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TITLE:

A High-Throughput Platform for the Screening of *Salmonella* spp./*Shigella* spp.

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KEYWORDS:

acute gastroenteritis; *Salmonella* spp.; *Shigella* spp.; screening; real-time PCR; guided culture

SUMMARY:

Salmonella spp./*Shigella* spp. are common pathogens attributed to diarrhea. Here, we describe a high-throughput platform for the screening of *Salmonella* spp./*Shigella* spp. using real-time PCR combined with guided culture.

ABSTRACT:

Fecal-oral transmission of acute gastroenteritis occurs from time to time, especially when people who handled food and water are infected by *Salmonella* spp./*Shigella* spp. The gold standard method for the detection of *Salmonella* spp./*Shigella* spp. is direct culture but this is labor-intensive and time-consuming. Here, we describe a high-throughput platform for *Salmonella* spp./*Shigella* spp. screening, using real-time polymerase chain reaction (PCR) combined with guided culture. There are two major stages: real-time PCR and the guided culture. For the first stage (real-time PCR), we explain each step of the method: sample collection, pre-enrichment, DNA extraction and real-time PCR. If the real-time PCR result is positive, then the second stage (guided culture) is performed: selective culture, biochemical identification and serological characterization. We also illustrate representative results generated from it. The protocol described here would be a valuable platform for the rapid, specific, sensitive and high-throughput screening of *Salmonella* spp./*Shigella* spp.

INTRODUCTION:

Diarrhea is still a common health issue with a high incidence rate globally[1,2]. Though the mortality is relatively low, some patients show various symptoms for weeks (such as loose and

watery stools, an urgency to go to the bathroom), which make the socioeconomic impact very high[3,4]. More seriously, some patients may even develop irritable bowel syndrome if left untreated[5]. There are various kinds of bacteria, viruses and parasites that can cause diarrhea[6]. *Salmonella* spp./*Shigella* spp. are among the most common bacteria for the transmission of acute gastroenteritis[7-11]. Therefore, many counties have issued laws or regulations for regular *Salmonella* spp./*Shigella* spp. screening among people who would handle food and water. For example, the Chinese government have issued laws for obligatory *Salmonella* spp./*Shigella* spp. screening once a year.

The gold standard method for *Salmonella* spp./*Shigella* spp. detection is bacteria culture. Through bacteria culture and successive biochemical identification and serological characterization, we can identify the species of bacteria, which could facilitate disease outbreak management and antimicrobial profiling to aid the treatment of patients[12]. It could also help trace the source of infection during the *Salmonella* spp./*Shigella* spp. outbreak[13]. However, this method is labor-intensive (requiring manual operation) and time-consuming (taking several days), especially for the testing of large numbers of samples[7]. Moreover, viable but non-culturable (VBNC) *Salmonella* spp./*Shigella* spp. may exist in some stool samples[14]. In view of these drawbacks, many laboratories have tried to develop new techniques for the detection of *Salmonella* spp./*Shigella* spp.[15-25]. All these methods use the nuclear acid amplification test (NAAT), among which the polymerase chain reaction (PCR) is the most common. One major limitation of these NAAT based methods is that dead bacteria, even bacterial debris containing incomplete genomic DNA, could show positive results[26], which could largely influence the accurate diagnosis of disease. Blanco *et al.* showed that molecular assay is highly sensitive, not only to viable *Salmonella* in cultures, but also to partial genomes and dead or unviable bacteria from past infections or contamination[26]. Therefore, new technology should be developed.

Here, we described a novel method that combines the NAAT based method and culturing. As shown in **Figure 1**, this new method applies real-time PCR screening first and then positive samples are sent for bacteria culture and identification.

PROTOCOL

The protocol follows the guidelines set by the human research ethics committee of Zhuhai International Travel Healthcare Center. Please use standard sterile operation during the experiment.

1. Culture Media Composition and Preparation

1.1. Prepare Nutrient Broth: Dissolve 1% peptone, 0.3% beef extract, 0.5% sodium chloride, 0.1% glucose in H_2O, adjust pH to 7.5 and autoclave it in 121 °C for 15 min.

1.2. Prepare Selenite Cystine medium: Dissolve 0.5% peptone, 0.4% lactose, 1% Na_2HCO_3, 0.4% sodium hydrogen selenite, 0.001% L-Cystine in H_2O, adjust pH to 7.0 and boil it for 5 min.

1.3. Prepare the Xylose, Lysine, Deoxycholate agar (XLD) plate: Dissolve 0.3% yeast extract, 0.5% L-lysine, 0.375% xylose, 0.75% lactose, 0.75% sucrose, 0.5% sodium chloride, 0.008% phenol red, 0.68% sodium thiosulfate, 0.08% ammonium ferric citrate, 0.25% sodium deoxycholate, 1.5% agar in H_2O, and adjust pH to 7.4. Then boil it for 5 min and pour it into 90 mm plates.

1.4. Prepare the *Salmonella* chromogenic agar plate: Dissolve 1.5% agar, 0.7% peptone and yeast extract, 1.29% selective reagent in H_2O. Then boil it for 5 min and pour it into 90 mm plates.

1.5. Prepare the Nutrient agar plate: Dissolve 1% peptone, 0.3% beef extract, 0.5% sodium chloride, 1.5% agar in H_2O, and adjust pH to 7.3. Then autoclave it in 121 °C for 15 min and pour it into 90 mm plates.

1.6. Prepare the MacConkey agar (MAC) plate: Dissolve 2% peptone, 1% lactose, 0.5% sodium chloride, 0.5% OX Bile Salt, 0.0025% Neutral Red, 1.5% agar, 0.0001% crystal violet in H_2O, and adjust pH to 7.2. Then autoclave it in 115 °C for 20 min and pour it into 90 mm plates.

2. Real-time PCR

2.1. Sample collection

2.1.1. Insert an anal swab into the patient's anus 3-5 cm deep, and rotate it 360° around.

2.1.2. Put the anal swab into a sterile collection tube. Mark sample ID.

2.1.3. Send the sample to laboratory as soon as possible.

Note: Samples could be stored at 4 °C for no more than 24 h.

2.2. Pre-enrichment

2.2.1. Add 3 mL of Nutrient Broth into each sample in the collection tube.

2.2.2. Incubate at 36 °C for 6 h in an incubator.

2.3. Sample mixing (optional)

2.3.1. Collect 100 µL of each pre-enrichment culture and mix 8-10 samples of 1 specimen into a 1.5 mL tube if there are more than 10 samples.

2.3.2. Mark properly.

2.4. DNA extraction

2.4.1. Centrifuge the pre-enrichment culture at 800 × g for 2 min to allow large particles to settle down, and transfer the supernatant to a new tube and centrifuge at 12000 × g for 5 min. Discard the supernatant by aspiration.

2.4.2. Add 100 µL of DNA extraction solution (0.01 M pH 8.0 Tris-EDTA, 0.01% Nonidet P 40 (NP40)) to the pellet. Vortex vigorously for 1 min.

2.4.3. Boil at 100 °C for 5 min on a dry bath.

2.4.4. Centrifuge at 12000 × g for 5 min. Collect the supernatant using a new tube, which will be the template for the succeeding real-time PCR analysis.

2.5. Real-time PCR

2.5.1. Setup the reaction mixture as follow: for each sample, add 12.5 µL of 2x reaction mixture, 0.4 µM of each primer, 0.2 µM of each probe (sequences in **Table 1**)[27], 5 µL of the template as prepared in step 2.4.4, and use ddH_2O to add up to 25 µL total volume.

2.5.2. Setup the cycling program as follow: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 34 s. Collect fluorescent signals from 6-carboxy-fluorescein (FAM) and Hexachlorofluorescein (HEX) channels at the elongation step (72 °C) automatically by the fluorescent real-time PCR machine.

2.5.3. Perform real-time PCR on a fluorescent real-time PCR machine, according to instructions of the instrument.

2.5.4. 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.

2.5.5. 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.

Note: If sample mixing has been performed in step 2.3, then real-time PCR on individual samples which made up the positive one should be performed to screen out the real positive sample.

3. Guided Culture

3.1. *Salmonella* spp. PCR positive sample

3.1.1. Selective culture in medium

3.1.1.1. Add 100 µL of pre-enrichment culture into 5 mL of Selenite Cystine medium in a test tube. Incubate at 36 °C for 18-24 h in an incubator.

3.1.2. Separating culture on plate

3.1.2.1. Collect one loop of the culture with a micro-loop and spread onto a XLD plate or *Salmonella* chromogenic agar plate. Incubate at 36 °C for 18-24 h in an incubator.

3.1.3. Biochemical identification

3.1.3.1. Select suspicious colony on XLD plate (pink colony with/without dark heart; dark colony; yellow colony with/without dark heart) or *Salmonella* chromogenic agar plate (purple or prunosus colony, smooth and round) (**Figure 2**).

3.1.3.2. Subject suspicious colony for biochemical identification on automated microbial identification system, according to instructions of the instrument.

3.1.4. Serological characterization

3.1.4.1. O antigen characterization

3.1.4.1.1. Add one drop of the O antigen polyvalent sera onto a clean slide.

3.1.4.1.2. Collect one loop of the colony with a micro-loop and grind in the sera.

3.1.4.1.3. 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.

3.1.4.1.4. Use O antigen monovalent sera to repeat steps 3.1.4.1.1 to 3.1.4.1.3 until the specific O antigen is characterized.

3.1.4.1.5. Use Vi sera to repeat steps 3.1.4.1.1 to 3.1.4.1.3. Collect those Vi sera reactive colonies in a tube and boil at 100 °C for 5 min in a dry bath. Centrifuge at 12000 × g for 5 min. Collect the pellet and repeat steps 3.1.4.1.1 to 3.1.4.1.4 until specific O antigen is characterized.

3.1.4.2. H antigen characterization

3.1.4.2.1. Add one drop of the H antigen polyvalent sera onto a clean slide.

3.1.4.2.2. Collect one loop of the colony with a micro-loop and grind in the sera.

3.1.4.2.3. Go to step 3.1.4.2.4 if it looks like flowing sand, which means that the colony is reactive to the sera.

3.1.4.2.4. Use H antigen monovalent sera to repeat steps 3.1.4.2.1 to 3.1.4.2.3 until specific H antigen is characterized.

Note: Sometimes serum induction may be needed to characterize the second phase H antigen. If so, then the following optional steps should be performed.

3.1.4.2.5. (Optional) Add one drop of specific H antigen sera onto Nutrient agar plate. Wait until all the sera are absorbed.

3.1.4.2.6. (Optional) Collect one loop of the colony with a micro-loop and spread onto the plate where specific H antigen sera are absorbed. Incubate at 36 °C for 18-24 h in an incubator.

3.1.4.2.7. (Optional) Use H antigen monovalent sera to repeat steps 3.1.4.2.1 to 3.1.4.2.3 until second phase H antigen is characterized.

3.1.4.2.8. Go to step 4.

3.2. *Shigella* spp. PCR positive sample

3.2.1. Separating culture on plate

3.2.1.1. Collect one loop of the pre-enrichment culture with a micro-loop and spread onto a XLD plate or MAC plate. Incubate at 36 °C for 18-24 h in an incubator.

3.2.2. Biochemical identification

3.2.2.1. Collect suspicious colony on XLD plate (smooth, round, transparent and red colony) or MAC plate (smooth, round, transparent and colorless colony with 2-3 mm in diameter; *Shigella sonnei* may be bigger and turn to light pink as the elongation of incubation time) (**Figure 4**).

3.2.2.2. Subject suspicious colony for biochemical identification on automated microbial identification system, according to instructions of the instrument.

3.2.3. Serological characterization

3.2.3.1. Add one drop of the *Shigella* spp. polyvalent sera onto a clean slide.

3.2.3.2. Collect one loop of the colony with a micro-loop and grind in the sera.

3.2.3.3. Go to step 3.2.3.4 if it looks like flowing sand, which means that the colony is reactive to the sera.

3.2.3.4. Use *Shigella* spp. monovalent sera to repeat steps 3.2.3.1 to 3.2.3.3 until specific

Shigella spp. is characterized.

3.2.3.5. Go to step 4.

4. Report

4.1. Issue positive or negative reports according to the above results.

REPRESENTATIVE RESULTS

The protocol was applied for the screening of *Salmonella* spp./*Shigella* spp. in anal stool samples from people who would handle food and water.

In the real-time PCR step, as shown in **Figure 5A**, there was a successful amplification in HEX channel, which meant that the mixed sample was positive for *Salmonella* spp. Then a further real-time PCR was conducted on individual samples which made up the positive one. As shown in **Figure 5B**, sample 2 was positive. Therefore, sample 2 was chosen for the guided culture of *Salmonella* spp. In **Figure 5C**, there was a successful amplification in the FAM channel, which meant that the mixed sample was positive for *Shigella* spp. Then a further real-time PCR was conducted and sample 10 was found to be positive (**Figure 5D**). Therefore, sample 10 was chosen for the guided culture of *Shigella* spp.

In the guided culture of *Salmonella* spp., there were pink colonies and purple colonies on XLD plate and *Salmonella* chromogenic agar plate, separately, as shown in **Figure 2**. Then these colonies were subjected to biochemical identification on automated microbial identification system. Results showed that it was *Salmonella* spp., with species unknown. Therefore, serological characterization was performed (**Figure 3**) and it was reactive to O4, O12, Hb, H1,2. According to the White-Kauffmann-Le Minor scheme, sample 2 was finally reported as positive for *Salmonella paratyphi B*. While for the guided culture of *Shigella* spp., there were pink red and colorless colonies on XLD plate and MAC plate, separately, as shown in **Figure 4**. Then these colonies were also subjected to biochemical identification on automated microbial identification system. Results showed that it was *Shigella sonnei*. To confirm its specific serotype, serological characterization was performed (**Figure 3**) and it was reactive to sonnei phase II. Therefore, sample 10 was finally reported as positive for *Shigella sonnei phase II*.

FIGURE AND TABLE LEGENDS

Figure 1: The diagram of the protocol. Two major steps were shown and separated by dash line.

Figure 2: Representative culture results of *Salmonella* spp. on XLD plate and *Salmonella* chromogenic agar plate. On XLD plate, there were pink colonies with/without dark heart, while on *Salmonella* chromogenic agar plate, there were purple colonies.

Figure 3: Representative serological characterization result. If the colony was reactive to the sera, it looked like flowing sand (left). Otherwise, it was turbid (right).

Figure 4: Representative culture results of *Shigella* spp. on XLD plate and MAC plate. On XLD plate, there were red colonies, while on MAC plate, there were transparent and colorless colonies.

Figure 5: Representative real-time PCR results. A. HEX channel for mix samples. **B.** HEX channel for individual samples. **C.** FAM channel for mix samples. **D.** FAM channel for individual samples. PC, positive control. NC, negative control.

Table 1: Primers and probes used. The name and sequences of the primers and probes were provided.

DISCUSSION

Since *Salmonella* spp./*Shigella* spp. are often associated with food poisoning and fecal-oral transmission of acute gastroenteritis[28,29] and the routine method is either labor-intensive or time-consuming[7], we describe a high-throughput platform for the *Salmonella* spp./*Shigella* spp. screening, using real-time PCR combined with guided culture.

There are several steps that need consideration to maximize the capability of this platform. The first one is the pre-enrichment step of samples in Nutrient Broth (step 2.2). Though no pre-enrichment step is needed for stool samples collected from those patients with obvious symptoms, such as diarrhea, *etc.*, a general 6 h pre-enrichment step is still needed for anal swab samples, when collected during the pre-employment physical examination for people who would handle food and water, as those people were mainly healthy or at least asymptomatic adults. 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. The second critical step is the identification of suspicious colonies (step 3.1.3.1 and 3.2.2.1). There are many interfering background flora in the stool samples that may mask the detection and isolation of target pathogens[30]. Therefore, the characteristic of the suspicious colonies as defined in step 3.1.3.1 and 3.2.2.1 should be kept in mind during the experiment. The third critical step is the serological characterization of *Salmonella* spp. As a majority of *Salmonella* spp. contain a second phase for H antigen[31], serum induction needs to be performed. However, the serum induction is not always successful, and several rounds of induction may be needed to determine the correct second H phase.

The protocol is highly specific and sensitive as verified by our previous study[27]. The high specificity of the protocol is demonstrated by its ability, in which *Salmonella* spp./*Shigella* spp. could be distinguished from other related pathogens[27]. The lower limit of detection of the protocol is 10^4 CFU/mL and 10^3 CFU/mL for *Salmonella* spp. and *Shigella* spp., respectively[27], which are comparable to previous reports[7,32-34]. As we stated above, the sensitivity of *Salmonella* spp. detection could be further increased by Selenite Cystine pre-enrichment if the protocol was only applied for the detection of *Salmonella* spp. Moreover, the protocol could increase the positive rate by two folds and decrease the workload/median turnaround time significantly[27].

Similar to other NAAT assays, one major limitation of the protocol, when compared to classic bacteria culture method, is that the protocol could only identify *Salmonella* spp./*Shigella* spp., while other common diarrhea-causing bacteria are omitted[7]. In contrast, during classic bacteria culture, those bacteria could be identified in parallel if they existed. Another limitation of the protocol is that some of *Salmonella* spp./*Shigella* spp. could not be identified by the real-time PCR due to sequence variations[27]. However, 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. During a large outbreak, we may use a single sample instead of pooled samples for the first round of PCR screening.

In conclusion, the protocol provided here could serve as a valuable platform for the screening of *Salmonella* spp./*Shigella* spp.

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DISCLOSURES

The authors have nothing to disclose.

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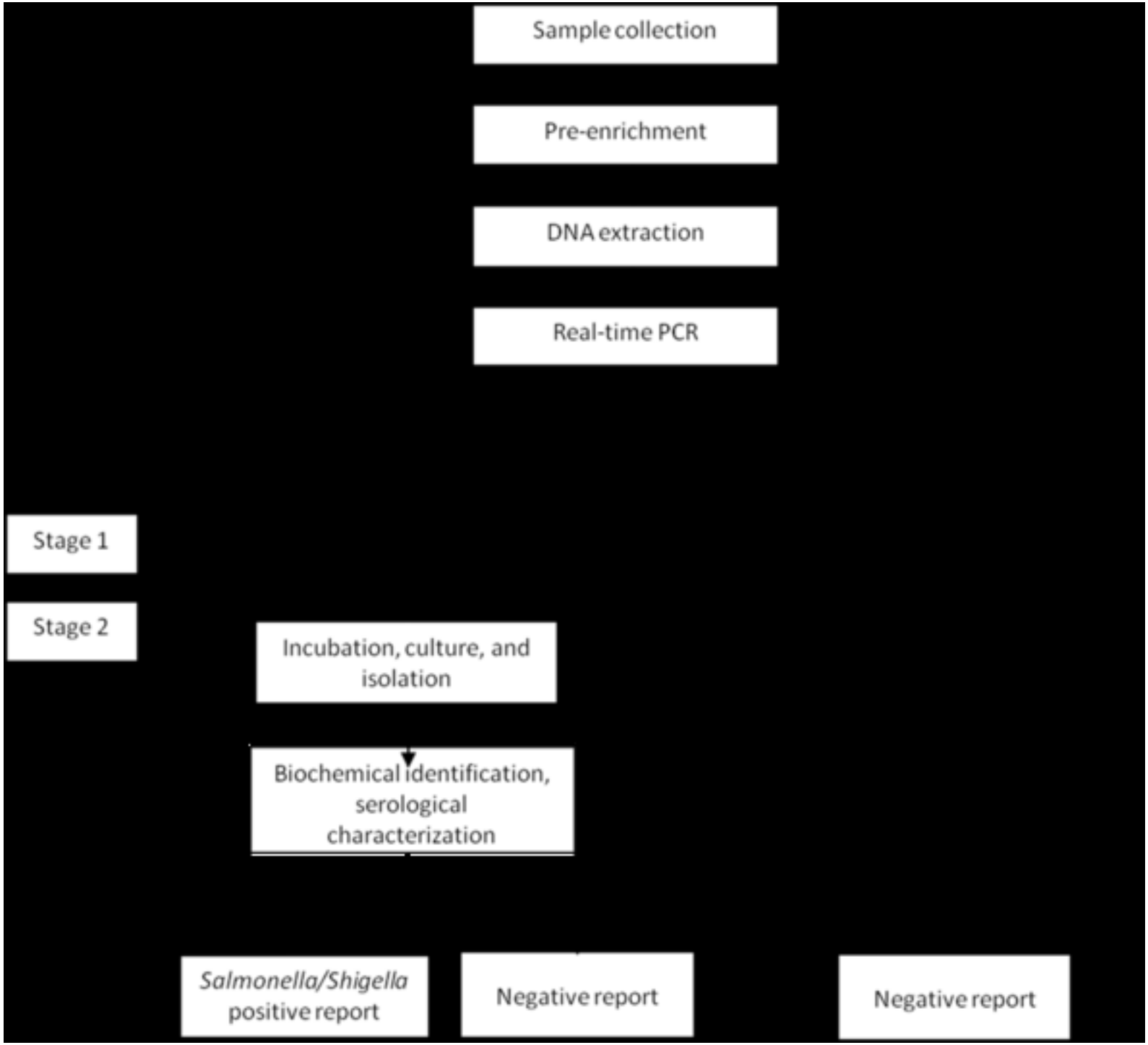

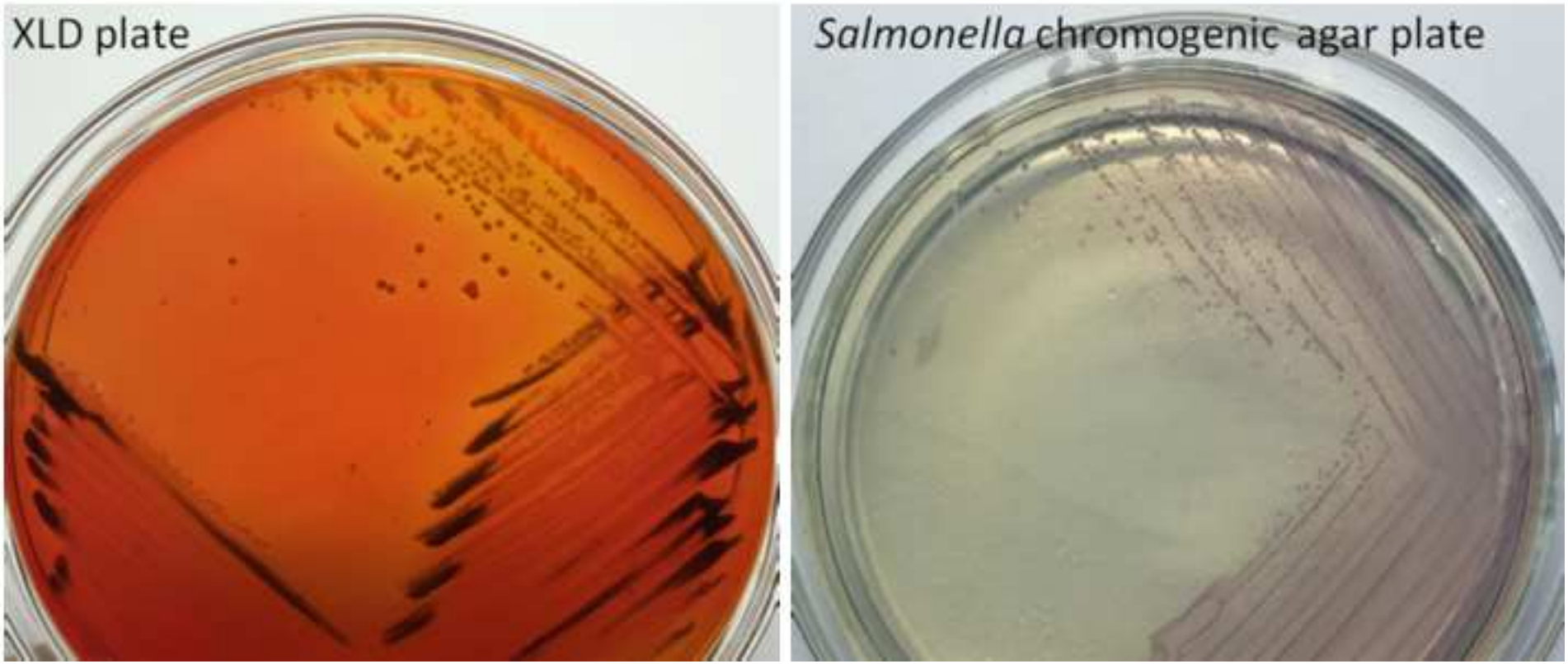

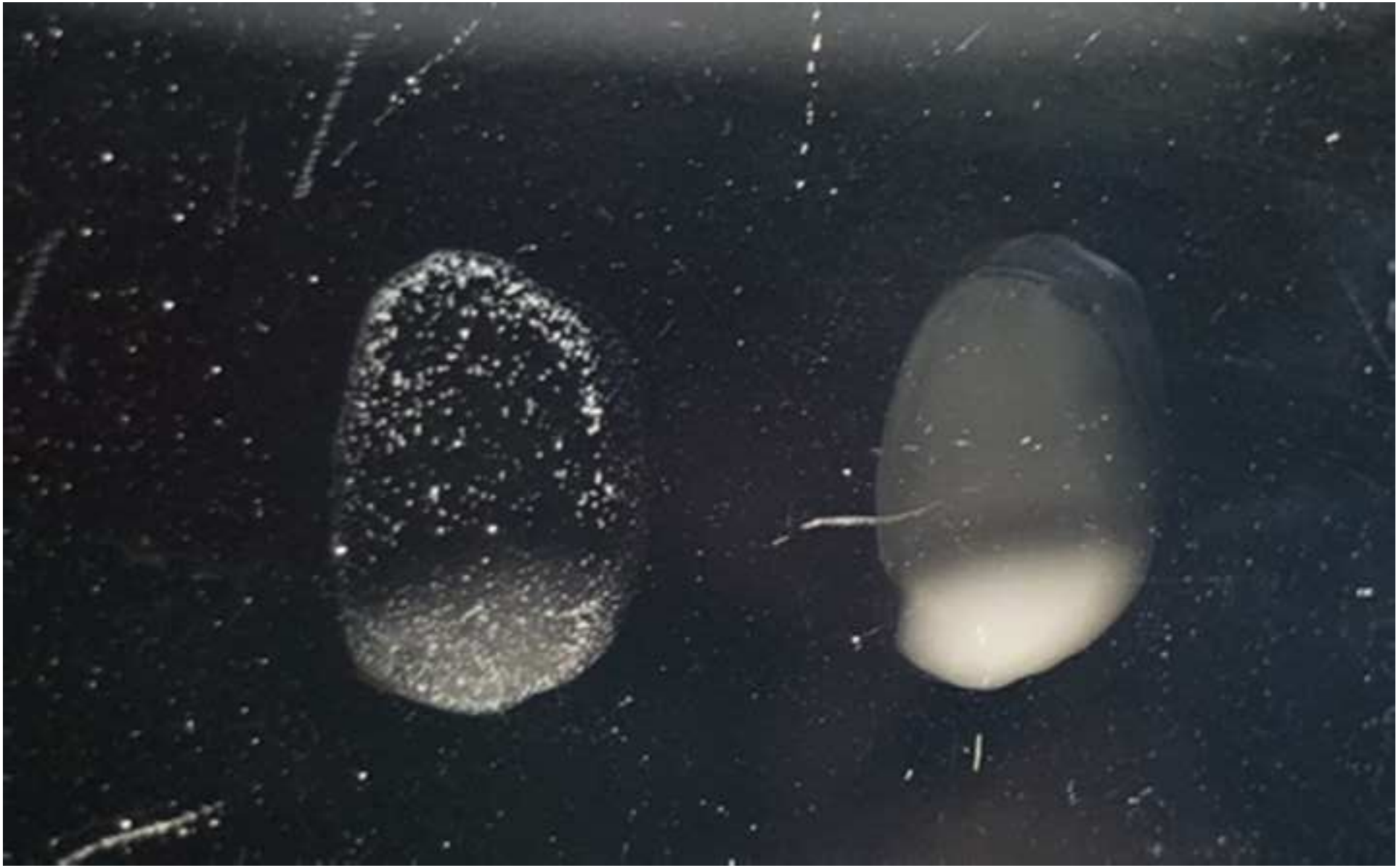

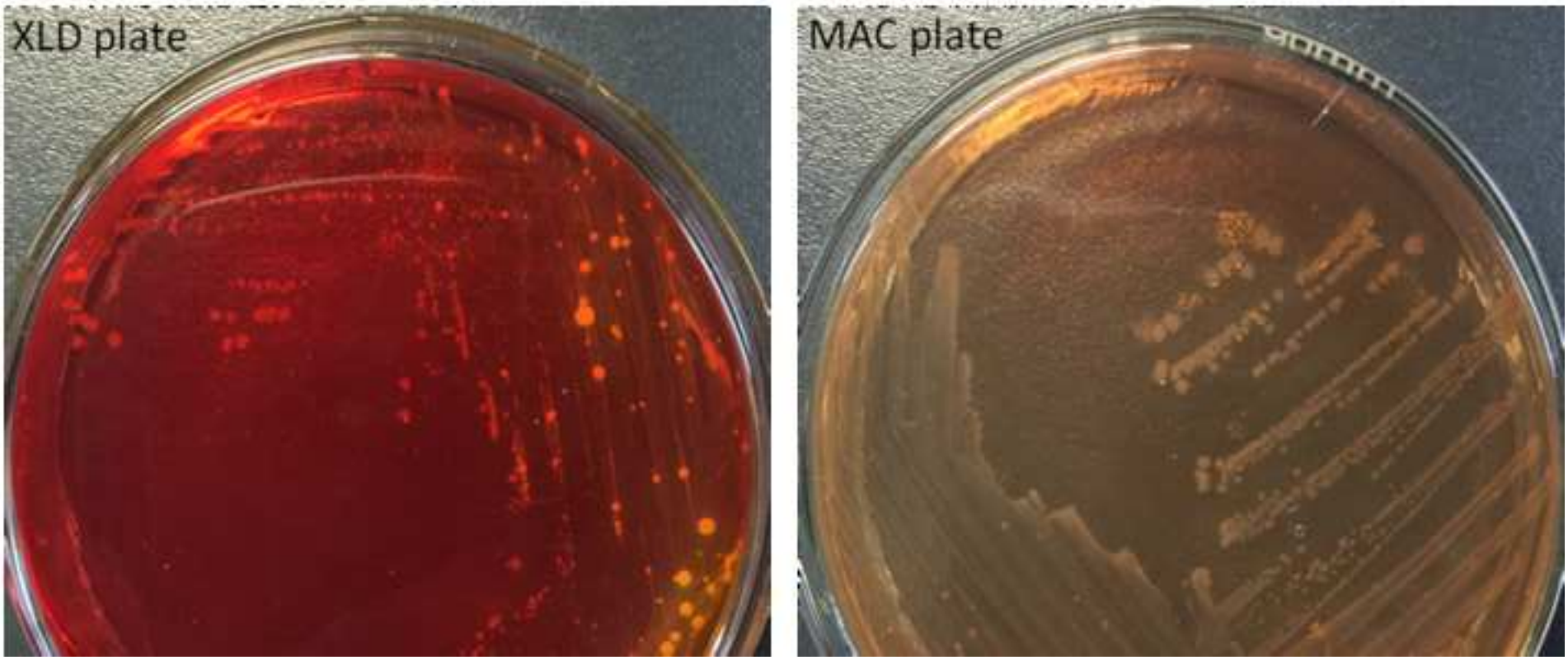

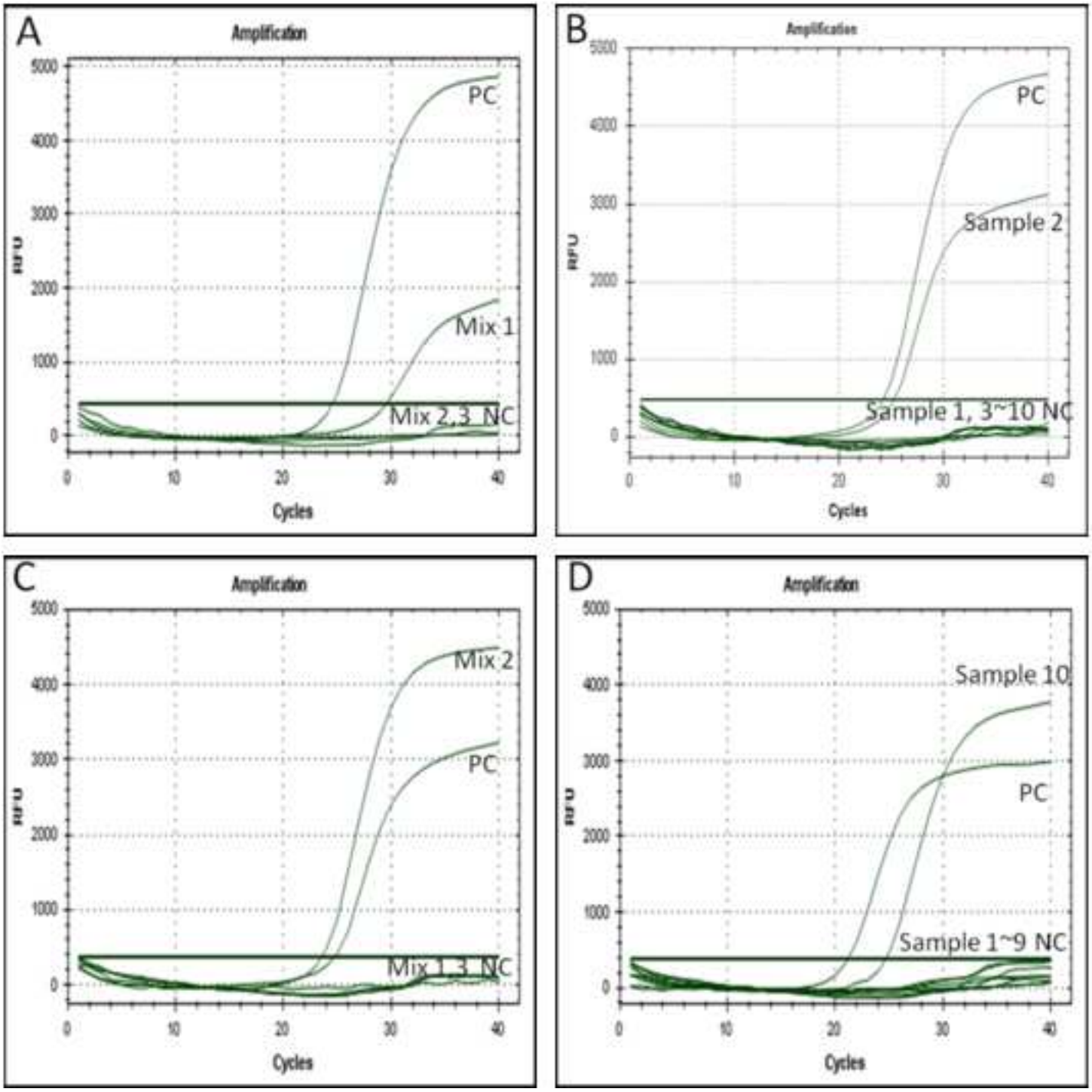

Table 1 Primers and probes used

Pathogen	Name	Sequence(5'-3')
Salmonella	Sal-F	gctcatattaattccggcatttac
	Sal-R	caggtcaatagccagaaagg
	Sal-probe	HEX-ataagtaatccaatccgaaatgcctgcgt-Eclipse
Shigella	Shi-F	ccgggataaagtcagaactc
	Shi-R	cagtggagagctgaagtttc
	Shi-probe	FAM-aggccaggtagacttctatctcatccac-Eclipse

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Tris	Sigma	10708976001	
EDTA	Sigma	798681	
NP40	Sigma	11332473001	
ddH_2O	Takara	9012	
PrimeSTAR HS (Premix)	Takara	R040Q	
Nutrient Broth	LandBridge	CM106	
Nutrient agar	LandBridge	CM107	
Selenite Cystine medium	LandBridge	CM225	
XLD	LandBridge	CM219	
MAC	LandBridge	CM908	
Salmonella chromogenic agar	CHROMagar	SA130	
Salmonella diagnostic serum	Tianrun	SAL60	
Shigella diagnostic serum	Tianrun	SHI54	
anal swab (collecting tube plus)	Huachenyang		
slide	Mingsheng	7102	
micro-loop	Weierkang	W511	
incubator	Jinghong	DNP-9082	
autoclave	AUL	SS-325	
dry bath	Jinghong	KB-20	
automated microbial identification system	bioMérieux	VITEK2	other equivalent system could be used
fluorescent real-time PCR machine	ThermoFisher	ABI7500	other equivalent machine could be used

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Author(s): Ze Yang, Xin-Bin Chen, Cheng-Ning Tu, Ying Su, Hai-Bo WANG

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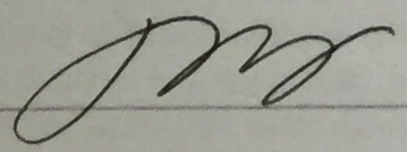
Signature: 

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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 H_2O, 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 "<u>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</u>".

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 10^1CFU/mL. However, in our previous paper, we tested 20664 stool samples but no *Shigella spp.* was detected 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.

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