Editorial comments:

Changes to be made by the Author(s):

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**Manuscript has been reviewed.**

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

**Short Abstract/Summary has been rephrased.**

3. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee.

**An ethics statement has been added.**

4. JoVWE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: NICE software

**Commercial language was removed and NICE software (which is freely available) was added to the Table of Materials and Reagents.**

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

**Protocol has been edited to use imperative tense.**

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

**Protocol steps have been modified.**

7. The Protocol should contain only action items that direct the reader to do something.

**Protocol steps have been modified.**

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

**Speeds were converted.**

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

**Details were added.**

10. 3.23: Please provide graphical user interface, button clicks etc to show how do you count the surviving colonies.

**Instructions were added to this section of the protocol to describe colony counting using NICE software. A figure was added to show the software interface.**

11. Please remove the redundancy in the protocol but not repeating the steps described before. E.g. solution preparation steps e.g. overlay agar, etc. Please write exactly how you perform your experiment with all specific details

**Redundancy in solution preparation was removed and details were added.**

12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please ensure that the highlighted steps is no more than 2.75 pages including heading and spacings.

**Highlighted protocol steps are less than 2.75 pages and flow was adjusted.**

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**No figures are being reproduced from previous publication and permission should not be needed.**

14. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable), abbreviations etc.

**Figures and legends have been modified to include definition of abbreviations.**

15. Please do not abbreviate journal title in the reference section.

**References were edited to include full journal titles.**

16. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: title of the manuscript, 37-38, 77-78, 119-120, 175-180, 226-227.

**The indicated sections were modified**.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript "A simple, high-throughput Shigella serum bactericidal assay" by Weerts et al. presents useful serum bactericidal assay to determine functionality of antibody against Shigella spp. in the serum. They developed and optimized the assay using simple colony-counting method. It is interesting assay protocol because serum bactericidal assay against Shigella spp. is not published.

Major Concerns:

1. This assay measures the functional activity of serum against Shigella spp. Therefore, the information of serum used in this study should be provided.

**Samples used in this assay include monoclonal antibodies, specific for *S. flexneri* 3a, described in a previous publication (Lin et al., 2016) and human serum samples from Control volunteers in a previous shigella vaccine study (Tribble et al., 2010). These serum were selected specifically for demonstration purposes as representative samples with activity against the strain being displayed. A reference to the origins of the sera shown in the assay was added to the discussion.**

**Lin, J., Smith, M. A., Benjamin, W. H., Jr., Kaminski, R. W., Wenzel, H., & Nahm, M. H. (2016). Monoclonal Antibodies to Shigella Lipopolysaccharide Are Useful for Vaccine Production. *Clinical Vaccine Immunolgy, 23*(8), 681-688. doi:10.1128/CVI.00148-16**

**Tribble, D., Kaminski, R., Cantrell, J., Nelson, M., Porter, C., Baqar, S., . . . Oaks, E. (2010). Safety and immunogenicity of a Shigella flexneri 2a Invaplex 50 intranasal vaccine in adult volunteers. *Vaccine, 28*(37), 6076-6085. doi:10.1016/j.vaccine.2010.06.086**

2. Figure 1 only showed the serum bactericidal activity (SBA) against S. flexneri 2a. In the protocol, the assays for S. flexneri 3a and S. sonnei were also described. Authors need to provide the SBA data for two species. Furthermore, it needs to be clarified whether one species-specific serum show cross-reactivity against other Shigella spp.

**The figure shown is actually a representation of a *S. flexneri* 3a assay and the text has been modified to reflect this. This scope of this manuscript is to describe in detail how this shigellacidal assay is performed. A previous publication has specific information on development of the assay to accommodate additional serotypes of *Shigella* (*S. flexneri* 2a, *S. flexneri* 3a and *S. sonnei)*. With minor modifications, that are described in the protocol section, this single protocol can be used with all three serotypes.**

**Cross-reactivity of the serum used is entirely dependent on the source of the serum and the exposure history of the animal/individual from which it is collected. This assay has been used in pre-clinical and clinical work and some level of cross reactivity has been seen between *S. flexneri* 2a and *S. flexneri* 3a, potentially because of their closely related LPS structures. Much less cross-reactivity is seen to *S. sonnei,* even though all strains share highly conserved protein structures*.* This information and a reference have been added to the discussion section.**

3. As authors described in the 3rd paragraph of Discussion section, sources and lot-variation of complements are critical for SBA. Authors should provide the data why baby rabbit complements was selected for SBA against Shigella spp. but not complements derived from other animal (e.g, guinea pig, adult rabbit, human, etc). In addition, the SBA needs to be tested with a few different batches of complement to see the lot-variations.

**Baby rabbit complement has been widely used in many different functional assays for many different pathogens, notably *Haemophilus influenza* (Kim, Kim, Kim, & Nahm, 2016), *Nisseria meningitidis* (Maslanka et al., 1997), *Salmonella thypi* (Jang, Sahastrabuddhe, Yun, Han, & Yang, 2016). BRC is often selected for functional assays because it is widely available, can be purchased in large lot volumes and has reliable actively with low non-specific killing. The process for selecting appropriate lots of complements is detailed in a freely available protocol for the MOPA assay. A link to this assay has been added to the manuscript. (**[**https://www.vaccine.uab.edu/uploads/mdocs/UAB-MOPA.pdf**](https://www.vaccine.uab.edu/uploads/mdocs/UAB-MOPA.pdf)**)**

**Kim, H. W., Kim, K. H., Kim, J., & Nahm, M. H. (2016). A high throughput serum bactericidal assay for antibodies to Haemophilus influenzae type b. *BMC Infectious Disease, 16*, 473. doi:10.1186/s12879-016-1808-4**

**Jang, M. S., Sahastrabuddhe, S., Yun, C. H., Han, S. H., & Yang, J. S. (2016). Serum bactericidal assay for the evaluation of typhoid vaccine using a semi-automated colony-counting method. *Microbial Pathogenesis, 97*, 19-26. doi:10.1016/j.micpath.2016.05.013**

**Maslanka, S. E., Gheesling, L. L., Libutti, D. E., Donaldson, K. B., Harakeh, H. S., Dykes, J. K., . . . Carlone, G. M. (1997). Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clinical Diagnostic Lab Immunology, 4*(2), 156-167.**

4. The specificity of the assay needs to be provided using irrelevant enteric bacteria.

**The specificity of the assay is highly dependent on the source of the serum and the exposure history of the animal/individual that it has come from, and certainly in individuals in endemic regions we would expect to see reactivity of their serum with other enteric bacteria. In these cases it would be important to use this assay to look at fold increase of bactericidal titers before and after immunization/challenge. When using well defined monoclonal antibodies or pathogen free animals we would not expect any cross-reactivity with other enteric bacterial. A recent publication has used monoclonal antibodies specific for *Shigella* in assays targeting E. coli (Lin et al., 2016). This study shows that the antibodies against *S. flexneri* LPS do not bind to E. coli. The complement controls that are present in this assay also take into account non-specific killing (NSK) related to the activity of complement alone, and this NSK should be routinely monitored as part of the assay to ensure specificity.**

**Lin, J., Smith, M. A., Benjamin, W. H., Jr., Kaminski, R. W., Wenzel, H., & Nahm, M. H. (2016). Monoclonal Antibodies to Shigella Lipopolysaccharide Are Useful for Vaccine Production. *Clinical Vaccine Immunolgy, 23*(8), 681-688. doi:10.1128/CVI.00148-16**

5. Advantages and limitations should be described with previous literatures in the Discussion section.

**Pervious literature citations were added to the advantages and limitations sections of the manuscript. This now includes comparisons to other bactericidal assays using different formats, and references to manuscripts showing the use of the assay in additional laboratories and with clinical samples.**

Minor Concerns:

1. Reference should be provided for the sentence from line 318 to 325.

**A reference was added to justify the incubation temperatures used.**

2. In this protocol (line 318 - 325), top agar was overlaid on the LBA plate after overnight incubation. However, in other SBA protocols, top agar was overlaid immediately after adsorption of serum-bacteria mixture on the LBA plate. Please provide the reason for this specific procedure.

**During development of this specific assay it was found that adding overlay directly after spotting caused *Shigella* to float to the surface of the agar and caused messy spots and faintly colored colonies. While this is not seen for other bacterial strains, such as *Streptococcus pneumoniae*, much more neat and consistent results were seen if the agar was added after an overnight incubation.**

Reviewer #2:

Manuscript Summary:

This described a detailed experimental protocol and related information on the newly established serum bactericidal assay (SBA) for shigella vaccine study.

Major Concerns:

1. Baby rabbit complements (BRCs) may have lot-to-lot variation in terms of complement components, their activities, and other serum components including natural antibodies, albumin, lipids, and lipoproteins, all of which might potentially influence on the SBA. Thus, a protocol section would better be added on how to select qualified BRCs and what criteria should be tested in new lot number BRC.

**The process for selecting appropriate lots of complements is detailed in a freely available protocol for the MOPA assay. A link to this assay has been added to the manuscript. (https://www.vaccine.uab.edu/uploads/mdocs/UAB-MOPA.pdf)**

2. The authors provided reagents and disposables in the Table of materials. Considering that potential users of the protocol are not only American scientists but also scientists in other countries, some scientists may not be able to purchase the same ones listed on the Table of materials. Thus, it would be better to provide equivalent reagents and disposables that are alternatively-usable.

**More detailed information has been added to the Table of Materials and Disposables. Many of the reagents used in this protocol are simple microbiological products and should be able to be obtained in many countries. The additional information included in the Table of Materials should enable laboratories across the globe to purchase similar/equivalent products.**

3. Vibriocidal antibody assay (i.e., SBA for Vibrio cholerae) uses guinea pig complements rather than BRCs. Have compare complements from different species? What is the scientific reason (or evidence) for the use of BRCs in the shigella SBA? Please add a paragraph on this issue.

**Two of the authors, Nahm and Yu, have been working with functional assays using different complement sources for many years and have found baby rabbit complement to be effective, widely available, can be purchased in large lot volumes and has reliable actively with low non-specific killing. BRC has been widely used in many different functional assays for many different pathogens, notably *Haemophilus influenza* (Kim et al., 2016), *Nisseria meningitidis* (Maslanka et al., 1997), *Salmonella thypi* (Jang et al., 2016). These citations and explanation have been added to the discussion section.**

**Kim, H. W., Kim, K. H., Kim, J., & Nahm, M. H. (2016). A high throughput serum bactericidal assay for antibodies to Haemophilus influenzae type b. *BMC Infectious Disease, 16*, 473. doi:10.1186/s12879-016-1808-4**

**Jang, M. S., Sahastrabuddhe, S., Yun, C. H., Han, S. H., & Yang, J. S. (2016). Serum bactericidal assay for the evaluation of typhoid vaccine using a semi-automated colony-counting method. *Microbial Pathogenesis, 97*, 19-26. doi:10.1016/j.micpath.2016.05.013**

**Maslanka, S. E., Gheesling, L. L., Libutti, D. E., Donaldson, K. B., Harakeh, H. S., Dykes, J. K., . . . Carlone, G. M. (1997). Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clinical Diagnostic Lab Immunology, 4*(2), 156-167.**

4. Please provide detailed information the machinery including Company/Manufacturer, catalog number, and alternatives.

**More detailed information has been added to the Table of Materials and Disposables.**

5. (line 136) "12 mm X 12 mm" might be "12 cm X 12 cm"

**This has been corrected to “120 mm x 120 mm”.**

Minor Concerns:

Typographic or editing errors that should be corrected are as follows:

(line 38) "serum bactericidal assay" should be SBA because it was already shortened above.

(line 41) "highthroughput" should be "high-throughput"

(lines 80, 169, 267, 365, 381, and 382) "heat inactivated" should be "heat-inactivated". In fact, both "heat-inactivated" and "heat inactivated" exist in the manuscript.

(lines 83) "colony forming" should be "colony-forming"

(line 85) Add hyphen at "labor intensive" and "time consuming"

(lines 97) "colony counting" should be "colony-counting"

(line 136) "1L" should be "1 L"

(line 147) Please provide the full name of HBSS once before use of HBSS.

(line 159) "use discard" should be "use and discard"

(line 161) Insert "and" before "thaw~"

(line 167) "Heat-inactivated" should be "heat-inactivated"

(line 182) Subscript 2 at "CO2"

(line 186) Subscript 600 at "OD600"

(line191) Insert "and" before "re-suspend~"

(line 437) Insert spaces before and after "plus/minus"

(line 441) Insert comma after "~community"

(Title of Table of Materials) "Name of Material/ Equipment" should be "Name of Materials and Disposables"

**All of the previously indicated errors have been corrected.**