SBA. The dashed line corresponds to the threshold of BioLux-SBA that separates killed and not killed bacteria by C-hSBA). Number under the X-axis refers to individual sera (n=48).

23.5. Terminal Complement Complex Deposition assay

We finally correlated the C-SBA and the BioLux-SBA titers to the levels of deposition of the C5b-C9 (the complement membrane attack complex, MAC) on the bacterial surface. The MAC deposition assay has been performed in all 48 sera samples using flow cytometry as described in Materials and methods. Three representative sera are depicted in Figure 7 with high, medium, and lower BioLux-SBA of 128, 32, and 2 titers, respectively. As reported in Figure 7, immune sera were able to promote C5b-C9 deposition on bacterial surface. C5b-C9 deposition levels correlated well with BioLux-SBA titers and ranged from 70.9% (BioLux-SBA of 128) to 47.4% (BioLux-SBA titer of 32) and 15.3% (BioLux-SBA titer of 2). The IC50 of 48 sera samples evaluated in BioLux SBA and the Mean Fluorescence Intensity (MFI) obtained using the C5b-C9 deposition assay showed a Spearman r correlation of 0.67 with a significant P-value <0.0001 (Figure 26).

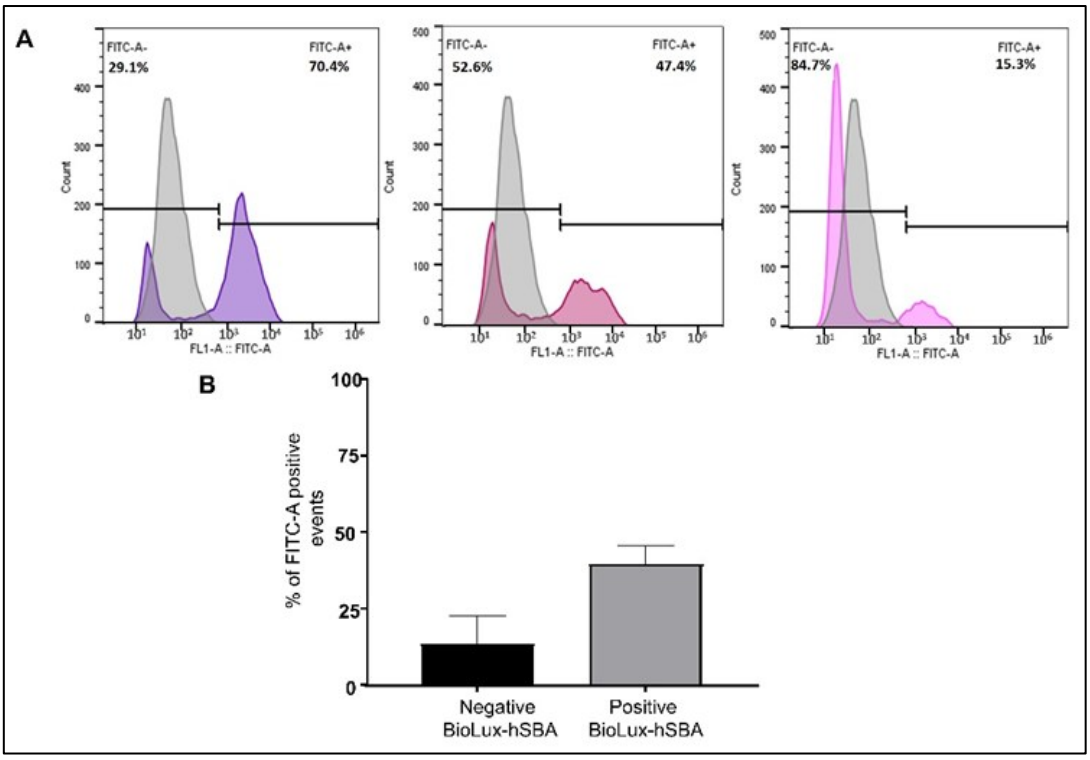

Figure 26 Binding of membrane attack complex on bacteria (A)The histograms show the result of three sera samples ((high (left), medium (middle) and low (right) bactericidal BioLux-SBA titers)) tested at the complement deposition assay. The grey curve stands for unstained bacteria. The colored curves correspond to bacteria stained with FITC-anti-human C5b-C9 antibody. The percentages of positive events (bacteria with disposition of C5b-C9 complex) are indicated at the upper right corner of each graph. (B). Combined levels of percentage of positive events (bacteria with disposition of C5b-C9 complex) for the 48 sera assessed. The black box depicts the mean and standard error of this percentage for sera with negative titer of BioLux-SBA (titer <4; n=13). The grey box depicts the mean and standard error of this percentage for sera with negative titre of BioLux-SBA (titer ≥4; n=35).

23.5.1. Measure of complement deposition on different serogroups of *N. meningitidis*

Invasive meningococcal disease (IMD) cases that are due to unusual groups appear to be associated with complement deficiencies (hereditary or acquired deficiencies). Only 6 of the 59 cases (10.2%) reported in this study were associated with such complement deficiencies. One case was associated with late terminal complement pathway deficiency (TPD). The other five cases were associated with acquired deficiency due to treatment for Paroxysmal *nocturnal* emoglobinuria (PNH) using monoclonal antibodies treatment (eculizumab or ravulizumab) against the C5 complement component (anti-C5 mAb). Of the six corresponding isolates, five belonged to group E, and one isolate was non-groupable (non-capsulated). One hypothesis is that the treatment with anti-C5 monoclonal antibodies impairs the formation of the MAC on the bacterial surface and therefore

enhances the survival of such isolates. To explore this hypothesis, we evaluated the deposition of the terminal complement pathway components on bacterial surfaces using flow cytometry and a monoclonal antibody directed against the membrane attack complex C5b-C9 (MAC), as described in the Methods section. Isolates of the serogroups E (n = 7), X (n = 6) and Z (n = 1) from the current study were tested, and we added invasive isolates of serogroups B (n = 16), C (n = 8), W (n = 14) and Y (n = 8) belonging to hyperinvasive isolates. Data were expressed as the means of fluorescence index for isolates of the same serogroup. Significantly higher levels of MAC deposition were observed for isolates of serogroup E when compared with serogroups B, C, and W ($p < 0.001$). Serogroup X isolates also showed higher levels compared with serogroups B, C, and W but did not reach statistical significance (Figure 27).

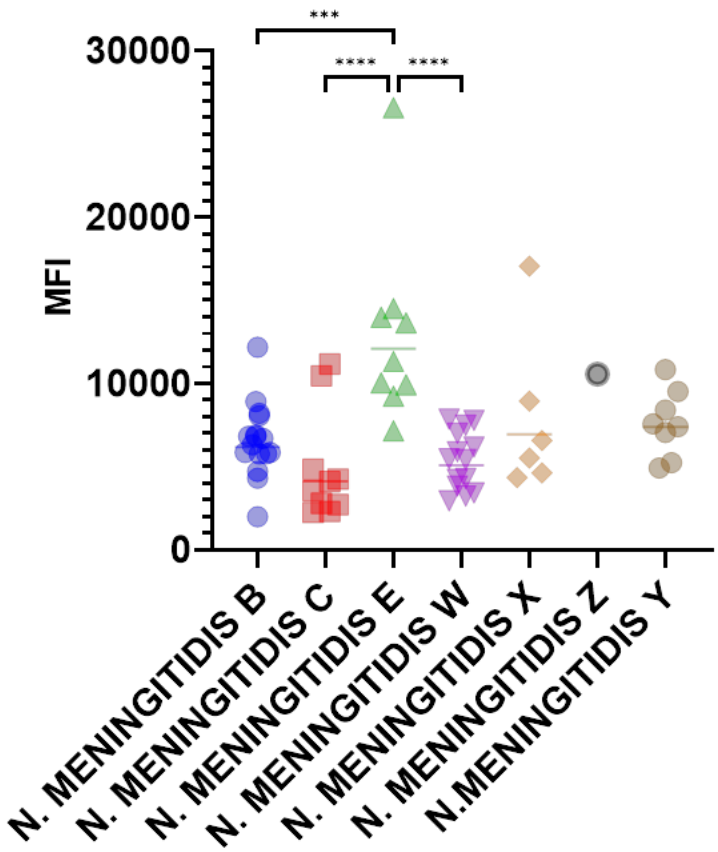

*Figure 27 shows flow cytometry analysis of deposition of membrane attack complex (MAC) at the surface of N. meningitidis isolates of several serogroups. Data are expressed as the mean of fluorescence index (MFI) for isolates of serogroups B (n = 16), C (n = 8), W (n = 14), E (n = 7), X (n = 6), Z (n = 1), and Y (n = 8). Significantly higher levels of MAC deposition were observed for isolates of serogroup E when compared to serogroups B, C, and W (*** p < 0.001 and **** p < 0.0001).*

24. FACS-Based Whole cell ELISA on live *N. Gonorrhoea*

The flow cytometry binding analysis of rabbit serum IgG to live *N. gonorrhoeae* strains are shown in Figure 28. The FACS-based whole cell ELISA evaluates the sera binding on the bacterial surface which is proportional to the fluorescence signal generated by the secondary antibody BV421-conjugated Goat Anti-Rabbit IgG. Figure 28 shows the median fluorescence intensity detected for the different sera samples: placebo pre- and post-immunization (negative control), 3 different pools of sera of different immunization with the candidate vaccine and the Bexsero control (mid-low positive control).

- Ng WHO F

The 10,000-strain specific count for the pooled samples were not reached as the bacterial cells lysed during the procedure. The protocol was amended where the time for all the centrifuge steps was decreased from 10 to 7 minutes, and the initial bacterial concentration was increased from the addition of 50 µL of bacterial suspension of OD_{600} 0.5 to 100 µL. With these changes, all samples successfully reached a 10000 count.

The negative control cells with secondary antibody did not bind (Figure 28). Staining with the Rb2C7 mAbs gave a high median fluorescence intensity (MFI) of 3,535,000. Sera from rabbits immunized with pool 1 also gave a high MFI value of 923,500. Low staining intensity was obtained for Control-pre (68,300), -post (68,935) and pool 3 (156,957). Bexsero gave an MFI value like pool 3.

- Ng WHO Z

The highest staining intensity was obtained for pool 1 pooled sera with an MFI value of 643,000. Staining with Rb2C7 mAbs gave a lower MFI value of 343,133 compared to pool 1. Similar staining intensity was obtained for pool 2 and Bexsero sera (213,351 and 199,751, respectively). Low binding was obtained for Control-pre (102,302) and -post (148,467).

- Ng WHO R

The highest staining intensity was obtained for pool 1 serum pool with an MFI value of 416,000. Staining with the Rb2C7 mAbs gave an MFI value of 128,952, like Control-post (122,469). The staining intensity for Bexsero (218,404) was like Control-pre (212,806) and pool 3 (184,857).

- Ng WHO K

The highest staining intensity was obtained for Rb2C7 mAb with an MFI value of 5,730,000, which was the highest obtained compared to all the strains assessed. Staining with pool 1 sera gave the second highest MFI value of 2,080,000. The staining intensity for the Control serum samples were for pre: 245,507 and post: 207,767. Similar staining intensity was obtained for pool 3 and Bexsero sera (506,500 and 513,500, respectively).

- Ng WT1291

The highest staining intensity was obtained for pool 1 with an MFI value of 5,730,000, the highest compared to all the strains evaluated. A low binding was recorded for the Rb2C7 mAb (MFI 69,877). The Control serum samples gave a high staining intensity (pre: 986,500; post: 1,305,000). A similar staining intensity was obtained for both the pool 3 (1,810,000) and Bexsero pooled sera (2,010,000).

- Ng WHO S2

The highest staining intensity was obtained for the pool 1 pooled sera (1,170,000) followed by Bexsero (882,500). Exceptionally low binding was recorded for the Rb2C7 mAb (18,498). Some background binding of Control sera (pre: 263,007; post: 236,781) was observed. pool 2 also showed binding to a higher degree than Control (882,500).

- Ng FA1090

As for strain WHO F, the 10,000-strain specific count for the pooled samples were not reached as the bacterial cells lysed during the procedure. The same protocol amendments as for strain WHO F were applied, and most samples, except for pool 3 and one Bexsero replicate, reached a strain positive

count of 10,000 (Figure 28). The highest staining intensity was obtained for Rb2C7 mAbs (2,765,000). High staining intensity was also observed for pool 1 (1,600,000) and Bexsero (365,474). Low background binding of Control sera (pre: 60,124; post: 88,313) and pool 3 (139,911) was observed.

MFI data from the assay development and the triplicate measurements were pooled and analysed (Figure 28). Highest MFI values for all groups were obtained for strain WT1291, while WHO R gave the lowest. For all strains, pool 2 gave a higher staining intensity compared to Bexsero and Control-post sera. Moreover, compared to Control-post, pool 2 showed an increase in staining intensity between 3.1 to 38.4-fold. While for Bexsero, the increase in staining intensity was only between 1.3 to 3.9-fold compared to Control-post. The highest fold difference in staining intensity for Pool 2compared to Control-post was obtained against strain FA1090 and the lowest for strain WHO R, while for Bexsero the highest fold difference was obtained against strains FA1090 and the lowest for WHO Q. Analysis of the MFI ratio suggests that the 1:200 dilution of the secondary antibody to be optimal for all the Ng strains

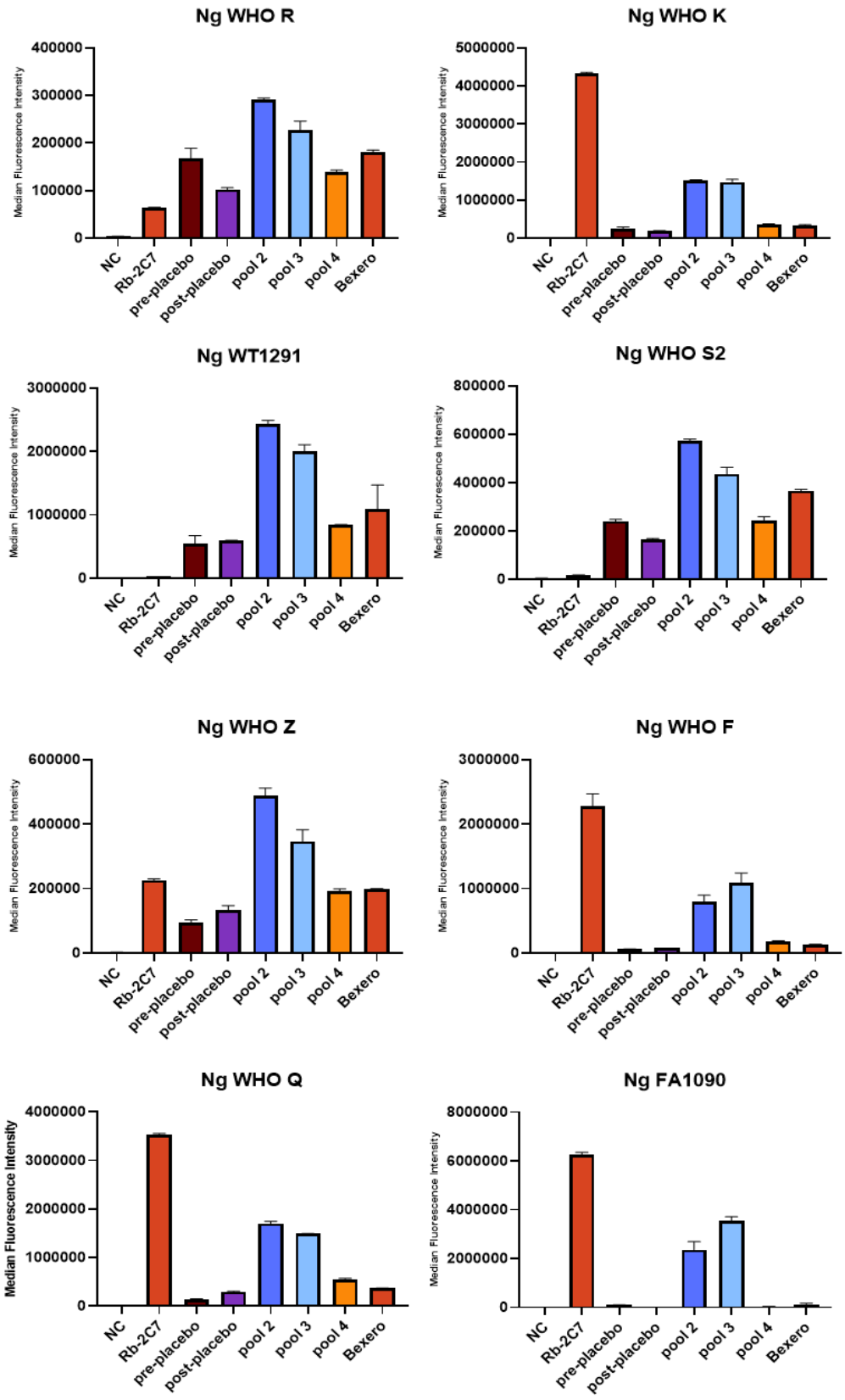

Figure 28 shows the Median fluorescence intensity (MFI) at 10,000 counts was read on fixed cells using the 525/40 BP channel (B525) for Alexa 488. Bars indicate median and +/-95% confidence interval. Data were analysed using FlowJo and GraphPad PRISM v10.3.1.

CONCLUSION AND FUTURE PROSPECTIVES

In recent years, the field of vaccinology has achieved significant advances. These innovations have indirectly led to the improvement of serological assays to assess the efficacy of vaccines and to better understand the mechanisms behind protective immunity. The immunogenicity is evaluated during all the steps performed during the approval process of a novel vaccine candidate, starting from the pre-clinical until the phase III clinical studies. The primary goal of a candidate vaccine is the prevention of the disease by inducing a persistent immunologic memory and the antibodies play a fundamental role. Antibodies prevent infection by enhancing different mechanisms, such as pathogens and toxin neutralization, complement activation, and cellular phagocytosis. Currently, the ELISA assay is the most widely used assay to measure the antibody concentration. However, the binding assay cannot evaluate the functionality of the antibodies. SBA and OPA are the most used assays for the assessment of neutralizing and opsonophagocytic antibodies. However, all the conventional versions of these assays are labour-intensive, time-consuming, and not applicable to a large number of samples. For these reasons, high throughput versions of these assays have been proposed to overcome some of the drawbacks. Luminescence-SBA has been demonstrated as a suitable alternative to the CFU-based method for several pathogens; however, it is not yet considered the gold standard like conventional SBA for meningococcal strains. The availability of biological standards or pathogen-specific positive controls will be also critical to assess vaccine-induced immune responses, promote standardization, minimize inter and intra-laboratory variability, and support comparability between vaccine candidates. Despite numerous improvements, further optimizations and development are needed to reduce assay variability and increase the applicability. The final goal is the employment of functional assays as part of the vaccine immunogenicity assessment process for all bacterial species, especially considering the need for new vaccines to fight antimicrobial resistance that represent the future worldwide challenge.

BIBIOLOGRAPHY

1. Baquero, F. Threats of antibiotic resistance: an obliged reappraisal. *Int. Microbiol.* **24**, 499–506 (2021).
2. Rosini, R., Nicchi, S., Pizza, M. & Rappuoli, R. Vaccines Against Antimicrobial Resistance. *Front. Immunol.* **11**, 1048 (2020).
3. Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* **18**, 318–327 (2018).
4. Mix, E., Goertsches, R. & Zett, U. K. Immunoglobulins—Basic considerations. *J. Neurol.* **253**, v9–v17 (2006).
5. Mix, E., Goertsches, R. & Zett, U. K. Immunoglobulins—Basic considerations. *J. Neurol.* **253**, v9–v17 (2006).
6. Goulet, D. R. & Atkins, W. M. Considerations for the Design of Antibody-Based Therapeutics. *J. Pharm. Sci.* **109**, 74–103 (2020).
7. Lu, L. L., Suscovich, T. J., Fortune, S. M. & Alter, G. Beyond binding: antibody effector functions in infectious diseases. *Nat. Rev. Immunol.* **18**, 46–61 (2018).
8. Heesterbeek, D. A. C., Angelier, M. L., Harrison, R. A. & Rooijakkers, S. H. M. Complement and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. *J. Innate Immun.* **10**, 455–464 (2018).
9. Xie, C. B., Jane-Wit, D. & Pober, J. S. Complement Membrane Attack Complex. *Am. J. Pathol.* **190**, 1138–1150 (2020).
10. Nandakumar, K. S. *et al.* A Recombinant Vaccine Effectively Induces C5a-Specific Neutralizing Antibodies and Prevents Arthritis. *PLoS ONE* **5**, e13511 (2010).
11. Nicaise-Roland, P., Mehlal, S., Bouz, C. & Chollet-Martin, S. Biomarqueurs et tests fonctionnels dans l'hypersensibilité immédiate aux vaccins ARNm dirigés contre le SARS-CoV-2. *Rev. Fr. Allergol.* **62**, 624–627 (2022).

12. Salerno-Gonçalves, R. & Sztein, M. B. Cell-mediated immunity and the challenges for vaccine development. *Trends Microbiol.* **14**, 536–542 (2006).
13. Jiskoot, W., Kersten, G. F. A., Mastrobattista, E. & Slütter, B. Vaccines. in *Pharmaceutical Biotechnology* (eds Crommelin, D. J. A., Sindelar, R. D. & Meibohm, B.) 281–304 (Springer International Publishing, Cham, 2019). doi:10.1007/978-3-030-00710-2_14.
14. Li, Y. *et al.* The Immunoregulation of Th17 in Host against Intracellular Bacterial Infection. *Mediators Inflamm.* **2018**, 1–13 (2018).
15. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat. Immunol.* **9**, 503–510 (2008).
16. Corrado, M. & Pearce, E. L. Targeting memory T cell metabolism to improve immunity. *J. Clin. Invest.* **132**, e148546 (2022).
17. Pidwill, G. R., Gibson, J. F., Cole, J. Renshaw, S. A. & Foster, S. J. The Role of Macrophages in Staphylococcus aureus Infection. *Front. Immunol.* **11**, 620339 (2021).
18. Montero, D. A. *et al.* Two centuries of vaccination: historical and conceptual approach and future perspectives. *Front. Public Health* **11**, 1326154 (2024).
19. Del Giudice, G., Rappuoli, R. & Didierlaurent, A. M. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. *Semin. Immunol.* **39**, 14–21 (2018).
20. De Mot, L. *et al.* Transcriptional profiles of adjuvanted hepatitis B vaccines display variable interindividual homogeneity but a shared core signature. *Sci. Transl. Med.* **12**, eaay8618 (2020).
21. Osterloh, A. Vaccination against Bacterial Infections: Challenges, Progress, and New Approaches with a Focus on Intracellular Bacteria. *Vaccines* **10**, 751 (2022).
22. Song, J. Y., Moseley, M. A., Burton, R. L. & Nahm, M. H. Pneumococcal vaccine and opsonic pneumococcal antibody. *J. Infect. Chemother.* **19**, 412–425 (2013).
23. Osterloh, A. Vaccination against Bacterial Infections: Challenges, Progress, and New Approaches with a Focus on Intracellular Bacteria. *Vaccines* **10**, 751 (2022).

24. Rappuoli, R. Glycoconjugate vaccines: Principles and mechanisms. *Sci. Transl. Med.* **10**, eaat4615 (2018).

25. Thisyakorn, U. *et al.* Immunogenicity and safety of 23-valent pneumococcal polysaccharide vaccine as a booster dose in 12- to 18-month-old children primed with 3 doses of 7-valent pneumococcal conjugate vaccine. *Hum. Vaccines Immunother.* **10**, 1859–1865 (2014).

26. Mbaeyi, S. A. *et al.* Meningococcal Vaccination: Recommendations of the Advisory Committee on Immunization Practices, United States, 2020. *MMWR Recomm. Rep.* **69**, 1–41 (2020).

27. Slack, M. *et al. Haemophilus influenzae* type b disease in the era of conjugate vaccines: critical factors for successful eradication. *Expert Rev. Vaccines* **19**, 903–917 (2020).

28. Domenech De Cellès, M., Rohani, P. & King, A. A. Duration of Immunity and Effectiveness of Diphtheria-Tetanus–Acellular Pertussis Vaccines in Children. *JAMA Pediatr.* **173**, 588 (2019).

29. Fraser, A., Paul, M., Goldberg, E., Acosta, C. J. & Leibovici, L. Typhoid fever vaccines: Systematic review and meta-analysis of randomised controlled trials. *Vaccine* **25**, 7848–7857 (2007).

30. Roy, A. *et al.* Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. *BMJ* **349**, g4643–g4643 (2014).

31. Ii, J. F. M., Smith, L. L., Brantley, P. & Locke, D. Vaxchora: The First FDA-Approved Cholera Vaccination in the United States.

32. Castilla, J. *et al.* Effectiveness of a Meningococcal Group B Vaccine (4CMenB) in Children. *N. Engl. J. Med.* **388**, 427–438 (2023).

33. Poolman, J. T. Expanding the role of bacterial vaccines into life-course vaccination strategies and prevention of antimicrobial-resistant infections. *Npj Vaccines* **5**, 84 (2020).

34. North, J. *Challenges to Tackling Antimicrobial Resistance: Economic and Policy Responses*. (Cambridge University Press, 2020). doi:10.1017/9781108864121.

35. Pollard, A. J. & Bijker, E. M. A guide to vaccinology: from basic principles to new developments. *Nat. Rev. Immunol.* **21**, 83–100 (2021).

36. Trombetta, C., Perini, D., Mather, S., Temperton, N. & Montomoli, E. Overview of Serological Techniques for Influenza Vaccine Evaluation: Past, Present and Future. *Vaccines* **2**, 707–734 (2014).

37. Manenti, A. *et al.* Evaluation and correlation between SARS-CoV-2 neutralizing and binding antibodies in convalescent and vaccinated subjects. *J. Immunol. Methods* **500**, 113197 (2022).

38. Feavers, I. & Walker, B. Functional Antibody Assays. in *Vaccine Adjuvants* (ed. Davies, G.) vol. 626 199–211 (Humana Press, Totowa, NJ, 2010).

39. Plotkin, S. A. Vaccines: Correlates of Vaccine-Induced Immunity. *Clin. Infect. Dis.* **47**, 401–409 (2008).

40. Plotkin, S. A. Updates on immunologic correlates of vaccine-induced protection. *Vaccine* **38**, 2250–2257 (2020).

41. Boero, E., Vezzani, G., Micoli, F., Pizza, M. & Rossi, O. Functional assays to evaluate antibody-mediated responses against Shigella: a review. *Front. Cell. Infect. Microbiol.* **13**, 1171213 (2023).

42. Herrera, C. M., Schmitt, J. S., Chowdhry, E. I. & Riddle, M. S. From Kiyoshi Shiga to Present-Day Shigella Vaccines: A Historical Narrative Review. *Vaccines* **10**, 645 (2022).

43. Holmgren, J. *et al.* Correlates of protection for enteric vaccines. *Vaccine* **35**, 3355–3363 (2017).

44. Oosterhuis-Kafeja, F., Beutels, P. & Van Damme, P. Immunogenicity, efficacy, safety and effectiveness of pneumococcal conjugate vaccines (1998–2006). *Vaccine* **25**, 2194–2212 (2007).

45. 1307.

46. Toh, Z. Q. *et al.* Evaluating Functional Immunity Following Encapsulated Bacterial Infection and Vaccination. *Vaccines* **9**, 677 (2021).

47. Dunbar, S. A. Multiplexed suspension array immunoassays for detection of antibodies to pneumococcal polysaccharide and conjugate vaccines. *Front. Cell. Infect. Microbiol.* **13**, 1296665 (2023).

48. LaFon, D. C. & Nahm, M. H. Measuring immune responses to pneumococcal vaccines. *J. Immunol. Methods* **461**, 37–43 (2018).

49. Wernette, C. M. *et al.* Enzyme-Linked Immunosorbent Assay for Quantitation of Human Antibodies to Pneumococcal Polysaccharides. *Clin. Vaccine Immunol.* **10**, 514–519 (2003).

50. Aruta, M. G., Carducci, M., Micoli, F., Necchi, F. & Rossi, O. Increasing the High Throughput of a Luminescence-Based Serum Bactericidal Assay (L-SBA). *BioTech* **10**, 19 (2021).

51. Borrow, R. *et al.* Methods to evaluate serogroup B meningococcal vaccines: From predictions to real-world evidence. *J. Infect.* **81**, 862–872 (2020).

52. Findlow, J., Lucidarme, J., Taha, M.-K., Burman, C. & Balmer, P. Correlates of protection for meningococcal surface protein vaccines: lessons from the past. *Expert Rev. Vaccines* **21**, 739–751 (2022).

53. Borrow, R. *et al.* Interlaboratory Standardization of the Measurement of Serum Bactericidal Activity by Using Human Complement against Meningococcal Serogroup B, Strain 44/76-SL, before and after Vaccination with the Norwegian MenBvac Outer Membrane Vesicle Vaccine. *Clin. Vaccine Immunol.* **12**, 970–976 (2005).

54. Donnelly, J. *et al.* Qualitative and quantitative assessment of meningococcal antigens to evaluate the potential strain coverage of protein-based vaccines. *Proc. Natl. Acad. Sci.* **107**, 19490–19495 (2010).

55. Vogel, U. *et al.* Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment. *Lancet Infect. Dis.* **13**, 416–425 (2013).

56. McNeil, L. K. *et al.* Predicting the Susceptibility of Meningococcal Serogroup B Isolates to Bactericidal Antibodies Elicited by Bivalent rLP2086, a Novel Prophylactic Vaccine. *mBio* **9**, e00036-18 (2018).

57. Plotkin, S. A. Correlates of Protection Induced by Vaccination. *Clin. Vaccine Immunol.* **17**, 1055–1065 (2010).

58. Borrow, R. *et al.* Interlaboratory Standardization of the Measurement of Serum Bactericidal Activity by Using Human Complement against Meningococcal Serogroup B, Strain 44/76-SL,

before and after Vaccination with the Norwegian MenBvac Outer Membrane Vesicle Vaccine. *Clin. Vaccine Immunol.* **12**, 970–976 (2005).

59. Necchi, F., Saul, A. & Rondini, S. Development of a high-throughput method to evaluate serum bactericidal activity using bacterial ATP measurement as survival readout. *PLOS ONE* **12**, e0172163 (2017).
60. Heuser, E., Becker, K. & Idelevich, E. A. Evaluation of an Automated System for the Counting of Microbial Colonies. *Microbiol. Spectr.* **11**, e00673-23 (2023).
61. Mancini, F. *et al.* Exploring the Role of GMMA Components in the Immunogenicity of a 4-Valent Vaccine against Shigella. *Int. J. Mol. Sci.* **24**, 2742 (2023).
62. Shimanovich, A. A. *et al.* Functional and Antigen-Specific Serum Antibody Levels as Correlates of Protection against Shigellosis in a Controlled Human Challenge Study. *Clin. Vaccine Immunol.* **24**, (2017).
63. Stazzoni, S. *et al.* High-throughput bactericidal assays for monoclonal antibody screening against antimicrobial resistant Neisseria gonorrhoeae. *Front. Microbiol.* **14**, 1243427 (2023).
64. Mak, P. A. *et al.* Development of an Automated, High-Throughput Bactericidal Assay That Measures Cellular Respiration as a Survival Readout for Neisseria meningitidis. *Clin. Vaccine Immunol.* **18**, 1252–1260 (2011).
65. Petzke, M. & Schwartz, I. Borrelia burgdorferi Pathogenesis and the Immune Response. *Clin. Lab. Med.* **35**, 745–764 (2015).
66. Strnad, M., Rudenko, N. & Rego, R. O. M. Pathogenicity and virulence of *Borrelia burgdorferi*. *Virulence* **14**, 2265015 (2023).
67. Simon, M. M., Schaible, U. E., Wallich, R. & Kramer, M. D. A mouse model for Borrelia burgdorferi infection: approach to a vaccine against Lyme disease. *Immunol. Today* **12**, 11–16 (1991).
68. Nigrovic, L. E. & Thompson, K. M. The Lyme vaccine: a cautionary tale. *Epidemiol. Infect.* **135**, 1–8 (2007).

69. Bézay, N. *et al.* Optimisation of dose level and vaccination schedule for the VLA15 Lyme borreliosis vaccine candidate among healthy adults: two randomised, observer-blind, placebo-controlled, multicentre, phase 2 studies. *Lancet Infect. Dis.* **24**, 1045–1058 (2024).

70. Nisa, I., Qasim, M., Yasin, N., Ullah, R. & Ali, A. Shigella flexneri: an emerging pathogen. *Folia Microbiol. (Praha)* **65**, 275–291 (2020).

71. Sadredinamin, M., Yazdansetad, S., Alebouyeh, M., Yazdi, M. M. K. & Ghalavand, Z. higella Flexneri Serotypes: O-antigen Structure, Serotype Conversion, and Serotyping Methods. *Oman Med. J.* **38**, e522–e522 (2023).

72. Pakbin, B., Brück, W. M. & Brück, T. B. Molecular Mechanisms of Shigella Pathogenesis; Recent Advances. *Int. J. Mol. Sci.* **24**, 2448 (2023).

73. Ranjbar, R. & Farahani, A. Shigella: Antibiotic-Resistance Mechanisms And New Horizons For Treatment. *Infect. Drug Resist.* **Volume 12**, 3137–3167 (2019).

74. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. (2023).

75. Raso, M. M., Arato, V., Gasperini, G. & Micoli, F. Toward a Shigella Vaccine: Opportunities and Challenges to Fight an Antimicrobial-Resistant Pathogen. *Int. J. Mol. Sci.* **24**, 4649 (2023).

76. Martin, P. & Alaimo, C. The Ongoing Journey of a Shigella Bioconjugate Vaccine. *Vaccines* **10**, 212 (2022).

77. Phalipon, A. & Mulard, L. A. Toward a Multivalent Synthetic Oligosaccharide-Based Conjugate Vaccine against Shigella: State-of-the-Art for a Monovalent Prototype and Challenges. *Vaccines* **10**, 403 (2022).

78. Mo, Y. *et al.* Safety and Immunogenicity of a Shigella Bivalent Conjugate Vaccine (ZF0901) in 3-Month- to 5-Year-Old Children in China. *Vaccines* **10**, 33 (2021).

79. Micoli, F., Nakakana, U. N. & Berlanda Scorza, F. Towards a Four-Component GMMA-Based Vaccine against Shigella. *Vaccines* **10**, 328 (2022).

80. Fierer, J. Invasive Non-typhoidal *Salmonella* (iNTS) Infections. *Clin. Infect. Dis.* **75**, 732–738 (2022).

81. Lu, J. *et al.* Salmonella: Infection mechanism and control strategies. *Microbiol. Res.* **292**, 128013 (2025).

82. Takaya, A., Yamamoto, T. & Tokoyoda, K. Humoral Immunity vs. Salmonella. *Front. Immunol.* **10**, 3155 (2020).

83. Sears, K. T., Galen, J. E. & Tennant, S. M. Advances in the development of *Salmonella* -based vaccine strategies for protection against Salmonellosis in humans. *J. Appl. Microbiol.* **131**, 2640–2658 (2021).

84. Baliban, S. M., Lu, Y.-J. & Malley, R. Overview of the Nontyphoidal and Paratyphoidal Salmonella Vaccine Pipeline: Current Status and Future Prospects. *Clin. Infect. Dis.* **71**, S151–S154 (2020).

85. MacLennan, C. A., Martin, L. B. & Micoli, F. Vaccines against invasive *Salmonella* disease: Current status and future directions. *Hum. Vaccines Immunother.* **10**, 1478–1493 (2014).

86. Tennant, S. M., MacLennan, C. A., Simon, R., Martin, L. B. & Khan, M. I. Nontyphoidal salmonella disease: Current status of vaccine research and development. *Vaccine* **34**, 2907–2910 (2016).

87. De Simone, D. *et al.* GMMA-based vaccine candidates against invasive nontyphoidal salmonellosis elicit bactericidal antibodies against a panel of epidemiologically relevant Salmonellae. *Front. Immunol.* **16**, 1610067 (2025).

88. Hollingshead, S. & Tang, C. M. An Overview of Neisseria meningitidis. in *Neisseria meningitidis* (eds Seib, K. L. & Peak, I. R.) vol. 1969 1–16 (Springer New York, New York, NY, 2019).

89. Borrow, R. *et al.* Neisseria meningitidis group B correlates of protection and assay standardization—International Meeting Report Emory University, Atlanta, Georgia, United States, 16–17 March 2005. *Vaccine* **24**, 5093–5107 (2006).

90. Caugant, D. A. & Maiden, M. C. J. Meningococcal carriage and disease—Population biology and evolution. *Vaccine* **27**, B64–B70 (2009).

91. Acevedo, R. *et al.* The Global Meningococcal Initiative meeting on prevention of meningococcal disease worldwide: Epidemiology, surveillance, hypervirulent strains, antibiotic resistance and high-risk populations. *Expert Rev. Vaccines* **18**, 15–30 (2019).

92. Rosain, J. *et al.* Strains Responsible for Invasive Meningococcal Disease in Patients With Terminal Complement Pathway Deficiencies. *J. Infect. Dis.* **215**, 1331–1338 (2017).

93. McNamara, L. A. *et al.* High Risk for Invasive Meningococcal Disease Among Patients Receiving Eculizumab (Soliris) Despite Receipt of Meningococcal Vaccine.

94. Biolchi, A. *et al.* 4CMenB Immunization Induces Serum Bactericidal Antibodies Against Non-Serogroup B Meningococcal Strains in Adolescents. *Infect. Dis. Ther.* **10**, 307–316 (2021).

95. Langereis, J. D. *et al.* Eculizumab impairs Neisseria meningitidis serogroup B killing in whole blood despite 4CMenB vaccination of PNH patients. *Blood Adv.* **4**, 3615–3620 (2020).

96. Ladhani, S. N. *et al.* Invasive meningococcal disease in patients with complement deficiencies: a case series (2008–2017). *BMC Infect. Dis.* **19**, 522 (2019).

97. Quillin, S. J. & Seifert, H. S. Neisseria gonorrhoeae host adaptation and pathogenesis. *Nat. Rev. Microbiol.* **16**, 226–240 (2018).

98. Petousis-Harris, H. *et al.* Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *The Lancet* **390**, 1603–1610 (2017).

99. Unemo, M. & Shafer, W. M. Antimicrobial Resistance in Neisseria gonorrhoeae in the 21st Century: Past, Evolution, and Future. *Clin. Microbiol. Rev.* **27**, 587–613 (2014).

100. Ngampasutadol, J. *et al.* Human Factor H Interacts Selectively with *Neisseria gonorrhoeae* and Results in Species-Specific Complement Evasion. *J. Immunol.* **180**, 3426–3435 (2008).

101. Edwards, J. L., Jennings, M. P. & Seib, K. L. Neisseria gonorrhoeae vaccine development: hope on the horizon? *Curr. Opin. Infect. Dis.* **31**, 246–250 (2018).

102. Belcher, T., Rollier, C. S., Dold, C., Ross, J. D. C. & MacLennan, C. A. Immune responses to Neisseria gonorrhoeae and implications for vaccine development. *Front. Immunol.* **14**, 1248613 (2023).

103. ICH Q2(R2) Guideline on validation of analytical procedures - Step 5.

104. Sevestre, J. *et al.* Durability of immunogenicity and strain coverage of MenBvac, a meningococcal vaccine based on outer membrane vesicles: Lessons of the Normandy campaign. *Vaccine* **35**, 4029–4033 (2017).

105. Carlin', N. I. A. & Lindberg, A. A. Monoclonal Antibodies Specific for Shigella flexneri Lipopolysaccharides: Clones Binding to Type IV, V, and VI Antigens, Group 3,4 Antigen, and an Epitope Common to All Shigella fexneri and Shigella dysenteriae Type 1 Strains. *INFECT IMMUN* **55**, (1987).