The procedure and method of enteric pathogens detection applied in the study

Stool samples (collected from diarrhea cases and healthy controls, >= 3 g or 3 ml)

Each stool specimen was delivered to the laboratory in Cary-Blair (C-B) culture medium within 12 h. And then each stool sample was divided into **three aliquots**

The **first stool** was used for isolating, culturing and identifying bacterial (DEC, NTS, *Shigella* spp., *Vibrio cholera*, *Vibrio parahaemolyticus*, *Aeromonas* spp. and *Plesiomonas* spp.).

The **second** stool to detect viral pathogens (RVA, NoV and As) (There are all RNA viruses).

The **last stool** to find three intestinal protozoa (*Cryptosporidium*.spp; *G. lamblia* and *B. hominis*) and *Adenovirus*(Ad, DNA virus).

The first stool were further divided into four small parts. In order to detect seven bacterial.

Nucleic acid was extracted from each stool with QIAamp Viral RNA Kit (Qiagen, Hilden, Germany).

The genomic DNA from each stool sample with QIAamp DNA stool mini kit (Qiagen, Hilden, Germany)

The one small stool was inoculated onto alkaline peptone water to enrich the *Vibrio cholera*, *Vibrio parahaemolyticus*, *Aeromonas* spp. and *Plesiomonas* spp.. 37 ℃ for 18 hours.

The second small stool was directly inoculated into xylose, lysine and deoxycholate agar (XLD) to culture Shigella spp. . 37 ℃ for 18 hours.

The third small stool was was inoculated into the selenite brilliant green sulfa enrichment broth to enrich NTS. 37 ℃ for 18 hours.

The forth small stool was directly inoculated into MacConkey agar to culture **DEC** . 37 ℃ for 18 hours.

Each stool was directly inoculated on to TCBS, 37 ℃ for 18 hours.

Select the suspicious colony (yellow, median and big colony), and inoculated on to nutrient agar. 37 ℃ for 18 hours.

And then, all isolation were detected with Oxidase test. When change into purple within 15s,The colony was suspicious isolation.

Select the suspicious colony (no color or black, and small and median colony), and inoculated on to TSI. 37 ℃ for 18 hours. And select the suspicious Colony into nutrient agar. 37 ℃ for 18 hours.

Select these colnoy to detect with serum agglutination with four multivalentserum and Baumannii serum. When the serum were agglutination. The colony was suspicious Shigella isolation.

Select the suspicious colony (no color or black, and small and median colony), and inoculated on to TSI. 37 ℃ for 18 hours. And select the suspicious Colony into nutrient agar. 37 ℃ for 18 hours.

Select the suspicious colony, and the inoculated on to Chromagar salmonena medium (37 ℃ for 18 hours), when the color was red. And then select these colnoy to detect with serum agglutination with A-F serum.When the serum were agglutination. The colony was suspicious Salmonella isolation.

Select all suspicious isolation, and then was mix with physiological saline to 0.5 Nephelometric Turbidity Unit (NTU), and then, the systematic biochemical identification (Gram negative identification card, bioMerieux, Marcyl'Etoile, France) of was performed using the VITEK® 2 Compact instrument (bioMerieux, Marcyl'Etoile, France) to ensure these **six** kinds bacterium. 37 ℃ for 18 hours.

Each stool was directly inoculated on to Salmonella-Shigella agar, 37 ℃ for 18 hours.

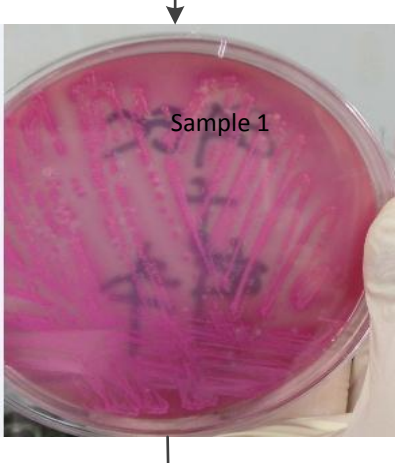
Here, the RNA was obtained, and then, the RNA was reverse transcripted in to cDNA with PrimeScript™ RT reagent Kit (Takara Bio Inc, Shlga, Japan)

Here, RVA, NoV and SaV were detected the PCR by primes, respectively. The primes and the reaction condition were all from references published.
1: RVA was detected by 1 ul cDNA and specific primer.
2: NoV was detected by 1 ul cDNA and specific primer.
3: SaV was detected by 1 ul cDNA and specific primer.
All the PCR amplicons were analyzed in a 1 % agarose gel electrophoresis at 120 V for 40 min and observed under UV light after ethidium bromide staining. Positive PCR products were kept at −70 ℃, and were then sequenced to ensure there enteric pathogen infection.

Here, the DNA of each stool sample was obtained,

Here, *Cryptosporidium*.spp; *G. lamblia* , *B. hominis* and Ad were detected the PCR by respective primes. The primes and the reaction condition were all from references published.
1: *Cryptosporidium*.spp was detected with nest-PCR by 1 ul DNA and specific primer in first round, and then the 1 ul products of first round and specific primer was used to detected *Cryptosporidium*.spp.
2: *G. lamblia* was detected with nest-PCR by 1 ul DNA and specific primer in first round, and then the 1 ul products of first round and specific primer was used to detected G. lamblia.
3: *B. hominis* was detected with by 1 ul DNA and specific primer.
4: Ad was detected with by 1 ul DNA and specific primer.
All the PCR amplicons (the second products of *Cryptosporidium*.spp.and *G.lamblia*; and the products of *B.hominis* and Ad) were analyzed in a 1 % agarose gel electrophoresis at 120 V for 40 min and observed under UV light after ethidium bromide staining. Positive PCR products were kept at −70 ℃, and were then sequenced to ensure there enteric pathogen infection.

We take sample 1 for example.

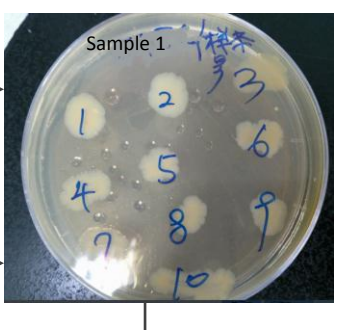


In most common study, the DEC was divided into EAEC.EPEC.EIEC and EHEC. In the study, we select the genes of "eae, stx1, stx2, elt, estA, estB, aggR and ipaH" to divide DEC.

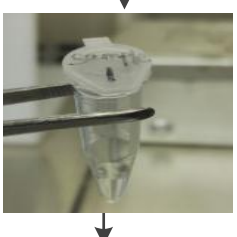
Yes! "There is no a particular visual feature of DEC colonies on MC which could be used as clue to identify them"

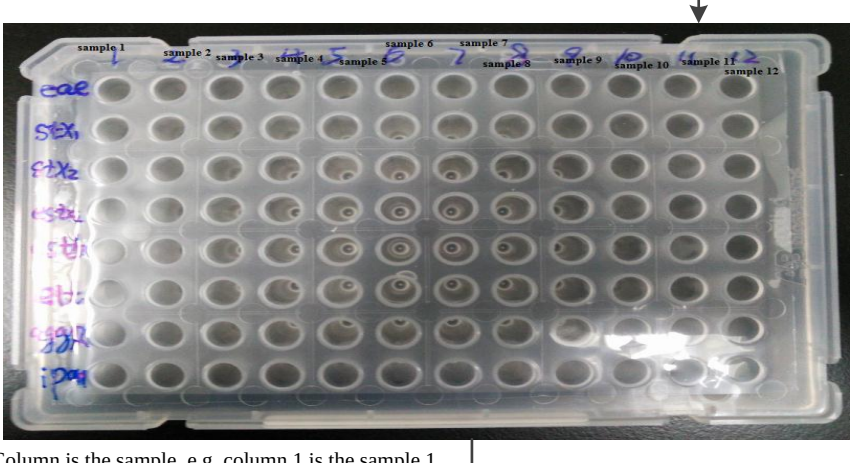
Select six to ten separate colonies (Suspicious colony: medium-size or small size, smooth edge, in addition, the 3 to 6 isolation of colorless and transparent must be selected(not to lactose fermenters) , and 3 to 6 pink isolation must be selected (lactose fermenters)), mix with dd H_2O. and then, adjust to the concentration of bacteria to 4.0 NTU in EP trub, and then, extract 1.5 ml Bacterial suspension to 1.5 ml EP tube, and then heat to 100℃ to extract the bacterial genome ,and then, centrifuged for 10 min at 12000 g, and then, absorb supernatant for qPCR.

Meantime, select these six to ten single clonnies in to nutrient agar, and then culture 37 ℃ for 18 hours.



For sample 1, we added these eight pair primes (1 μL forward primer (10 μmol), 1 μL reverse primer (10 μmol)) of eight gene into eight wells respectively, and then, in each wells, we added 1 ul DNA template, 10 μL master mix and 7 μL H_2O.

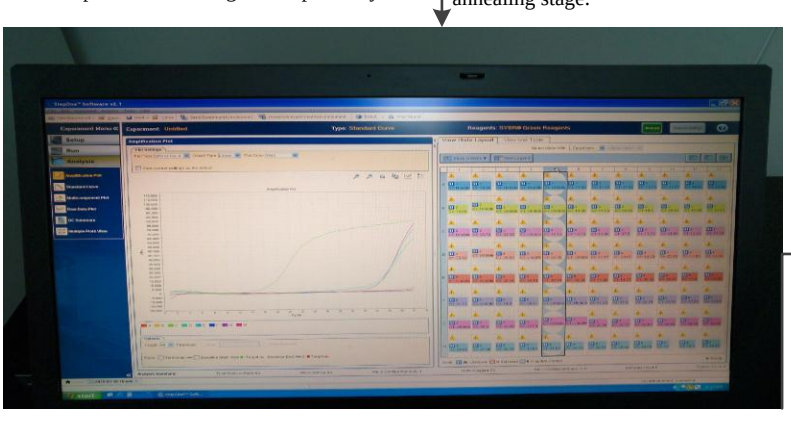




Column is the sample, e.g. column 1 is the sample 1, and the column 2 is the sample 2. so as to other samples (3 to 12). Rows is the primes' s targeted gene, e.g. in row 1, we add the primes for the eae gene. In row 2, we add the primes for the stx1 gene. So as to other primes for other gene, respectively.

We take sample 1 for example.

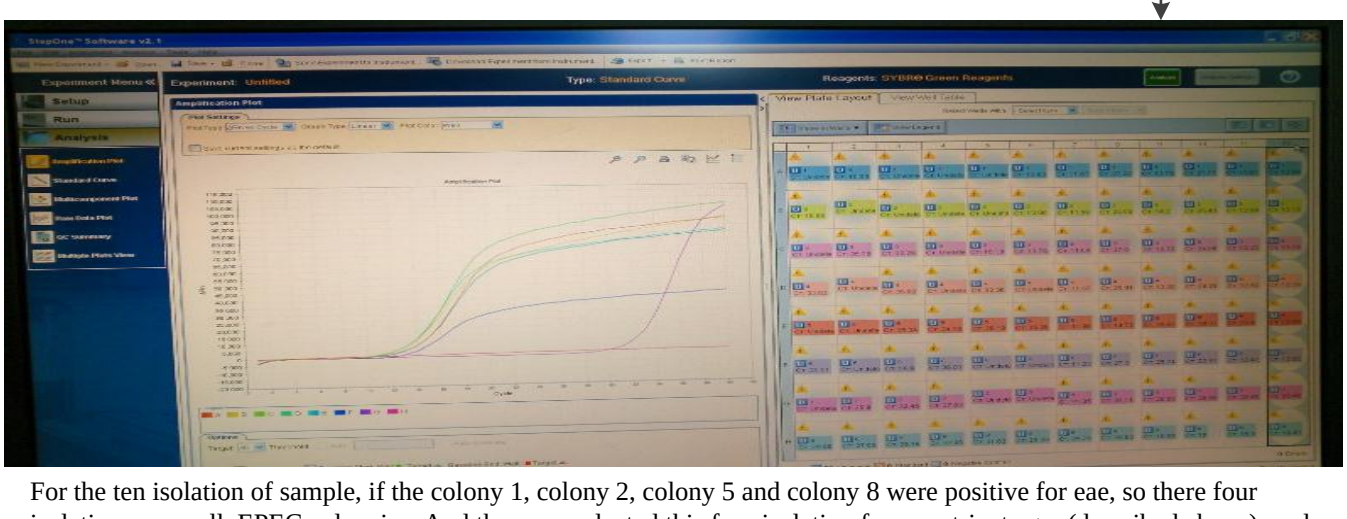
The cycling conditions was 95 ℃ for 5 min, 40 cycles of 95 ℃ for 5 s, 60 ℃ for 30 s. The fluorescence recorded was at the annealing stage.



For sample 1, for eight genes, if only eae was positive (CT<28), which means that sample 1 was EPEC infection, and in there six to ten colonies, there had one EPEC isolation at least.
Select these ten separate colonies respectively, mix with dd H_2O. and then, adjust to the concentration of bacteria to 4.0 NTU in EP trub, and then, extract 1.5 ml Bacterial suspension to 1.5 ml EP tube, and then heat to 100℃ to extract the bacterial genome ,and then, centrifuged for 10 min at 12000 g, and then, absorb supernatant for qPCR.

For sample 1, for eight genes, if only eae was positive (CT<28), in meantime, stx1 and stx2 were all negative, which means that sample 1 was EPEC infection, and in there six to ten colonies, there were one EPEC isolation at least.

For sample 1, we added the pair primes of eae (1 μL forward primer (10 μmol), 1 μL reverse primer (10 μmol).) in ten wells respectively, and then, in each wells, we added 1 ul DNA template of ten isolation, respectively, 10 μL master mix and 7 μL H_2O.The cycling conditions was 95 ℃ for 5 min, 40 cycles of 95 ℃ for 5 s, 60 ℃ for 30 s. The fluorescence recorded was at the annealing stage.



For the ten isolation of sample, if the colony 1, colony 2, colony 5 and colony 8 were positive for eae, so there four isolations were all EPEC coloneies. And then, we selected this four isolation from nutrient agar (described above), and kept at -70 ℃ with 25% glycerin bouillon.

DECs	Judging standard
EPEC	eae+
EAEC	aggR+
EIEC	ipaH+
EHEC	eae+, and (stx1+, and/or stx2+)
ETEC	hlt+, and/or estA, and/or estB+

We take sample 1 for example, and suppose sample 1 is EAEC infection, the others subtypes of DEC and other stool samples were detected with the same method and process.

The detection methods of enteric pathogens applied in this work were all from papers published previously .