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Serum bactericidal assay for the evaluation of typhoid vaccine using a semi-automated colony-counting method

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ABSTRACT

Typhoid fever, mainly caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), is a life-threatening disease, mostly in developing countries. Enzyme-linked immunosorbent assay (ELISA) is widely used to quantify antibodies against *S.* Typhi in serum but does not provide information about functional antibody titers. Although the serum bactericidal assay (SBA) using an agar plate is often used to measure functional antibody titers against various bacterial pathogens in clinical specimens, it has rarely been used for typhoid vaccines because it is time-consuming and labor-intensive. In the present study, we established an improved SBA against *S.* Typhi using a semi-automated colony-counting system with a square agar plate harboring 24 samples. The semi-automated SBA efficiently measured bactericidal titers of sera from individuals immunized with *S.* Typhi Vi polysaccharide vaccines. The assay specifically responded to *S.* Typhi Ty2 but not to other irrelevant enteric bacteria including *Vibrio cholerae* and *Shigella flexneri*. Baby rabbit complement was more appropriate source for the SBA against *S.* Typhi than complements from adult rabbit, guinea pig, and human. We also examined the correlation between SBA and ELISA for measuring antibody responses against *S.* Typhi using pre- and post-vaccination sera from 18 human volunteers. The SBA titer showed a good correlation with anti-Vi IgG quantity in the serum as determined by Spearman correlation coefficient of 0.737 ($P < 0.001$). Taken together, the semi-automated SBA might be efficient, accurate, sensitive, and specific enough to measure functional antibody titers against *S.* Typhi in sera from human subjects immunized with typhoid vaccines.

1. Introduction

Typhoid fever is a gastrointestinal infectious disease caused by *Salmonella enterica* serovar Typhi that is transmitted through the ingestion of contaminated food or water. Risk factors for the disease are high in developing countries due to poor hygiene and sanitation, and the global burden of typhoid fever in 2010 was estimated at 26.9 million cases [1]. Typhoid fever is treated with antibiotics but it has become complicated by the emergence of multi-drug resistant strains of *S.* Typhi [2]. Moreover, the average cost of medical care for typhoid fever is estimated at $4500 per patient in the United States [3]. Thus, reasonably-priced vaccine would be a cost-effective approach to prevent typhoid fever [4].

Currently, two licensed typhoid vaccines, the oral live attenuated *S.* Typhi Ty21a and the parenteral Vi capsular polysaccharide (PS), are known to be safe and efficacious for people aged over 2 years [5]. These two vaccines offer similar levels of protection against typhoid fever and showed similar typhoid-specific humoral responses to Vi and Ty21a in field trials [6]. Although both vaccines confer protective immunity after the vaccination, booster immunization is recommended every 3 years and neither is effective nor licensed for the use in children under 2 years of age [7]. Thus, to overcome these limitations, attempts to develop next-generation typhoid vaccines that confer higher immunogenicity and long-lasting protective immunity in all age groups have been made. Several Vi-conjugated vaccines are under development, including a Vi-diphtheria toxoid [8], Vi-tetanus toxoid [9], Vi-CRM$_{197}$ [10,11],

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and live attenuated *S.* Typhi, CVD 909 [12,13].

For evaluation of typhoid vaccines, a passive hemagglutination assay has been previously used to quantify anti-Vi antibodies in serum after the vaccination [14,15]. However, it is rarely used due to tedious steps including a requirement for pre-absorption of test sera with sheep erythrocytes [16]. Enzyme-linked immunosorbent assay (ELISA) provides more practical tool to determine serum antibodies against *S.* Typhi in clinical trials [17,18] but it does not assess functional antibody levels. The serum bactericidal assay (SBA) measures functional *S.* Typhi-specific antibodies capable of complement-mediated bacterial killing. This has been accepted as an *in vitro* surrogate assay for the evaluation of immunogenicity of bacterial vaccines against cholera [19] and meningococcal disease [20] due to its close correlation with protection. Several factors including source and quantity of exogenous complements, bacterial strain, test sera, and antigen expression in target bacteria are important for obtaining reliable SBA results. However, conventional SBA is not appropriate for testing a large number of samples because it is time-consuming and labor-intensive. Here, we describe a simple and convenient SBA against *S.* Typhi using a semi-automated colony-counting system that has been developed for the measurement of cholera [19] and pneumococcal vaccine-induced functional antibody responses [21].

2. Materials and methods

2.1. Bacteria and reagents

S. Typhi Ty2, *S.* Typhi Ty21a, *S.* Paratyphi A, and *S.* Typhimurium were obtained from the American Type Culture Collection (Manassas, VA). *Vibrio cholerae* O1 El Tor Inaba (strain T19479) and *Shigella flexneri* 5a M90T were kindly provided by Prof. Jan Holmgren (University of Gothenburg, Sweden) and Prof. Dong Wook Kim (Hanyang University, Ansan, Korea), respectively. Luria-Bertani (LB) broth and agar were purchased from Conda (Madrid, Spain) and Junsei (Tokyo, Japan), respectively. 2,3,5-triphenyl tetrazolium chloride (TTC) and 4-nitrophenyl phosphate disodium hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Baby rabbit (3- to 4-week-old) and adult rabbit (8- to 12-week-old) complements were purchased from Pel-Freez Biologicals (Rogers, AR). Guinea pig and human complements were purchased from Rockland (Gilbertsville, PA) and Valley Biomedical (Winchester, VA), respectively. All complements were stored in aliquots at −80 °C until used. Phosphate-buffered saline (PBS) was purchased from Gibco-BRL (Gaithersburg, MD) and used for serum dilution.

2.2. Serum samples

Human sera were randomly chosen from healthy volunteers who had received the Vi PS vaccine during clinical trials and convalescent sera were obtained from cholera and typhoid patients [22,23]. Use of these samples was approved from the institutional review board of the International Vaccine Institute. The clinical sera, collected prior to and 4 weeks after the vaccination, were examined for SBA and antibody titers. To inactivate complements in the serum, human serum was heated at 56 °C for 30 min before use. Inactivated serum samples were kept at 4 °C until used.

2.3. Semi-automated SBA

The original SBA was kindly provided by Dr. Beth Kirkpatrick (University of Vermont, Burlington) and modified by using an automated colony-counting system as previously described [19]. In brief, each serum sample in triplicate was serially-diluted seven times with PBS in two-fold increments. Each well of a 96-well plate (Nunc, Roskilde, Denmark) was filled with 50 μl of serum sample. A single colony of *S.* Typhi Ty2 grown on an LB agar plate was inoculated and cultured in 5 ml of LB broth overnight at 37 °C with shaking. Cultured bacteria were harvested by centrifugation and resuspended in PBS. To adjust the bacteria to 7×10^3 CFU/ml, bacteria were further diluted with PBS, and a 50 μl mixture of bacteria and 10% baby rabbit complement was added to the 96-well microtiter plate containing 50 μl of 2-fold-serially-diluted test serum. After incubation for 1 h at 37 °C, 5 μl of reaction mixture was taken from each well and plated at 3 × 8 (24 samples in total) on a square LB agar plate using an eight-channel micropipette to enumerate colonies in triplicate of 8 samples prepared by 2-fold serial dilution. Once the mixtures were absorbed into the bottom agar, the agar plate was overlaid with a top agar (LB medium containing 2% agar and 100 μg/ml of TTC dye). The plates were then incubated at 37 °C overnight and bacterial colonies on the plates were counted using a colony counting system (FluorChem™ IS-9900; Alpha Innotech, San Leandro, CA). Viability of bacteria was calculated by comparing samples to the number of CFU of complement control in the absence of antibody. SBA titer was determined as the reciprocal highest dilution fold that had ≥50% of bactericidal capacity.

2.4. Microtiter plate-based SBA

The initial procedures used in the microtiter plate assay were the same as those in the semi-automated SBA as described above with minor modifications in bacterial numbers (1×10^6 CFU/ml). At the end of incubation, 150 μl of LB media were added to each well and the microplate was incubated for an additional 4, 6, 8, or 16 h and the optical density (OD) of the plate was read at 600 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The microtiter plate assay against *V. cholerae* was performed as previously described [19].

2.5. ELISA

A microwell plate (Nunc) was coated with 2 μg/ml of Vi PS and incubated at 37 °C overnight with gentle shaking. The plate was washed six times with a washing buffer containing 0.85% NaCl and 0.1% Brij 35. To prevent non-specific binding, 200 μl of 1% bovine serum albumin (BSA) in PBS was added and incubated for 1 h at 37 °C with shaking. After washing the plate, 100 μl of pre-diluted sera in 1% BSA/0.1% Brij 35/PBS was added and incubated for 1 h at 37 °C. After washing the plate, 100 μl of alkaline phosphatase (AP)-conjugated anti-human IgG or IgM (1:5000) (Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 1 h at 37 °C. The plate was washed three times with washing buffer. Then, 100 μl of 4-nitrophenylphosphate substrate (1 mg/ml) in 1 M Tris-HCl supplemented with 3 mM $MgCl_2$ at pH 9.8 was added and incubated for 30 min at room temperature. Fifty microliters of 3 M NaOH was added to stop the reaction and OD was measured at 405–490 nm using a microplate reader. Endpoint titers were expressed as the last dilution giving an OD 0.1 higher than the background [24,25].

2.6. Statistical analysis

Mean value ± standard deviation (SD) or standard error (SEM) was determined from at least triplicate in each sample [26]. Statistical significance of serum antibody titers and SBA titers between pre- and post-vaccinated groups was determined by a two-tailed Student's *t*-test. To examine the relationship between SBA titers and anti-Vi IgG or anti-Vi IgM titers, the results were plotted against each other and Spearman correlation coefficient (r) and P value