Comparison of the Identification Methods of Diarrheagenic *E. coli* in Taiwan by O Serotyping and Single-tube Multiplex Real-time PCR

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Abstract

Differentiation of the 5 major types of diarrheagenic *E. coli* [EHEC (enterohemorrhagic *E. coli*), ETEC (enterotoxigenic *E. coli*), EPEC (enteropathogenic *E. coli*), EIEC (enteroinvasive *E. coli*), and EaggEC (enteroaggregative *E. coli*)] is very difficult even with the tools available today. In order to improve the ability of the Centers for Disease Control (CDC) to detect these pathogenic bacteria, the Research and Diagnosis Center established a set of real-time PCR methods to rapidly identify the following 4 types: ETEC, EHEC, EPEC, and EIEC. Six types of virulent genes were chosen for examination; specifically, *lt* and *st* for ETEC, *stx1* and *stx2* for EHEC, *eae* for EPEC, and *ipaH* for EIEC. In addition, we compared results of the traditional serotyping method with real-time PCR and found the association between the virulent genes and specific serotypes. Among 137 suspected strains of diarrheagenic *E. coli*, 15 strains were confirmed to carry virulent genes by real-time PCR, including 10

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strains of ETEC with *st*, 3 strains of EIEC with *ipaH* and 2 strains of EPEC with *eae*. The positive rate of virulent genes was 10.9%, indicating that this method could substantially reduce the high number of false positive results that occur when using traditional serotyping for identification. In addition, after reviewing the literature, our study is the first to discover 6 new strains, including 2 strains of ETEC (O15:H16 and O1:H6), 2 strains of EPEC (O111:H9 and O63:H6), and 2 strains of EIEC (O63:H9 and O169:H9).

Keyword: Diarrheagenic *E. coli*, real-time PCR, serotyping, virulence gene, diarrhea

Introduction

Most types of *Escherichia coli* (*E. coli*) are present in the human body's gastrointestinal tract as part of the normal flora and are considered non-pathogenic. However, certain strains of *E. coli* can cause illness in humans, including urinary tract infections, bacteremia, meningitis, and gastrointestinal diseases such as diarrhea and abdominal pain. These types of *E. coli* are considered diarrheagenic *E. coli* (DEC) [1, 2, 3]. These pathogenic *E. coli* are of specific serotypes. Currently, at least 5 major types have already been identified according to their serotype and virulence factor: enterohemorrhagic *E. coli* (EHEC) or shiga-toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EaggEC) [14]. Each type includes different O and H antigens.

In the past, these types of bacteria have been identified phenotypically. A single type of bacteria is isolated through cell culture, and immunologic or serotyping methods are used for further analysis. For example, the identification of ETEC has always depended on special immunological methods such as enzyme immunoassay (EIA) to detect the production of ST or LT toxins [8]. The

detection of EPEC depends on cell culture and immunofluorescence microscopy [8, 9], while the identification of EaggEC relies on observing adhesion to Hep-2 cells [8]. All of these methods require professional training and experience, and identification kits currently available on the market focus on diagnosing *E. coli* serotype O157. New assays for the other 4 types of DEC have not been extensively developed. Thus, easy and accurate methods for the identification of various DEC are urgently needed. With the advances of molecular biology, current trends are gradually moving towards diagnostic tests based on targeting specific virulent genes. For example, PCR and blotting methods can be used to detect specific factors related to virulence, including genes for toxins and invasive proteins. Since the virulence factors of DEC utilize different levels of DNA control and expression, ranging from chromosomal, plasmid, to phage DNA, PCR gene sequencing was used in our study.

Research from other countries on the proportion of diarrheal diseases caused by DEC has shown that the annual rate is approximately 12% in the US [21] and 15% in Japan [17]. However, Taiwan does not have similar data available. CDC is the nation's highest-level agency for preparedness and response, which must be able to diagnose various pathogens in order to respond to outbreaks, including DEC. To overcome for the current lack of diagnostic and identification techniques for DEC, this study established a real-time PCR system targeting ETEC, EHEC, EPEC, and EIEC to obtain diagnostic results for these 4 major types of DEC within a short time frame. This method will reduce the labor and financial cost, required in preparing large batches of serum assays. In addition, we used this research to compare results of the traditional serotyping method with real-time PCR and to determine associations between virulent genes and specific serotypes. From a prevention standpoint, the CDC might improve the current

under-diagnosis of DEC, provide physicians with additional epidemiologic data, and clarify the causal relationship between DEC and diarrheal diseases.

Materials and Methods

1. Source of specimens

   We tested the specificity of the PCR using clinical isolates from Taiwan CDC. Hospitals, health bureaus, and doctors participating in sentinel surveillance contributed samples from patients with abdominal pain. Colonies from anal swabs and stool samples tested negative for cholera, typhoid, *Salmonella*, *Shigella*, *Staphylococcus aureus*, and *Bacillus cereus* were used.

2. Growth and isolation of *E. coli*

   The culture isolation of *E. coli* was similar to the method described by Hitchins [12]: the patients' anal swabs or stool samples were plated directly on MacConkey or MacConkey sorbitol media and incubated at 35℃ for 18-24 hours, after which suspected colonies were chosen for further culture. Triple sugar iron agar (TSIA), lysine iron agar (Creative Micro, Ltd.) and Biotest No. 1 assay kit (Eiken Co., Tokyo, Japan) were used to identify *E. coli*.

3. Standard curve calculations for bacteria colonies

   The *E. coli* was cultured in 5ml of BHI liquid media, and 300 μl of the solution served as the stock for a series of 10-fold dilutions. We used 100 μl of extracted chromosomal DNA as the template for the real-time PCR. We took 40 μl of the bacterial solution and added it to 360 μl of BHI liquid media (1/10X), after which the 10^0 solution was further diluted to a factor of 10^{-8}. Afterwards, 100 μl was taken from each diluted solution and spread over MacConkey media with a sterilized glass rod. The plates were incubated overnight at 35℃, and calculations were made by multiplying the number of

colonies at each dilution by the dilution factor to get the number of bacteria in the original stock solution.

4. Extraction of bacterial DNA

   The *E. coli* cultured on MacConkey or MacConkey sorbitol media was incubated again at 35℃ in 5 ml of TSB or BHI liquid media (Creative Micro, Ltd.) for 18-24 hours, after which the QIAamp DNA Mini Kit (QIAGEN, Cat. No. 51304) was used to extract chromosomal DNA. Afterwards, we performed agarose gel electrophoresis to confirm that the quality of the DNA products could serve as a template for real-time PCR.

5. Single-tube multiplex real-time PCR

   (1) Real-time PCR primers, probes, reagents, and reaction conditions

   The primers and probes used for ETEC and EIEC were designed based on its virulence gene sequence. For EHEC and EPEC, the type and position of the fluorescence label were modified after consulting the literature (Table 1) [4, 15]. We were able to amplify 6 types of virulent gene segments: *lt* and *st* in the ETEC chromosome, *stx1* and *stx2* for EHEC, *eae* for EPEC, and *ipaH* for EIEC. Fluorescence labels for the probes were Red 640 for *lt* and Red 705 for *st*, Red 640 for *stx2* and Red 705 for *stx1*, and Red 640 for both *eae* and *ipaH*. Thus, the different virulent genes could be placed within the same PCR reaction tube and detected simultaneously under the same reaction conditions. Our study used the Roche LightCycler 1.0 instrument paired with the Roche LightCycler FastStart DNA Master^{PLUS} HybPROBE (Cat. No. 03 515 575 001) reagent to quantify DNA. The total volume for the PCR reaction was 20 μl, which included 1 μl of 10 μM for each primer (final concentration was 0.5 μM), 0.4 μl of 10 μM for each of the FL and LC probes (final concentration was

0.2 μM), 4 μl of LightCycler FastStart DNA MasterPlus HybProbe 5X concentration reagent, and 4.8 μl of template DNA. The reaction conditions were: 1. Denaturing: 95℃ for 10 minutes; 2. Cycling: 95℃ for 5 seconds, 48℃ for 10 seconds, and 72℃ for 20 seconds for a total of 45 cycles; and 3. Cooling: 40℃ for 45 seconds. The total reaction time was approximately 60 minutes.

Table 1. Primer and probe sequences for real-time PCR

Target gene	Primer or probe	Sequence (5'-3')	Amplicon size (bp)	*Tm* (℃) of primer or probe	Reference
lt	LT S	TTT GTC AGA TAT GAT GAC GGA	253	50.6	This study
	LT A	TCA CAC CAA AAT TAA CAC GA		50.9	
	LT FL	ATT AGG CGT ATA CAG CCC TCA CCC-FL		62.2	
	LT LC	640-TAT GAA CAG GAG GTT TCT GCG TTA GGT G-p		64.4	
st	ST S	GAT GCT AAA CCA GTA GAG TCT TC	152	50.7	This study
	ST rev short	CCG GTA CAA GCA GGA TT		59.1	
	ST FL	AAA GTG GTC CTG AAA GCA TGA AT-FL		57.5	
	ST LC	705-GTA GCA ATT ACT GCT GTG AAT TGT GTT G-p		61.3	
stx1	stxA1 598	AGT CGT ACG GGG ATG CAG ATA AAT	418	60.2	14
	stxA1 1015	CCG GAC ACA TAG AAG GAA ACT CAT		58.7	
	stxA1 FL724	CTG TCA CAG TAA CAA ACC GTA ACA TCG CTC-FL		65.5	
	stxA1 LC	705-TGC CAC AGA CTG CGT CAG TGA GGT-p		67.3	
stx2	stxA2 679	TTC CGG AAT GCA AAT CAG TC	264	55.9	14
	stxA2 942	CGA TAC TCC GGA AGC ACA TTG		58.4	
	ALL FL	MAG AGC AGT TCT GCG TTT TGT CAC TGT CA-FL		65.6	
	ALL LC	640-AGC AGA AGC CTT ACG CTT CAG GC-p		63.3	
eae	eaeAF	GAC CCG GCA CAA GCA TAA GC	384	61.5	15
	eaeAR	CCA CCT GCA GCA ACA AGA GG		61.2	
	eae FL	CCT GGT CAG CAG ATC ATT TTG CCA CTC A-FL		68.4	
	eae LC	640-ACT TCC CTT TGA ATA CAG TGC CTT ACC A-p		63.4	
ipaH	ipaH-S	GAT AAT GAT ACC GGC GCT CTG	221	57.1	This study
	ipaH-R	CGG AGG TCA TTT GCT GTC A		56.5	
	ipaH FL	CCT TCC AGA CCA TGC TCG CAG AGA A-FL		67.0	
	ipaH LC	640-TCAGCT CTC CAC TGC CGT GAA GGA A-p		67.9	

(2) Real-time PCR sensitivity testing

To test the sensitivity of the real-time PCR reaction and the accuracy of

the quantification, we incubated the DEC in 5 ml of BHI liquid media at 35℃ and calculated the number of colonies. We used the QIAamp DNA mini kit to extract chromosomal DNA from the liquid media and then used the DNA from the various 10-fold serial dilutions as the template. We conducted a series of real-time PCR analysis, and the positive PCR products at different concentrations were confirmed with gel electrophoresis. We performed calculations to determine the Cp values at various dilutions and made crude estimates of the bacteria count for the standard curve, which served as a reference for other Cp values later in the qualitative and quantitative analysis.

(3) The criteria for the isolation of DEC using real-time PCR were as follows: ETEC, if bacteria have *lt* or *st*; EHEC, if bacteria have *stx1* or *stx2*; EPEC, if bacteria have *eae*; and EIEC, if bacteria have *ipaH*.

6. Serotyping of Somatic O and Flagella H Antigens

We used commercially available O and H antigen antiserum for DEC (Denka Seiken, Tokyo, Japan) (Table 2) and conducted agglutination tests in *E. coli* strains to determine the O and H antigen type.

Table 2. Definitions for diarrheagenic *E. coli* serotypes from commerically-sold antiserum

Mixed serogroup	Serotype
1	O1、O6、O86a、O111、O119、O127a、O128
2	O44、O55、O125、O126、O146、O166
3	O18、O114、O142、O151、O157、O158
4	O6、O27、O78、O148、O159、O168
5	O20、O25、O63、O153、O167
6	O8、O15、O115、O169
7	O28ac、O112ac、O124、O136、O144
8	O29、O143、O152、O164
9	O74、O91、O103、O121、O145、O161、O165

(1) Analysis of O antigen: The bacteria strain was suspended in 3 ml of salt water and boiled at 100℃ for 1 hour. After centrifugation, the O

antiserum was mixed with the supernatant for 1 minute and examined for the presence of agglutination.

(2) Analysis of H antigen: The bacteria was cultured in a Craigie's test tube to increase its flagella antigen expression and fixed with 1% formalin. The solution was further mixed with the H antiserum and incubated at 50℃ for 1 hour before being examined for the presence of agglutination.

Results

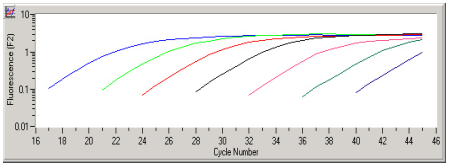
1. Sensitivity and Specificity of Real-time PCR for Detection of Virulent Genes

To test the specificity of real-time PCR, we used *E. coli* BCRC 15376 (EHEC), *E. coli* BCRC 15372 (ETEC), *E. coli* BCRC 15536 (EPEC), and *E. coli* ATCC 43893 (EIEC) as positive controls. In addition, other types of Enterobacteria, such as *Salmonella enteritidis*, *Bacillus cereus*, *Salmonella typhi*, *Salmonella paratyphi*, *Shigella flexneri*, *Shigella sonnei*, *Vibrio parahemolyticus*, *Staphylococcus aureus*, and purified chromosomal DNA from 2 non-pathogenic *E. coli* strains were used for the 6 virulent genes in the real-time PCR reaction: *lt* and *st* in ETEC, *stx1* and *stx2* in EHEC, *eae* in EPEC, and *ipaH* in EIEC. The results (Table 3) show that each pathogenic *E. coli* had different signals after reaction with the complementary primers and probes. Only *ipaH* from EIEC, *Shigella flexneri* and *Shigella sonnei* had a cross-reaction. To test the sensitivity of the single-tube multiplex real-time PCR method, we used serial dilutions of chromosomal DNA from each DEC. Calculations indicated that the lowest detectable concentration was 10 CFU/PCR reaction (Figure 1).

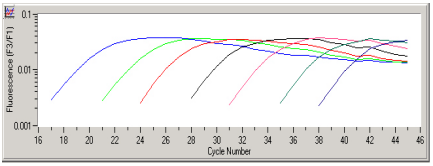
Table 3. Specificity for 6 types of virulent genes by real-time PCR

Organism	Results of real-time PCR					
	lt	*st*	*stx1*	*stx2*	*eae*	*ipaH*
Escherichia coli BCRC 15376 (EHEC)	-	-	+	+	+	-
Escherichia coli BCRC 15372 (ETEC)	+	+	-	-	-	-
Escherichia coli BCRC 15536 (EPEC)	-	-	-	-	+	-
Escherichia coli ATCC 43893 (EIEC)	-	-	-	-	-	+
flora E. coli-1	-	-	-	-	-	-
flora E. coli-2	-	-	-	-	-	-
Shigella flexneri	-	-	-	-	-	+
shigella sonnei	-	-	-	-	-	+
Salmonella enteritidis	-	-	-	-	-	-
Salmonella typhi	-	-	-	-	-	-
Salmonella paratyphi	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-
Vibrio parahemolyticus	-	-	-	-	-	-

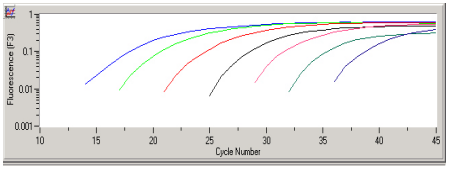
(A) ETEC *lt*



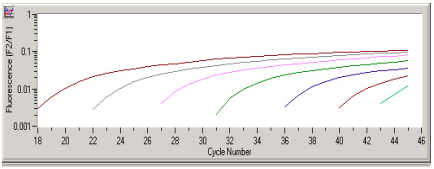
(B) ETEC *st*



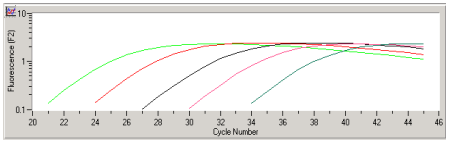
(C) EHEC *stx1*



(D) EHEC *stx2*



(E) EPEC *eae*



(F) EIEC *ipaH*

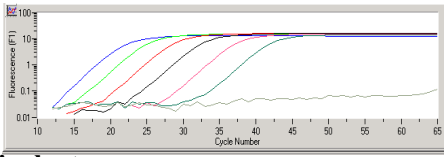


Figure 1. Sensitivity for the 6 types of virulent genes

2. Use of Antiserum to Identify Clinically Isolated DEC

The 261 specimens of *E. coli* from patients with diarrhea were analyzed using O antiserum. Among them, 137 isolates (52%) agglutinated with the antiserum and were classified as suspected DEC (sDEC). The O serotype for each of the sDEC are shown in Table 4, and the 3 most frequent serotypes were O1 (12/137; 8.7%), O25 (9/137; 6.5%) and O44 (9/137; 6.5%).

Table 4. Serotypes for suspected strains of diarrheagenic *E. coli*

Serogroup	No. of isolate	Serogroup	No. of isolate	Serogroup	No. of isolate	Serogroup	No. of isolate
O1	12	O28ac	1	O111	1	O151	1
O6	7	O29	1	O112ac	4	O152	2
O8	8	O44	9	O114	3	O153	4
O15	8	O55	4	O124	1	O159	6
O18	7	O63	2	O125	4	O164	6
O20	1	O74	2	O128	2	O166	4
O25	9	O86a	7	O144	4	O168	2
O26	2	O91	3	O146	3	O169	2
O27	3	O103	2				

3. Detection of Virulent Genes in sDEC with Real-Time PCR

To confirm that the 137 isolates of sDEC were pathogenic, real-time PCR was used to detect the presence of specific virulent genes as the basis for classification. The sDEC isolates with virulent genes were classified as true DEC (tDEC). The results (Table 5) show that 15 isolates (10.9%, 15/137) carried virulent genes. In order of frequency, 10 isolates (66.7%, 10/15) had the *st* gene, and were classified as ETEC; 3 isolates (20%, 3/15) had the *ipaH* gene, and were classified as EIEC; and 2 isolates (13.3%, 2/15) had the *eae* gene, and were classified as EPEC. Our study did not find any EHEC. Among the 261 specimens from patients with diarrhea, 5.7% (15/261) were tDEC. The proportions of ETEC, EPEC and EIEC were 3.8% (10/261), 0.8% (2/261) and 1.1% (3/261), respectively.

Table 5. Real-time PCR and classification results for confirmed diarrheagenic *E. coli*

Source	Result of real-time PCR						Category
	lt	*st*	*stx1*	*stx2*	*eae*	*ipaH*	
Inbound traveler-809P	-	+	-	-	-	-	ETEC
Indigenous patient-7864	-	+	-	-	-	-	ETEC
Indigenous patient-86482	-	+	-	-	-	-	ETEC
Inbound traveler-559P	-	+	-	-	-	-	ETEC
Inbound traveler-463P	-	-	-	-	+	-	EPEC
Emergency patient- J63	-	+	-	-	-	-	ETEC
Emergency patient- J80	-	+	-	-	-	-	ETEC
Emergency patient- J91	-	+	-	-	-	-	ETEC
Inbound traveler-896P	-	+	-	-	-	-	ETEC
Inbound traveler-051P	-	+	-	-	-	-	ETEC
Emergency patient- J94	-	+	-	-	-	-	ETEC
Indigenous patient-008629	-	-	-	-	-	+	EIEC
Indigenous patient-019882	-	-	-	-	-	+	EIEC
Indigenous patient-019871	-	-	-	-	-	+	EIEC
Emergency patient- J113	-	-	-	-	+	-	EPEC

4. Serotyping of tDEC

The 15 isolates of tDEC carrying virulent genes were further analyzed with H antiserum to determine their serotype. The results (Table 6) show that 10 isolates of ETEC belonged to 6 different O antigen groups: O1, O6, O15, O25, O44, and O159, and 5 H antigen groups: H6, H11, H16, H18, and H34. There were two O15:H11 strains and two O25:NM strains. The O antigens for the 3 isolates of EIEC were O63, O124 and O169; the H antigens were H7 and H9. The O antigens for the 2 EPEC isolates were O63 and O111; the H antigens were H6 and H9.

Table 6. Serotypes for confirmed diarrheagenic *E. coli* by real-time PCR

Categories	Total no. of isolates	Serotypes (no. of isolates)
ETEC	10	O15:H11 (2), O15:H16 (1), O1:H6 (1) O25:NM (2), O159:H34 (1), O44:H18 (1) O6:H16 (1), O1:NM (1)
EPEC	2	O111:H9 (1), O63:H6 (1)
EIEC	3	O124:NM (1), O169:H9 (1), O63:H7 (1)

Discussion

Distinguishing pathogenic strains from non-pathogenic strains of *E. coli* remains a challenge in a clinical setting. In the past, biochemical and serotyping methods were used to identify DEC. However, these methods have their limitations. Among the 5 major types of DEC, with the exception of the O157:H7 serotype that can be isolated on sorbitol MacConkey media, the appearance of colonies from the remaining 4 types are similar [1, 2]. In addition, other than O157, the biochemical characteristics of the other 4 types are also very similar. Thus, it is not possible to use biochemical identification to distinguish ETEC, EPEC, EIEC, and EAEC from each other. Although serotyping can use agglutination tests to detect a bacteria's O and H antigens, several antigens for the various DEC are similar [10], leading to false-positive results. Thus, serotyping cannot prove the pathogenicity of strains [10]. In addition, since genotyping requires the use of different antiserums, most hospital laboratories do not have all of them because of cost. Furthermore, because the commercial reagents currently available are not very popular, most clinical laboratories do not perform DEC identification.They usually send suspected strains that have been isolated to larger reference laboratories to request follow-up tests.

Our study performed serotyping on 261 specimens from patients with diarrhea and classified 137 strains as sDEC. We then used real-time PCR to detect the positivity rate for 6 types of virulent genes. The results indicated that 15

strains (10.9%, 15/37; including 10 ETEC, 3 EIEC and 2 EPEC) carried virulent genes. The remaining 122 strains were non-pathogenic *E. coli*. The 2 methods showed a difference in the results for identifying DEC. Relying solely on serotyping for identification could result in a much higher positive rate. The rate of DEC isolated among all of the specimens was 5.7% (15/261). The proportions of ETEC, EPEC and EIEC were 3.8% (10/261), 0.8% (2/261) and 1.1% (3/261), respectively.

Among the 10 strains identified as ETEC, there were 6 types of O serogroups: O15 (3 strains), O1 (2 strains), O25 (2 strains), O159 (1 strain), O44 (1 strain), and O6 (1 strain). The first 3 types made up 70% of the total sample. The positive rate of virulence genes for the O antigen groups were 37.5% (3/8), 16.7% (2/12), 22.2% (2/9), 16.7% (1/6), 11.1% (1/9), and 14.3% (1/7). Compared with the data presented by Tamaki et al, the positive rate of virulence genes for the strains in the O15 and O1 serogroups were different (37.5% vs 0% and 16.7% vs 0%). Comparisons for the remaining 4 groups were: O6 (14.3% vs 8.1%), O25 (22.2% vs 12.5%), O44 (11.1% vs. 13.3%), and O159 (16.7% vs. 25%). In addition, the proportion of ETEC among sDEC specimens was 7.3% (10/137) in our study, compared with 7.0% (79/1130) as reported by Tamaki. After detection of the H antigen, the 10 strains of ETEC had 8 different serotypes. Among them, there were two O15:H11 strains and two O25:NM strains. Compared to the finding reported in the literature [17], O44 has always been defined as being part of the EPEC serogroup. Our study detected that the O44:H18 strain carried the *st* gene. Thus, it was confirmed to be ETEC, which is different from previous reports. When searching for the serogroup of two strains of ETEC (O15:H16 and O1:H6) in the literature, we could not find similar reports. Thus, our study may be the first to discover these new serotypes. Our study did not isolate EHEC from

patient specimens. Since there have already been reports that the plasmid carrying virulent genes in EHEC could be lost during the bacteria growth or preservation stages [14], we aim to improve diagnostic procedures for this type of *E. coli* in the future to increase the isolation rate.

Previously, the WHO defined the following 12 O serogroups of *E. coli* as EPEC [3]: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158. Among the EPEC isolated in our study, only one strain (O111) was of the types described above, indicating that of the 23 strains considered to be part of the 12 serogroups of sDEC, the confirmed rate of EPEC was only 4.4% (1/23). This was lower compared to other studies, which reported 16.9%, 62% and 18.6% [17, 3, 18], suggesting that the serotypes of EPEC may vary by country. In addition, the positive rate of the *eae* gene for O111 in our study was 100% (1/1), which is the same as other reports that described O111 as the main serogroup of EPEC [19]. Furthermore, our study is the first to isolate the 2 strains of EPEC with the O111:H9 and O63:H6 serotype.

We report the identification of the following O serogroups for EIEC: O124, O63 and O169, which were different compared to other reports. The positive rate for the *ipaH* gene in O124 (100%, 1/1) was the same as that reported by Levine et al [20]. However, reports from Tamaki and Sunabe gave a different result (proportion was 0% in both cases) [17, 18]. In addition, the serogroups O63 and O169 were not considered EIEC in the 3 reports above, indicating that the EIEC serotype varies by country. Compared to the literature, our study isolated O124:NM as classic EIEC, and O63:H9 and O169:H9 serotypes were the first to be discovered.

The positive rate of virulence genes in DEC made up a large proportion of certain O serogroups (O63, O111 and O124 were all 100%), suggesting that

serotyping can be used to identify these 3 groups of DEC. For the other O serogroups, using only serotyping could result in higher positive rates. In addition, different DEC can belong to the same O serogroup. Thus, although serotyping can identify a few of the major types of *E. coli*, it is unable to classify all of the strains. Combining the results of our study, real-time PCR can directly identify the 4 major types of DEC (ETEC, EHEC, EPEC, and EIEC) within 60 minutes when serotyping is not done. This method meets the needs for timely prevention measures, especially when outbreaks occur, making it worthy of widespread implementation.

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