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Screening procedure from cattle feces and the prevalence of *Escherichia coli* O157:H7 in Taiwan dairy cattle

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A procedure has been established for screening *Escherichia coli* O157:H7 from bovine feces. It consists of four steps: enrichment, selective culture, phenotyping and genotyping. Modified trypticase soy broth (mTSB) containing 20 μg/mL of novobiocin was used for the enrichment step. The selective culture step was done using sorbitol MacConkey agar containing 0.05 μg/mL of cefixime and 2.5 μg/mL of potassium tellurite. The phenotyping steps include: species confirmation as *E. coli*, serotyping to O157 and H7, and the detection of verocytotoxin (VT) production. Genotyping was made up of confirmation of the presence of the O157 antigen (*rfb*), the H7 antigen (*ficC*), the adherence factor (*eaeA*), hemolysin (*hlyA*), and VT production (*vt* I and *vt* II) by polymerase chain reaction. mTSB gave better enrichment for *E. coli* O157 than gram-negative (GN) broth. The detection limit for this screening procedure was 0.85 ±0.5 cfu/g. Using the screening procedure described above, *E. coli* O157 was found in four out of 3062 (0.13%) fresh bovine fecal specimens, and in two out of 78 (2.56%) dairy herds in Taiwan. Three out of the four detected strains were *vt* II producing *E. coli* O157:H7, while the other was *E. coli* O157:H7 which lacked VT-producing ability.

Key words: *Escherichia coli* O157:H7, fecal samples, prevalence

Escherichia coli O157:H7 has emerged as an important food-borne human gastrointestinal pathogen since it was first reported in 1982 when it was classified as an enterohaemorrhagic *E. coli* (EHEC) of the enterovirulent *E. coli* [1-4]. EHEC produces two distinct lysogenic bacteriophage-encoded toxins, verocytotoxin I (*vt* I) and verocytotoxin II (*vt* II), which inhibit protein synthesis and are active against Vero and HeLa cells. Thus, EHEC, also called verocytotoxin-producing *E. coli* (VTEC) is very similar to the Shiga toxin produced by *Shigella dysenteriae*. Therefore, verocytotoxin (VT) has been called Shiga-like toxin. Patients infected with EHEC may develop serious clinical conditions such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. Death may even result [1-5]. Worldwide outbreaks have been reported and these incidents have been attributed to foods of bovine origin such as ground beef, raw meat products, untreated and pasteurized milk, and water [6-9]. Other nonbovine foods also have become contaminated and implicated as sources of infection. Ruminants are thought to be reservoirs of this organism, and bovine feces are now recognized as an important original contamination source [1,8-13].

Several screening procedures for *E. coli* O157:H7 are available [1,8,14-17]. Foods or human fecal specimens can be directly plated on selective or differential agar. Samples can also be selectively enriched in broth media. However, exclusive procedures for detection in bovine feces have been few because of complex matrix interference. Before applying the screening procedure, enrichment and selectivity have to be established. Gram-negative (GN) broth containing vancomycin and cefsulodin, and modified trypticase soy broth (mTSB) containing novobiocin are two possible enrichment broth [6,8,12-14]. The selective agar, sorbitol MacConkey agar containing cefixime and potassium tellurite (CT-sMAC), has been used as an important screening step [18,19]. Over 94% of *E. coli* has the ability to ferment sorbitol and this makes the colony on CT-sMAC appear pink to red. *E. coli* O157:H7, on the contrary, cannot ferment sorbitol, resulting in a colorless colony on CT-sMAC agar [1,14,18,19]. The addition of cefixime and potassium tellurite successfully increases the selectivity of sorbitol MacConkey agar. Various researchers have used polymerase chain reaction (PCR) techniques to confirm the genetic profiles of *E. coli* O157:H7. The primers specific for the VT genes and O157 gene are published, and can be used for the confirmation of *E. coli* O157:

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H7 and the presence of a VT gene [9,15-17,24-27].

The prevalence of *E. coli* O157:H7 in beef and dairy cattle on farms has been reported as varying from 0 to 68%, with a herd prevalence from 1.8 to 100% worldwide [28,29]. It has also been reported that 10 out of 3570 (0.28%) fecal samples from dairy cattle in five out of 60 herds (8.3%) in Washington State (USA) were positive for *E. coli* O157:H7 and the prevalence of *E. coli* O157:H7 in New York State was 0.96% [30]. No mass screening survey for *E. coli* O157 in animal farm in Taiwan has been conducted before. Therefore, the purpose of this study was to establish a screening process for the detection of *E. coli* O157:H7 from bovine fecal samples with phenotypes and genotypes, and to investigate the prevalence of *E. coli* O157:H7 in the eastern and northern parts of Taiwan.

Materials and Methods

Preparation of the bovine fecal samples with *E. coli* O157:H7

Bovine feces with a known amount of *E. coli* O157:H7 was made by mixing *E. coli* O157:H7 and *E. coli* O157:H7 free bovine feces. *E. coli* O157:H7 ATCC 43895 (American Type Culture Collection, USA) was grown overnight in TSB (Difco, Detroit, MI, USA) at 37 °C. The culture was then subjected to 10-fold serial dilution from 10^{-1} to 10^{-10} and plated on plate count agar (PCA) (Difco) to estimate the number of organisms. Two milliliter of each diluted suspension was mixed with 2 g bovine feces by vortex (MSI minishaker, IKA, Germany) at 1800 rpm, thus producing a series bovine fecal samples containing different concentrations of *E. coli* O157:H7 (BFEs). The original ATCC 43895 strain and the original *E. coli* O157:H7-free feces were also processed as positive and negative controls, respectively.

Determination of enrichment capability of media

GN broth (Difco) containing 8 μg/mL of vancomycin and 10 μg/mL of cefsulodin and mTSB (Merck, KgaA, 64271 Darmastadt, Germany) containing 20 μg/mL of novobiocin were used to enrich for *E. coli* O157:H7. To compare the enrichment ability of these two media, the BFEs were enriched in either GN broth or mTSB at 42 °C overnight. Ten-fold serial dilutions of the two enriched suspensions were spread on CT-sMAC and PCA, respectively. CT-sMAC, which contains 0.05 μg/mL of cefixime and 2.5 μg/mL of potassium tellurite (Merck, KgaA), was used to select for *E. coli* O157:H7, and PCA was used to measure the total enrichment ability of these two kinds of media.

Identification of *E. coli* O157:H7 colony in fecal samples

The flow chart of the screening process for the identification of *E. coli* O157:H7 is presented in Fig. 1. BFEs and the control samples were prepared as described above, then those enriched in mTSB were spread on CT-sMAC plates and cultivated at 42 °C overnight to detect the presence of *E. coli* O157:H7. Up to five suspected colonies, which were pale-gray to colorless on CT-sMAC agar plate and did not ferment sorbitol, were selected and then rescreened by subculture on the same medium (CT-sMAC) to confirm the phenotype of the selected colonies. Identification of the strains as *E. coli* was confirmed by API ID 32E (bioMerieux sa, Marcy l'Etoile, France) biochemical tests.

Serotyping of *E. coli* isolates

A colorless *E. coli* single colony on CT-sMAC was mixed with a drop of O157 antiserum (Seiken, Denka Seiken Co., Tokyo, Japan) on a glass slide. The slide was tilted back and forth to observe the agglutination reaction. Granular agglutination patterns occurring within 1 min were taken as a positive result. Heated cells were also tested. A reference *E. coli* O157:H7 strain and normal saline were used as positive and negative controls, respectively. For the agglutination test for H antigen, the test strain was serially passed through the semi-liquid medium in Craigy's tubes three times to enrich for highly motile organisms. The organism was then cultured in brain heart infusion (Difco) at 37 °C for 8 h. To an aliquot of this infusion, an equal volume of 1% (vol/vol) formalin in normal saline solution was added, and the resulting suspension was used as an antigen solution. Two drops of H7 antiserum (Seiken, Denka Seiken Co.) were added to 0.5 mL of antigen solution in a small test tube. After thorough mixing of the antigen solution and the H antiserum, the test tube was incubated in a 50 °C water bath for 1 h and observed for the agglutination reaction. Positive and negative controls were similarly tested at the same time.

Detection limit of the screening procedure

Detection limit was determined from the BFEs. After counting each of the 10-fold serial dilutions of the BFEs on PCA, the samples were inoculated into mTSB and incubated at 42 °C overnight for enrichment. The enriched broth was then plated on CT-sMAC at 42 °C for 18 to 20 h. Suspected *E. coli* O157 colonies were then further identified and confirmed by the screening procedure as shown in Fig. 1. The lowest concentration of confirmed *E. coli* O157:H7 in BFEs was defined as the detection limit.

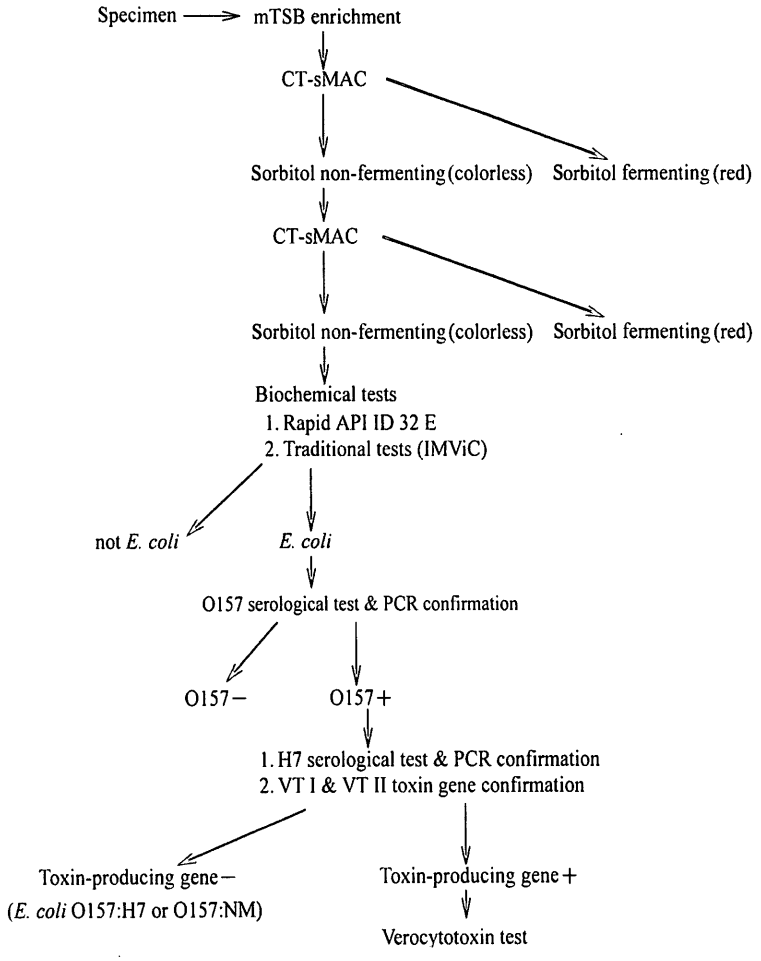

Fig. 1. The flow chart of the *E. coli* O157:H7 screening for bovine fecal specimens.

Detection of VT in bacterial culture supernatant

All the confirmed *E. coli* O157:H7 antigen strains were screened for the production of *vt* I and *vt* II using a verotox-F reversed passive latex agglutination (RPLA) kit (Seiken, Denka Seiken Co., Japan), according to the manufacturer's recommendation. Isolated strains were enriched in casamino acid-yeast extract broth at 37 °C for 18 to 20 h, and the enriched broth was centrifuged at 1000 x *g* for 30 min. Supernatant (25 μL) was pipetted into the well of a 96-well microtiter plate and then serially diluted in two-fold steps. To every dilution well 25μL of *vt* I or *vt* II sensitized latex was added and mixed. The mixture was sensitized at room temperature for 20 h before agglutination.

Genotype analysis by PCR

PCR was used to analyze the genotypes of the isolated strains and the reference *E. coli* O157:H7 strains by detection of DNA fragments of the O157 antigen *rfb* gene, the H7 antigen *ficC* gene, the adherence factor *eaeA* gene, the hemolysin *hly* gene, and the VT-producing gene *vt* I and *vt* II (Table 1). The test strains were grown in Luria-Bertani broth (Difco) at 37 °C overnight. The DNA template for PCR was prepared by boiling the culture in water for 10 min [16]. The sequences of the primers used are shown in Table 1. PCR assay were performed in 25 μL containing 100 ng of DNA template, 10 mM tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM (each) primer set, 0.2 mM (each) deoxynucleotide triphosphate, and 0.5 U of *Taq* DNA polymerase (MDBio, Inc., Taipei, Taiwan). Four *E. coli* O157:H7 strains (ATCC 43895, ATCC 43890, ATCC 43889 and ATCC 43888) were selected as reference strains. The VT-production ability of these four reference strains is different (Table 2). Strain ATCC 43895 produces both *vt* I and *vt* II, ATCC 43890 produces only *vt* I, ATCC 43889 produces only *vt* II, and ATCC 43888 does not produce either. The optimized PCR cycles for the different genes are shown in Table 3.

Table 1. Sequences of PCR primers used

Oligonucleotide	Sequence (5′ to 3′)	Target gene or amplicon	Expected size of PCR products (bp)	Ref.
O157 PF8	CGTGATGATGTTGAGTTG	*rfb*	420	[20]
O157 PR8A	GATTGGTTGGCATTACTG			
FLICH7-F	GCGCTGTCGATTCTATCGAGC	*ficC*	625	[21]
FLICH7-R	CAACGGTGACTTTATCGCCATTCC			
eaeAF	GACCCGGCACAAGCATAAGC	*eaeA*	384	[22]
eaeAR	CCACCTGCAGCAACAAGAGG			
hlyAF	GCATCATCAAGCGTACGTTCC	*hlyA*	534	[22]
hlyAR	AATGAGCCAAGCTGGTTAAGCT			
NS 1	CAGTTAATGTGGTGGCGAAG	*vt* I	475	[16]
NS 2	CACAGACTGCGTCAGTGAGG			
NS 5	CTTCGGTATCCTATCCCCGG	*vt* II	862	[16]
NS 7	CGCTGCAGCTGTATTACTTTC			

Table 2. *E. coli* O157 reference strains used

ATCC no.	CCRC no.	Kind of verocytotoxin production
43895	14824	*vt* I, *vt* II
43890	15373	*vt* I
43889	14825	*vt* II
43888	15374	—[a]

Abbreviations: ATCC = American Type Culture Collection (Rockville, Maryland, USA); CCRC = Culture Collection and Research Center (Hsinchu, Taiwan, ROC)
[a]No verocytotoxin production.

Prevalence of *E. coli* O157:H7 in dairy herds

It was necessary to determine the number of cattle that needed to be sampled and this was done in two steps: the number of herds that was necessary to sample was calculated, and then the number of cattle in each of these herds necessary for sampling was estimated. The former was calculated using formula 1 [23] and we assumed that B was 5% and Z was 1.96. The assumed values were made according to previous studies and assumed a herd prevalence of 20%. The latter was calculated using formula 2 [23]. We assumed that the number of positives in the population was 1%, and α was 95%. Each animal was restrained, registered, counted and a fatty acid free cotton swabs was used for direct bovine rectal sampling of 1 to 2 g feces. Three fecal swabs were pooled in one test tube containing mTSB, which was transported at low temperature to the laboratory for bacteriological culture. Pooled fecal specimens were enriched in mTSB at 42 °C overnight. The enriched culture was spread on CT-sMAC and incubated at 42 °C for 18 to 20 h. The isolation and identification procedures for *E. coli* O157:H7 are shown in Fig. 1.

Formula 1.
$$n = \frac{Npq}{\frac{(N-1)\,B^2}{Z^2} + pq}$$

n is the number of the herds that need to be sampled. N is the total number of herds in an area. p is the expected herd prevalence; q is 1-p; B is the limitation of the sampling error; Z is the coefficient of normal distribution.

Formula 2.
$$n = [1 - (1-\alpha)^{1/d}]\,(N - d/2) + 1$$

n is the number of sampled animals; N is the population size; d is the number of positives in the population; α is the desired confidence level.

Results

Enrichment capability of GN broth and mTSB

GN broth and mTSB were inoculated with same

Table 3. PCR programs used for the detection of the various genes in *E. coli* O157

Stage	Gene			
	rfb	*ficC*	*eaeA/hlyA*	*vt I/vt II*
Denaturing	94 °C, 1 min	94 °C, 15 sec	95 °C, 1 min	94 °C, 1 min
Annealing	55 °C, 1 min	65 °C, 15 sec	60 °C, 2 min	62 °C, 1 min
Extension	74 °C, 1 min	72 °C, 75 sec	72 °C, 2.5 min	72 °C, 1 min
Cycle no.	30	35	30	30
Final extension	74 °C, 10 min	72 °C, 10 min	72 °C, 7 min	72 °C, 5 min

concentration (63.6 ±12.1 cfu/mL) of bacteria (n = 5). The total bacterial counts by plating on PCA after incubation were (5.7 ±0.9) x 10^8 and (2.2 ±0.8) x 10^8 for GN and mTSB, respectively. The number of colony forming *E. coli* O157 measured by plating on CT-sMAC was (4.9 ±0.9) x 10^2 for GN and (8.2 ±0.7) x 10^2 for mTSB. These results indicated that GN broth was a better overall medium for PCA plating ($p < 0.05$), while, in contrast, mTSB was a more effective selective medium for *E. coli* O157:H7 than GN broth ($p < 0.05$).

The detection limit of the screening procedure

The serial dilution method was used to count the number of *E. coli* O157:H7 in the mTSB-enriched broth of BFEs. The results showed that *E. coli* O157:H7 could be detected at levels as low as 10^{-7} dilution and the organism was easily detected on the medium. The detection limit was calculated as 85.19 ±50.32 cfu/g of BFEs. With further enrichment on mTSB, the detection limit was reduced by 100 fold to 0.85 ±0.5 cfu/g of BFE.

E. coli O157:H7 prevalence in Taiwanese dairy cattle farm

A total of 3062 bovine fecal specimens were collected from 78 dairy herds in Taipei city (one herd), Taipei county (12 herds), Taoyuan county (57 herds), and Hualien county (eight herds). Only four *E. coli* O157:H7 positive fecal samples were detected and these were from Hualien county. Thus, all of the isolated strains were from the eastern part of Taiwan and none from the northern part. Cattle prevalence was 0.13% (4/3062), and herd prevalence was 2.56% (2/78). Three isolates were from the herd Hualien 1 and produced *vt* II, but the strain isolated from the herd Hualien 8 did not produce either form of VT. VT-producing strains of *E. coli* are a major public health concern and our results show a dairy cattle prevalence for VTEC of 0.1% (3/3062) and a herd prevalence of 1.28% (1/78).

PCR confirmation of *E. coli* O157:H7 strain

In the PCR assay, four reference strains of *E. coli* O157:H7 were used as positive controls. The size of PCR products amplified from the *rfb* gene, the *ficC* gene, the *eaeA* gene, the *hlyA* gene, the *vt* I gene, and the *vt* II gene were 420 bp, 625 bp, 384 bp, 534 bp, 475 bp, and 826 bp, respectively (Fig. 2-5). *E. coli* O157:H7 isolates from Hualien herd 1 and Hualien herd 8 gave PCR products of the same size as the reference strains for the *rfb* gene, the *ficC* gene, the *eaeA* gene, and the *hlyA* gene. The three *vt* II positive strains from the Hualien herd 1 gave a band of 862 bp when amplified from the *vt* II gene. The remaining *E. coli* O157:H7 strain which produce neither form of VT gave no PCR product when amplified for either *vt* I or *vt* II.

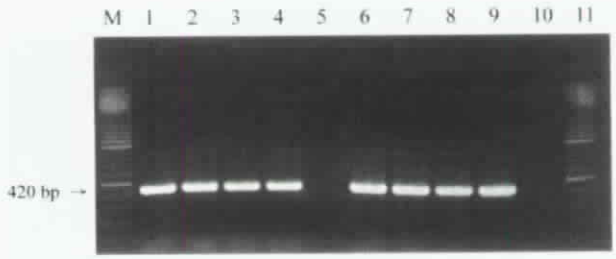

Fig. 2. *rfb* gene products by PCR amplification. M = markers of 100 bp DNA ladder; 1 = ATCC 43895; 2 = ATCC 43890; 3 = ATCC 43889; 4 = ATCC 43888; 5 = negative control (*E. coli* JM109) 6 = strain from herd Hualien 1-1; 7 = strain from herd Hualien 1-2; 8 = strain from herd Hualien 1-3; 9 = strain from herd Hualien 8; 10 = negative control: (distilled water); 11 = markers of 100 bp DNA ladder.

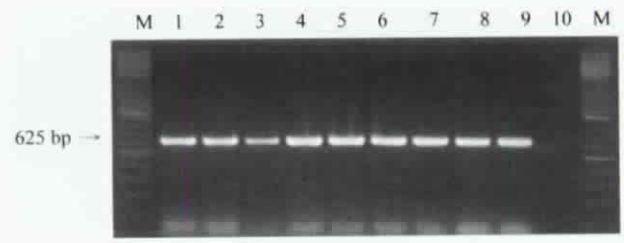

Fig. 3. *ficC* gene products by PCR amplification. M = markers of 100 bp DNA ladder; 1 = ATCC 43895; 2 = ATCC 43890; 3 = ATCC 43889; 4 = ATCC 43888; 5 = Y350-1 (An *E. coli* O157 : H7 strain isolated from a southern beef herd in Taiwan); 6 = strain from herd Hualien 1-1; 7 = strain from herd Hualien 1-2; 8 = strain from herd Hualien 1-3; 9 = strain from herd Hualien 8; 10 = negative control: (distilled water).

Discussion

The detection procedures for *E. coli* O157:H7 that are customarily used for contaminated foods or infected patients are not very suitable for the screening of bovine feces. There are known to be far fewer infectious bacteria present in food samples [6,10,25]. The fecal specimens from patients infected with *E. coli* O157:H7 are enriched in the human gastrointestinal tract, thus making them easier to identify. Some procedures for the screening *E. coli* O157:H7 from bovine feces has been published [9,11,12,26,28], but it was necessary to determine the reliability of these procedures and to modify the methods to ensure they are suitable for this study. A screening procedure for *E. coli* O157:H7 from bovine feces was established by current study using mTSB enrichment and CT-sMAC for selective

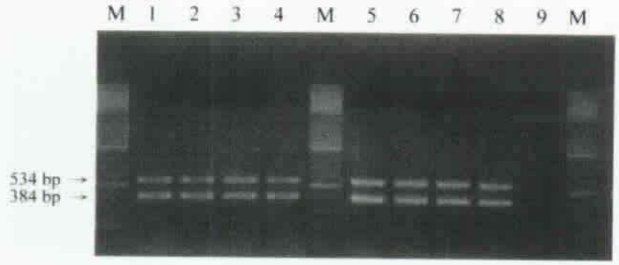

Fig. 4. *eaeA* and *hlyA* genes products by PCR amplification. M = markers of 100 bp DNA ladder; 1 = ATCC 43895; 2 = ATCC 43890; 3 = ATCC 43889; 4 = ATCC 43888, M: 100 bp DNA marker; 5 = strain from herd Hualien 1-1; 6 = strain from herd Hualien 1-2; 7 = strain from herd Hualien 1-3; 8 = strain from herd Hualien 8; 9 = negative control (distilled water).

Fig. 5. *vt* I and *vt* II gene products by PCR amplification. M = markers of 100 bp DNA ladder; 1 = ATCC 43895; 2 = ATCC 43890; 3 = ATCC 43889; 4 = ATCC 43888; 5 = negative control (distilled water); 6 = strain from herd Hualien 1-1; 7 = strain from herd Hualien 1-2; 8 = strain from herd Hualien 1-3; 9 = negative control (distilled water).

screening. Although mTSB was not as effective as GN at enrichment of total bacteria within the specimens, its ability to enrich for *E. coli* O157:H7 was superior at a detection limit of 0.85 ±0.50 cfu/g and about 100-fold greater than that derived from directly culturing on CT-sMAC. To avoid problems associated with high dilution factors, BFEs or so-called "contaminated samples" were used to establish the protocols [31]. The specimens were collected on a single day in enrichment broth and were incubated directly by pooled sample and this reduced the time needed for specimen preparation.

Antiserum agglutination tests were used to serotype O157 and H7. The slide agglutination of O157 antigen gave results within 1 min, but the tube agglutination of H7 antigen takes 2 to 3 days (including the inoculation period). Although the reliability of these tests is adequate, it is not perfect and false agglutination reactions for both the O157 and the H7 antigens were inevitable [16,20]. Thus, PCR was used to specifically amplify the target genes of VTs for final confirmation and it was relatively speedy (± 5 h).

Using PCR as a final confirmation, we concluded that only four isolated strains were *E. coli* O157:H7, based on the presence of the associated pathogenic genes, *eaeA* and *hlyA*. Although the PCR cycle programs differ for the various genes and the published procedures [11,16,20,24], using a multiplex PCR method would be a major improvement to this protocol and allow the profile to be asserted in a single test [17,25,27].

The use of pooled samples in this screening procedure permitted more efficient detection [27,30], and reduced the time and work needed in current procedure. Three specimens were pooled although some researchers have pooled up to 10 specimens [30], and to avoid false negative, three replicates on CT-sMAC were plated.

Seventy-eight dairy herds were sampled to provide a reasonable statistic estimation within these areas and *E. coli* O157 was rarely found in the current study. Only three *vt* II-producing strains were identified in the Hualien dairy herds and none elsewhere. It can be suggested that this could be due to poor feed sanitation. The current results indicate that the dairy herds in northern Taiwan areas are not seriously infected.

This first published report on *E. coli* O157:H7 in Taiwan showed a low prevalence of bovine shedding of these organisms. It is not surprising that such a low prevalence of bovine shedding has been reported elsewhere [30,33]. Even though *E. coli* O157:H7 was not found very frequently, the 0.13% estimate is probably lower than the true prevalence. Firstly, the bacterial concentrations in bovine fecal specimens would be too low to detect as the level of *E. coli* O157:H7 in a reservoir animal is an unknown quantity, and this would give rise to false negatives [27-29]. Secondly, seasonal variation is recognized as an important factor. *E. coli* O157:H7 in bovine feces has been shown to be more frequent during summer months, suggesting that ecological and management factors may influence cattle shedding [29]. This seasonal variation in *E. coli* O157 shedding could influence detection in some herds, which were sampled during the cooler months. The seasonal variation in *E. coli* O157:H7 excretion by cattle seems to parallel the seasonal variation in the sporadic cases of human *E. coli* O157:H7 infection.

One further useful approach would be to compare the *E. coli* O157 to similar strains found elsewhere over the world for epidemiological and evolutionary purposes. Pulsed-field gel electrophoresis is one technique which can be used for such a purpose [17].

Even though the current *E. coli* O157:H7 screening procedure have been applied successfully to bovine fecal specimens, other procedures might increase the

sensitivity of screening but it is critical that the VTEC screening program be done at the original source of infection such as the bovine reservoir and not limited to contaminated foods or already infected patients. Such efforts are too late. Prior screening for *E. coli* O157:H7 at the animal level is an immediate step that will reduce VTEC food poisoning.

Acknowledgment

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