**Reply to comments on JoVE58200R1**

We thank the editor and reviewers for their valuable comments and suggestions. All the changes are tracked in the revised manuscript.

**Editor:**

**Q1**: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**A1**: We have sent the manuscript to a native English-speaking scientist again for proofreading. The manuscript has now been amended to ensure that there are no spelling or grammar issues.

**Q2**: JoVE cannot publish manuscripts containing commercial language. This includes company names of 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.

**A2**: We have deleted all commercial language related to instrument and reagents, such as VITEK2, ABI7500, PrimeStar HS. The information could be found in the Talbe of Materials and Reagents.

**Q3**: Step 1.1-1.6: Please write each step in complete sentences and in imperative tense.

**A3**: Steps 1.1-1.6 have been re-written in complete sentences and in imperative tense. For example, in Step 1.1, we now say “Nutrient Broth: Dissolve 1 % peptone, 0.3 % beef extract, 0.5 % sodium chloride, 0.1 % glucose in H2O, adjust pH to 7.5 and autoclave it in 121 °C for 15 min”.

**Q4**: 2.5.4: Please ensure that all text is written in imperative tense.

**A4**: 2.5.4 has been re-written in imperative tense. We now say “Go to Step 4 directly and issue negative reports if negative results occur on the FAM/HEX channels, which means that samples are negative for Salmonella spp./Shigella spp.”.

**Q5**: 2.5.5: Please ensure that all text is written in imperative tense.

**A5**: 2.5.5 has been re-written in imperative tense. We now say “Go to step 3.1 and/or 3.2 if positive results occur on the HEX and/or FAM channels, which means that the sample may be positive for *Salmonella spp.* and/or *Shigella spp*., respectively”.

**Q6**: 3.1.4.1.3: Please ensure that all text is written in imperative tense.

**A6**: 3.1.4.1.3 has been re-written in imperative tense. We now say “Go to step 3.1.4.1.4 if it looks like flowing sand, which means that the colony is reactive to the sera (Figure 3). Otherwise, go to step 3.1.4.1.5.”.

**Q7**: 3.1.4.2.3: Please ensure that all text is written in imperative tense.

**A7**: 3.1.4.2.3 has been re-written in imperative tense. We now say “Go to step 3.1.4.2.4 if it looks like flowing sand, which means that the colony is reactive to the sera”. We also changed other similar sentences into imperative tense, such as 2.3.1, 3.1.4.1.4, 3.1.4.1.5, 3.1.4.2.4, 3.2.3.3, 3.2.3.4.

**Q8**: There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

**A8**: A 2.75 page of the protocol has been highlighted in yellow. It includes steps 2.2, 2.4, 2.5.1 to 2.5.3, 3.1 to 3.1.4.1.4, 3.1.4.2 to 3.1.4.2.4 and 3.2 to 3.2.3.4.

**Reviewer #4:**

**Q1**: Regarding the sensitivity and specificity, the authors report that there is some concern about the limitation of the primers/probes to detect all Salmonella spp/Shigella spp. due to sequence variations as they have reported previously. My question is why the primer/probe sets are not updated to compensate for this?

**A1**: This comment is well-taken. As reported previously, we admit that some of the *Salmonella* spp., such as *S. Wandsworth, S. Koessen,* and *etc,* could not be identified by Sal-probe while some of the *Sh. boydii* could not be identified by Shi-probe due to sequence variation. When we designed the method, we tried to design a set of primers and probes which could amplify all the serotypes of *Salmonella spp./Shigella spp.*. However, it failed. Therefore, with the aim to maximize the power of the method, we designed the primers/probes which majority of the *Salmonella spp/Shigella spp.* could be detected and several strains (the above mentioned ones) were sacrificed. However, as these serotypes were not the circulating ones in China [1-3], the protocol was still powerful for the screening of *Salmonella spp./Shigella spp.*. Ran *et al* showed that *S. Enteritidis* (31%) and *S. Typhimurium* (26%) were the most common in China[2] while Liang *et al* showed that *S. Typhimurium* (30%), *S. serotype 4,5,12:i:-* (14%), and *S. Enteritidis* (13%) were the most common in Guangdong Province where our laboratory located[1]. Results from Qiu *et al* indicated that the most common *Shigella spp.* was *Sh. flexneri* (55.3%), followed by *Sh. sonnei* (44.1%) in China[3]. We have discussed this in the manuscript by saying “if negative real-time PCR results appear for those samples from patients with obvious clinical symptoms, laboratory technicians should pay attention and may conduct other experiments to confirm the results”.

**Q2**: I have one minor concern regarding the authors' statement in the discussion that PCR directly from stool specimens does not require enrichment to obtain high sensitivity. In my own experience, for Salmonella spp. pre-enrichment in Selenite broth increases the sensitivity from 90% to 100% for both culture and PCR, with the last 10% of the specimens only positive after selenite enrichment. In addition, from my own experiments I have been able to reach sensitivities as low as 18 CFU/gr of stool for Salmonella spp by both PCR and culture, which is about 2-3 log more sensitive compared to what the authors report, assuming 1 gram of stool equals 1 ml of stool.

**A2**: We agree with the reviewer that pre-enrichment in Selenite broth would increase the sensitivity for both culture and PCR for *Salmonella spp.*. However, as these protocol was used for the simultaneous detection of *Salmonella spp./Shigella spp.*, pre-enrichment in Nutrient Broth was applied. If PCR positive results occur on the HEX channels, which means that the sample may be positive for *Salmonella spp.*, then Selenite Cystine medium was applied to selectively enrich *Salmonella spp.* for the purpose of increasing sensitivity. The difference between the reviewer and us regarding the initial pre-enrichment medium used could also explain the different sensitivities obtained. As Nutrition Broth was used, there were more interfering background flora cultured which may mask the detection and isolation of target pathogens[4]. To make the protocol more powerful, we added “If the protocol was only applied for the detection of *Salmonella spp.*, then pre-enrichment could be conducted in Selenite Cystine medium in order to increase the sensitivity”.

**Reviewer #5:**

**Q1**: There is a lack of references for various statements throughout the manuscript.

**A1**: This comment is well-taken. We have added a total of ten references (three existing and seven new references) to the Introduction and Discussion to backup various statements in the manuscript.

**Q2**: Provide more details in the introduction. e.g. why is it important to identify those pathogens at the species level?

**A2**: This suggestion is well-taken. The aim of identifying those pathogens to the species level is to facilitate the disease outbreak management and antimicrobial profiling for better treatment of the patients. We have added this to the Introduction. More other details have also been added in the Introduction part in track-change mode.

**Q3**: Species of Shigella are commonly seen in the human gut microbiota, both as colonizers and/or remnants of past infections. With that in mind, it is highly unlikely that the pooling strategy will reduce the workload for that pathogen, as most of the mixes composed of several samples will probably be positive, provided the qPCR is sufficiently sensitive.

**A3**: Indeed the sensitivity of qPCR for the detection of *Shigella* spp. was as low as 101CFU/mL. However, in our previous paper, we tested 20664 stool samples but no *Shigella spp.* wasdetected during the PCR stage [5]. The reason may be due to the target of PCR. When we designed the primers and probe for the detection of *Shigella spp.*, we chose invasive plasmid antigen H (ipaH) gene as target, which is one of the virulence genes of *Shigella spp.*. Therefore, the common *Shigella* without ipaH gene in the human gut micobiota would not be detected.

**References**

1. Liang, Z., et al., *Serotypes, seasonal trends, and antibiotic resistance of non-typhoidal Salmonella from human patients in Guangdong Province, China, 2009-2012.* BMC Infect Dis, 2015. **15**: p. 53.

2. Ran, L., et al., *Laboratory-based surveillance of nontyphoidal Salmonella infections in China.* Foodborne Pathog Dis, 2011. **8**(8): p. 921-7.

3. Qiu, S., et al., *Shift in serotype distribution of Shigella species in China, 2003-2013.* Clin Microbiol Infect, 2015. **21**(3): p. 252.e5-8.

4. Kumar, R., P.K. Surendran, and N. Thampuran, *Evaluation of culture, ELISA and PCR assays for the detection of Salmonella in seafood.* Lett Appl Microbiol, 2008. **46**(2): p. 221-6.

5. Xi-Jun Tang, Z.Y., Xin-Bin Chen, Wen-Fang Tian, Cheng-Ning Tu, Hai-Bo Wang, *Verification and large scale clinical evaluation of a national standard protocol for Salmonella spp./Shigella spp. screening using real-time PCR combined with guided culture.* Journal of Microbiological Methods, 2018. **145**: p. 14-19.