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Molecular Subtyping for *Escherichia coli* O157:H7 Isolated in Taiwan

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***Abstract*: Enterohaemorrhagic *Escherichia coli* O157:H7 is an important pathogen these days. Outbreaks of its infection have been reported all over the world, in Australia, Canada, Japan, the United States, south Africa, and various countries in Europe. In the summer of 2001, the first clinical infection by *E. coli* O157:H7 was identified in Taiwan. In this study, the standard procedures for molecular subtyping were applied to several strains collected in Taiwan as well as from elsewhere. The two molecular subtyping methods we used are pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). The isolates from the U.S.A., Canada, Japan, and Taiwan each showed a unique molecular fingerprinting pattern. The environmental strains isolated in Taiwan showed closer relationships with each other, and their similarity was in the range of 75–85%. The first clinical strain isolated in Taiwan in 2001 was similar to the strains from North America but not closely related to the Taiwanese environmental strains. Our surveys showed that some local *E. coli* O157:H7 strains did exist in Taiwan, but there had been only one official case report of the infection by local *E. coli* O157:H7. The eating habits of the people and the geographic distribution of the pathogen are considered crucial risk factors in Taiwan. The establishment of a database of our own and joining the global network database are important tasks if we want to control such agricultural and food-borne pathogens, and reduce the number of victims and amount sufferings, as well as the economic losses due to the infection.**

***Key words*: *Escherichia coli* O157:H7, Pulsed-field gel electrophoresis (PFGE), Amplified fragment length polymorphism (AFLP), Antimicrobial resistance**

There are more than 200 diseases which are transmitted through food (10). The cause of a food-borne illness can be a bacterium, virus, parasite, toxin, or metal. In the United States, food-borne diseases cause an estimated 6 to 81 million cases of infection, resulting in around 9,000 deaths each year (2, 3, 8, 20, 29). Between 1986 and 1995, a total of 852 outbreaks of food-borne diseases involving 26,173 cases, and resulting in 20 deaths was reported in Taiwan. Among the 852 reported outbreaks, 555 (65%) were caused by bacterial pathogens (24). Today enterohaemorrhagic *Escherichia coli* O157:H7 has become a pathogen of global concern. This strain, first recognized as a human pathogen in 1982, is now known to cause haemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (16, 25). Outbreaks of enterohaemorrhagic *E. coli* O157:H7 infection have occurred mostly as the result of the consumption of contaminated hamburgers in some fast-food restaurants in the Pacific Northwest of the United States (7). Other outbreaks of *E. coli* O157:H7 infection have been attributed to a variety of sources, including ground beef, milk, alfalfa sprouts, apple cider (9, 15, 21, 34), and water (11, 19, 30).

In the years after the discovery of this pathogen, *E. coli* O157:H7 has become increasingly prominent, and outbreaks have been reported in Australia, Canada, Japan, the United States, South Africa, and various

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Abbreviations: AFLP, amplified fragment length polymorphism; CDC, Centers for Disease Control and Prevention; dATP, deoxyadenosine 5′-triphosphate; dCTP, deoxycytidine 5′-triphosphate; dGTP, deoxyguanosine 5′-triphosphate; DNA, deoxyribonucleic acid; dTTP, deoxythymidine 5′-triphosphate; MIC, minimal inhibition concentration; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RAPD, random amplified polymorphic DNA; *stx*1, Shiga-like toxin 1; *stx*2, Shiga-like toxin 2; TE buffer, Tris-EDTA buffer; TSB, tryptic soy broth; UPGMA, unweighted pair-group method using arithmetic average.

countries in Europe. Today it is causing an estimated 20,000 illnesses and 250 deaths annually in the United States (1, 17). Japan had three major outbreaks in 1996, which resulted in more than 17,000 sick people and 13 deaths. In Taiwan, the infection of *E. coli* O157:H7 was made a statutory communicable disease in 1996, but no human cases were reported until 2001. In the summer of 2001, a 6-year-old boy who had come back from the United States about 6 weeks earlier, suddenly became ill and displayed symptoms that included bloody diarrhea, acute enterocolitis, acute renal failure, and suspected hemolytic uremic syndrome. The *E. coli* colonies were cultivated and isolated, and the serotypes and verotoxin were confirmed by our laboratory. Evidently the illness was induced by *E. coli* O157:H7, and we found that this particular infection was related to the infection in North America (32). In a previous study, we isolated and verified *E. coli* O157:H7 in specimens from dairy herds (18) and clinical cases (32). We also collected some clinical strains of *E. coli* O157:H7 isolated in the U.S.A., Canada, and Japan from patients. For epidemiological purposes, we, for the first time in Taiwan, applied the standard procedures for molecular subtyping, including pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) to examine *E. coli* O157:H7 strains isolated in Taiwan. Their molecular fingerprints and those of some foreign strains were compared by us to trace the source of infection in Taiwan.

Materials and Methods

Bacterial strains and DNA preparation. A total of 36 isolates originally from the U.S.A., Canada, Japan, and Taiwan were used for analysis (Table 1). A strain called *E. coli* O157:H7 ATCC43895 served in this study as the reference strain. Before testing, the bacterial strains were activated from the frozen storage and grown on a nutrient broth (NB; Difco, Detroit, Mich., U.S.A.) at 37 C for 18–24 hr. The cultures were then transferred to a tryptic soy broth (TSB; Difco) and incubated at 37 C until the optical density at 600 nm reached 1.2–1.4. Cells were subsequently harvested and used for DNA preparation, which was performed using a Wizard Genomic DNA purification kit (Promega Co., Madison, Wis., U.S.A) and following the exact procedures specified in the manufacturer's instructions.

Gene detection. Detection of selected genes in the isolates and the strains collected from abroad was performed by a multiplex polymerase chain reaction (PCR) whose primers were designed previously by our laboratory (23). The multiplex PCR was performed in a 0.2 ml eppendorf tube on a PCR Express™ thermal cycler (Thermo Electron Co., Ashford, U.K.). The DNA template was added to a 25-µl reaction mixture containing 0.2 mM each of dATP, dCTP, dTTP, and dGTP (Protech Technology, Taipei, Taiwan). A buffer solution was made up of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$; 1 µl PCR primers (Purigo, Taipei, Taiwan) containing *rfb* (O157), *fli*C (H7), *stx*1, and *stx*2 gene primer; and 2.5 U *Taq* polymerase (TaKaRa Bio, Inc., Otsu, Shiga, Japan). The PCR program was carried out according to our published paper (23) and the products were separated by agarose gel electrophoresis. The gel was stained with ethidium bromide, destained, visualized by exposing the gel to UV light, and then photographed.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis was performed with modification of the method of Barrett et al. (6). In brief, strains were grown in TSB (Difco) at 37 C to an optical density of 1.4–1.5 at 610 nm. Cells were washed twice in a sodium EDTA buffer (5 mM NaCl, 5 mM EDTA) and then re-suspended. The bacterial cell suspension was mixed with an equal volume of melted 0.9% Seakem® Gold agarose (Rockland, Me., U.S.A.), which was suspended in a Tris-EDTA buffer (10 nM Tris base, 1 mM EDTA, pH 8.0) containing 0.5 mg proteinase K (QIAGEN Gmbh, Germany). This mixture was then dispensed into 1.5-mm-thick block molds (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). After solidification, the plugs were transferred into a lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 1% *N*-laurylsarcosine, and 1 mg/ml proteinase K), and incubated at 54 C for 1 hr. The plugs were washed 4 times for 15 min each in a TE buffer. Four restriction enzymes, *Xba*I, *Not*I, *Sfi*I, and *Avr*II (New England Biolabs, Beverly, Mass., U.S.A.) were used for typing these strains. The enzyme concentration, buffer selection, and incubation temperature were recommended by the manufacturer for a 4-hr digestion of agarose-embedded DNA. Restriction fragments were separated by electrophoresis through a 1% PFGE agarose (Bio-Rad Laboratories) in a 0.5× Tris-borate-EDTA buffer at 14 C in a CHEF DR-II apparatus (Bio-Rad Laboratories) for 23 hr at a constant voltage rate of 6 volt/cm. Pulse times were ramped at 2.5 sec at the beginning and at 56.6 sec at the end. After PFGE, the gel was stained with ethidium bromide (0.2 µg/ml) and photographed under UV transillumination by Kodak Electrophoresis Documentation and Analysis System 290 (Kodak, Calif., U.S.A.).

Amplified fragment length polymorphism. Amplified fragment length polymorphism was examined by a modified version of the methods described in previous studies (5, 13, 31). In brief, the restriction-ligation

Table 1. *E. coli* O157:H7 strains used in this study

Strain	Toxin produced	Source			PFGE pattern	AFLP pattern
		Year	Location	Isolation origin		
TWE01	SLT II	1998	Pingdong, southern Taiwan	Cattle feces	P1	A1
TWE02	SLT II	2000	Hualien, eastern Taiwan	Cattle feces	P2	A2
TWE03	none	2000	Hualien, eastern Taiwan	Cattle feces	P2	A3
TWC01	SLT I, II	2001	Taipei, northern Taiwan	Human feces	P3	A4
TWE04	SLT II	2004	Taoyuan, northern Taiwan	Cattle feces	P4	A5
TWE05	SLT II	2004	Hualien, eastern Taiwan	Cattle feces	P5	A6
AME01	SLT I, II	NR	U.S.A.	Human feces	P6	A7
AME02	SLT I, II	NR	U.S.A.	Human feces	P7	A8
AME03	SLT II	NR	U.S.A.	Human feces	P8	A9
AME04	SLT I, II	NR	U.S.A.	Human feces	P9	A10
AME05	SLT I, II	1999	U.S.A.	Human feces	P10	A11
AME06	SLT II	1999	U.S.A.	Human feces	P11	A12
AME07	SLT I	1986	U.S.A.	Human feces	P12	A13
AME08	SLT I, II	1983	U.S.A.	Human feces	P13	A10
AME09	SLT I, II	1982	U.S.A.	Food	P13	A4
CAN01	SLT I, II	NR	Canada	Human feces	P14	A10
CAN02	SLT I, II	NR	Canada	Human feces	P15	A14
CAN03	SLT I	NR	Canada	Human feces	P16	A15
CAN04	SLT I, II	NR	Canada	Human feces	P17	A10
CAN05	SLT I	NR	Canada	Human feces	P18	A16
CAN06	SLT II	NR	Canada	Human feces	P19	A17
CAN07	SLT I	NR	Canada	Human feces	P20	A15
CAN08	SLT I, II	NR	Canada	Human feces	P17	A10
CAN09	SLT I, II	NR	Canada	Human feces	P21	A10
CAN10	SLT I, II	NR	Canada	Human feces	P22	A18
CAN11	SLT II	NR	Canada	Human feces	P23	A19
CAN12	SLT I, II	NR	Canada	Human feces	P24	A10
JPN01	SLT I, II	1996	Japan	Human feces	P25	A20
JPN02	SLT II	1996	Japan	Human feces	P26	A21
JPN03	SLT I, II	1996	Japan	Human feces	P25	A10
JPN04	SLT I, II	1996	Japan	Human feces	P25	A10
JPN05	SLT I, II	1996	Japan	Human feces	P27	A22
JPN06	SLT I, II	1996	Japan	Human feces	P27	A23
JPN07	SLT II	1996	Japan	Human feces	P26	A24
JPN08	SLT I, II	1996	Japan	Human feces	P28	A25
JPN09	SLT I, II	1996	Japan	Human feces	P29	A26

NR: No report.

reactions were performed for 3 hr in a total volume of 10 µl, which consisted of 10 ng genomic DNA, 5 U of *Eco*RI or *Pst*I, 1 U of *Mse*I (New England Biolabs), 1 U of T4 DNA ligase (Protech Technology), ligase buffer (33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol) and 0.2 µg of each adaptor (as shown in Table 2). The restriction-ligation products were diluted 10-fold as the PCR template and the reaction consisted of 200 µM dNTP, 1 µM each primer (shown in Table 2), 1 U of *Taq* polymerase (TaKaRa Bio, Inc.) and PCR reaction buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$). This reaction was performed in the PCR Express™ (Thermo Electron Co.) for the pre-amplified reaction, and the touch down PCR conditions were used. These conditions were 10 cycles at 94 C for 20 sec, touch down temperature from 66 C to 56 C for 30 sec and 72 C for 2 min, and 20 cycles at 94 C for 20 sec, 56 C for 30 sec and 72 C for 2 min. After these conditions were met, 60 C for 30 min and then storage

Table 2. The oligonucleotides used in this study to perform the AFLP

	Oligonucleotide name	Sequence
*Mse*I adapter	MAD1	5′-GACGATGAGTCCTGAG-3′
	MAD2	3′-TACTCAGGACTCAT-5′
*Eco*RI adapter	EAD1	5′-CTCGTAGACTGCGTACC-3′
	EAD2	3′-CTGACGCATGGTTAA-5′
*Pst*I adapter	PAD1	5′-CTCGTAGACTGCGTACATGCA-3′
	PAD2	3′-CATCTGACGCATGT-5′
*Mse*I primer	MSEP	5′-GATGAGTCCTGAGTAA-3′
*Eco*RI primer	ECOP	5′-GACTGCGTACCAATTC*-3′
*Pst*I primer	PSTP	5′-GACTGCGTACATGCAN*-3′

*Selected extension nucleotide.

at 4 C were carried out. The PCR products were diluted from 10- to 20-fold as the selective PCR templates. Then the selective PCR was carried out with one of the primer pairs *Mse*I+C/*Eco*RI+0, *Mse*I+G/*Eco*RI+G, *Mse*I+G/*Pst*I+G, and *Mse*I+G/*Pst*I+C, which were screened in 50 different combinations. The reaction consisted of 200 μM dNTP, 0.2 μM for *Mse*I+X selective primer, 0.04 μM for *Eco*RI+X or *Pst*I+X selective primer, 1 U of *Taq* polymerase (TaKaRa Bio, Inc.) and PCR reaction buffer (100 mM Tris-HCl, pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$. This reaction was performed in the PCR Express (Thermo Electron Co.) for selective amplified reaction. The PCR conditions were as follows: 33 cycles at 94 C for 1 min, 60 C for 1 min, and 72 C for 2.5 min. The PCR products were taken out for capillary electrophoresis by sequence analyzer (3100, Applied Biosystems, Foster City, Calif., U.S.A.) and Genescan TAMRA-500 (Applied Biosystems) was added as an internal standard in each sample.

Dendrograms for cluster analysis. Dendrograms for cluster analysis were performed by Bio-Profil® Image Analysis Software (Vilber Lourmat Co., Marne La Vallee, France) with the Pearson correlation coefficient and UPGMA (unweighted pair-group method using arithmetic average) algorithm.

Antimicrobial resistance analysis by MIC. In this study, we performed the antimicrobial resistance analysis by the ETEST® (AB Biodisk, Sweden) method and used 10 different antibiotics to analyze the antimicrobial resistance of these local isolates. The strains were cultured and harvested at 0.5 MacFarland (1.5×10^8 CFU/ml). The cell suspension was spread on a Mueller-Hinton agar plate (Difco) and the ETEST® strip was then pasted on it. After incubation for 24 hr at 37 C, the zone of the point of complete inhibition of growth according to the National Committee for Clinical Laboratory Standards guidelines was used to determine the minimum inhibitory concentration (MIC) value.

Results

Characterization and Determination of E. coli *O157:H7*

Characterization and determination of *E. coli* O157:H7 were performed prior to the molecular typing analysis. The results of the examination of Shiga-like toxin type and the source are shown for each strain in Table 1.

Pulsed-Field Gel Electrophoresis

Four restriction enzymes were employed for the analysis of the genomic DNA of a selected strain. The result indicated that the *Xba*I and *Avr*II had the best efficiency and ability to analyze genomic DNA (data not shown). In this study, we therefore used the *Xba*I and *Avr*II to examine the molecular type of the 36 test strains of *E. coli* O157:H7 isolated in Taiwan and other countries (Table 1). There were 36 strains which were restricted for 15–21 bands by *Xba*I (data not shown) and 29 different *Xba*I-digested fingerprint patterns detected by PFGE among the 36 tested strains. The comparison of the PFGE patterns of *Avr*II- or *Xba*I-digested DNA from the strains isolated from dairy herds (TWE01–TWE05) and a patient (TWC01) in Taiwan is shown in Fig. 1. Dendrogram analysis of these patterns (Fig. 2, A and B) shows that these environmental strains are quite similar. In particular, TWE02 and TWE03 are indistinguishable. The clinical strain, TWC01, however, had significantly different patterns from those of the environmental isolates, and there was only about 65% similarity between them (Fig. 2, A and B). As shown in Fig. 3, the similarity between isolates from Japan and the environmental strains from Taiwan, TWE01–TWE05, was about 60–80%. The environmental strains isolated in Taiwan were the closest to the two Japanese isolates, JPN02 and JPN07, and their similarity was 70%. On the other hand, one of the clinical strains isolated in Taiwan, TWC01, was significantly different from the isolates from Japan. The strains isolated from Canada and the United States had signifi-

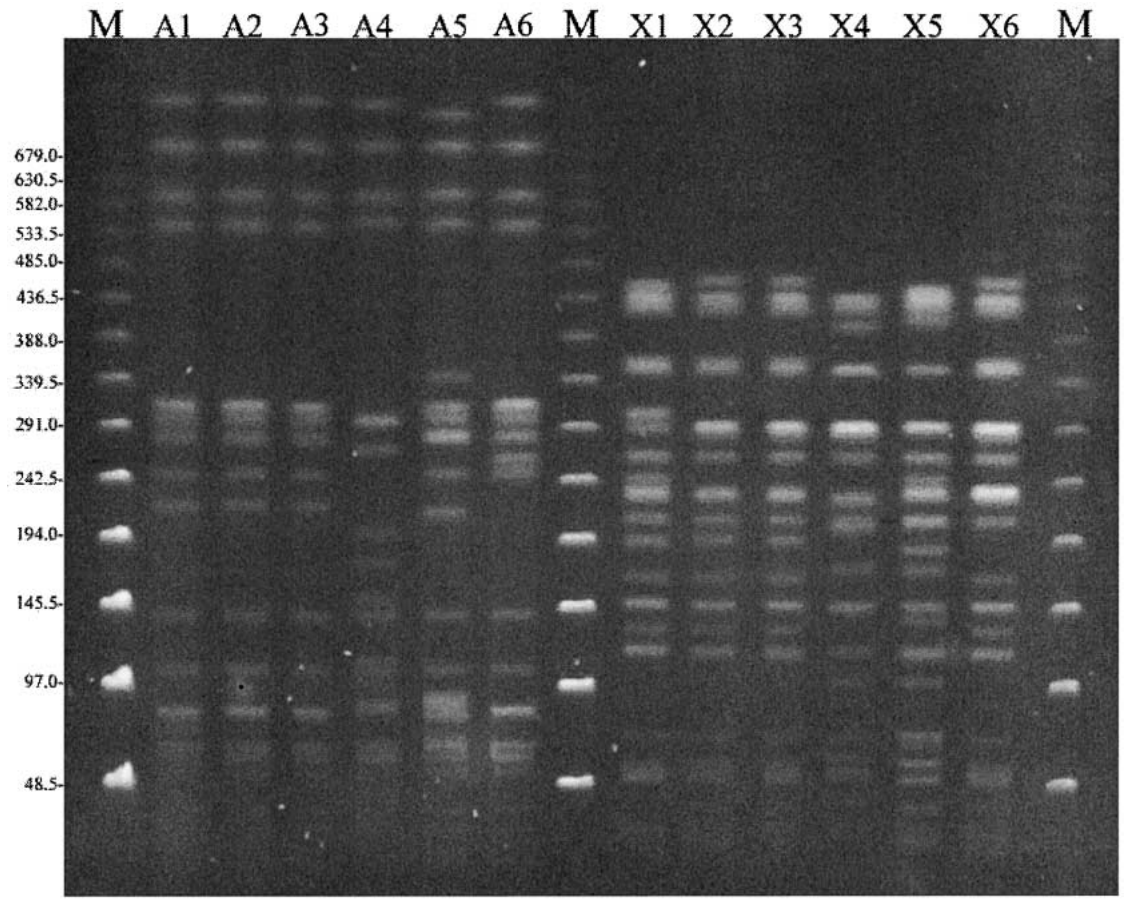

Fig. 1. PFGE patterns of *E. coli* O157:H7 strains isolated in Taiwan. Lane M is a Lambda ladder marker. Lane A1, A2, A3, A5, and A6 are TWE01–TWE05 and Lane A4 is TWC01 using *Avr*II as the restriction enzyme. Lane X1, X2, X3, X5, and X6 are TWE01–TWE05 and lane X4 is TWC01 using *Xba*I as the restriction enzyme.

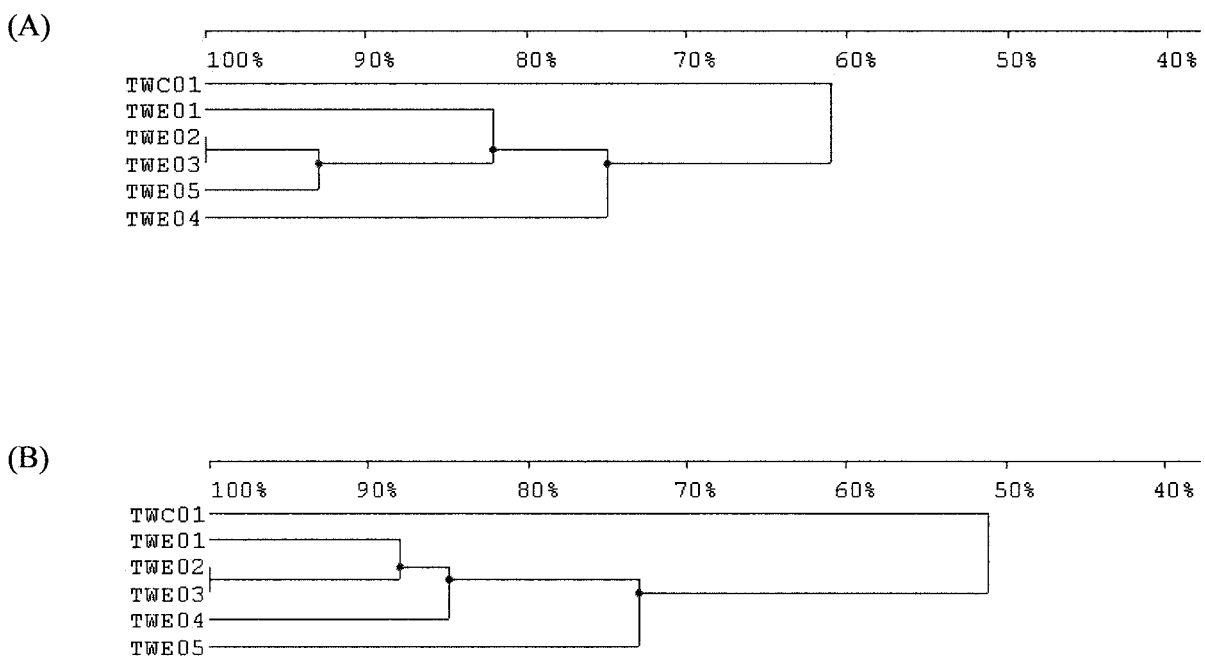

Fig. 2. The percentage of relationships for *E. coli* O157:H7 strains isolated in Taiwan by using the PFGE method with either (A) *Xba*I or (B) *Avr*II as the restriction enzyme.

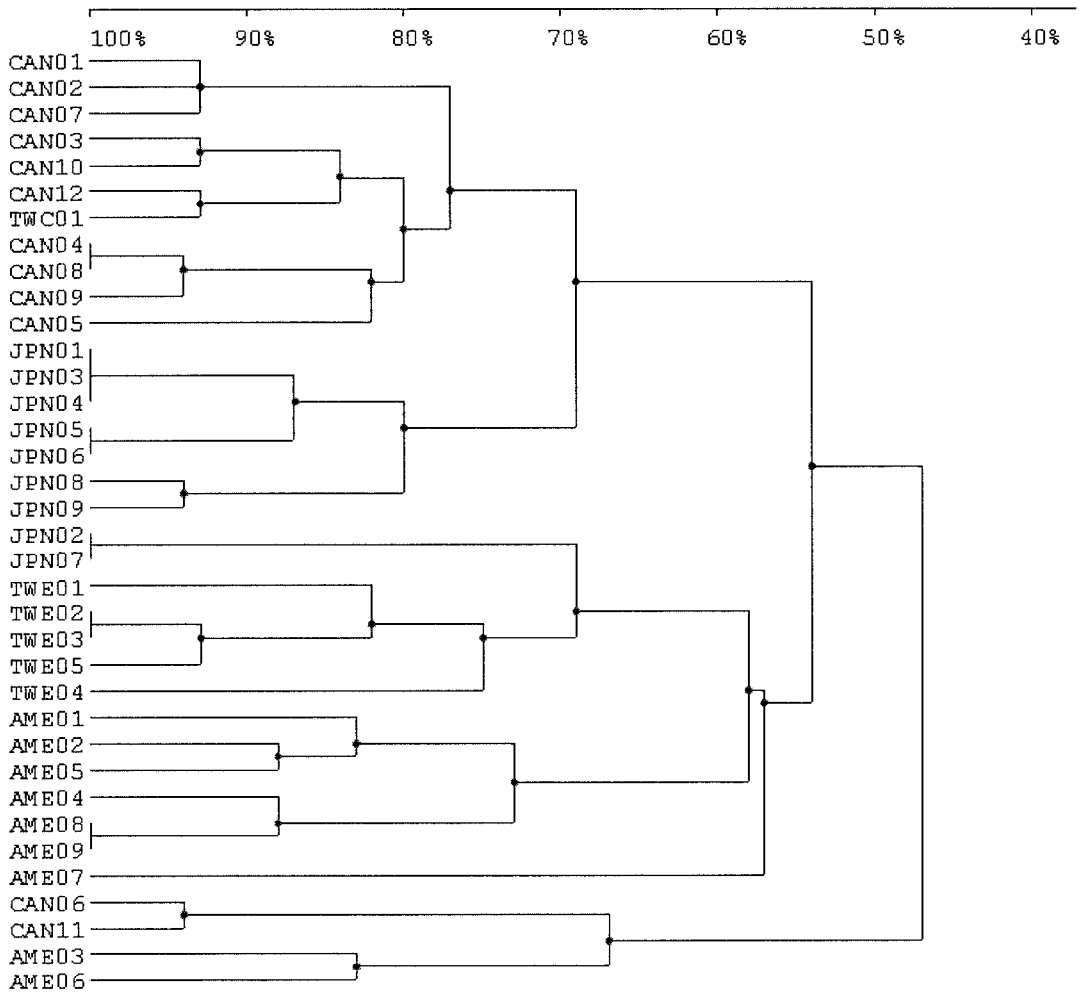

Fig. 3. The percentage of relationships for *E. coli* O157:H7 in this study by using the PFGE method with *Xba*I as the restriction enzyme.

cantly different PFGE patterns from the environmental strains from Taiwan (Fig. 3), especially from those isolated in the southern and eastern parts of Taiwan. However, the TWC01 had more similarity with the strain from Canada, CAN12. Accordingly, the environmental isolates from Taiwan are similar to each other and are altogether significantly different from those foreign strains. The isolate from the first infection case in Taiwan had a unique genotype among the isolates from Taiwan, but is similar to the strains isolated from Canada.

Amplified Fragment Length Polymorphism Analysis

AFLP analysis was performed with 2 sets of enzyme combinations (*Mse*I/*Eco*RI and *Mse*I/*Pst*I) and yielded amplified fragments ranging in size from 30 to 600 bp. Fifty different combinations were screened, and the selective primer pair combinations, *Mse*I+C/*Eco*RI+0, *Mse*I+G/*Eco*RI+G, *Mse*I+G/*Pst*I+G, and *Mse*I+G/*Pst*I+C, were performed to type these strains. There were 50–64 polymorphism fragments in these analyses, which were compared with the results of PFGE. The combination of selective primer pairs, *Mse*I+G/*Eco*RI+G, had very good resolution to illustrate the relationships between these isolated strains. Twenty-six different AFLP patterns were identified among the 36 strains. As shown in the results in Fig. 4, there was a similarity of about 80–90% among the environmental isolates from Taiwan, and the TWE02 and TWE03 showed different patterns only when analyzed by AFLP. The clinical strain, TWC01, had a fairly distant relationship with other environmental isolates from Taiwan with the similarity being just 50–60%. We also found that TWC01 had high similarities with the isolates from the United States and Canada. The results of the AFLP analysis are therefore similar to those of the PFGE analysis, and both methods have good resolutions for use in the epidemiology investigation.

Antimicrobial Resistance Analysis

Ten antibiotics, including those used for clinical and veterinary purposes in Taiwan, were selected to evaluate

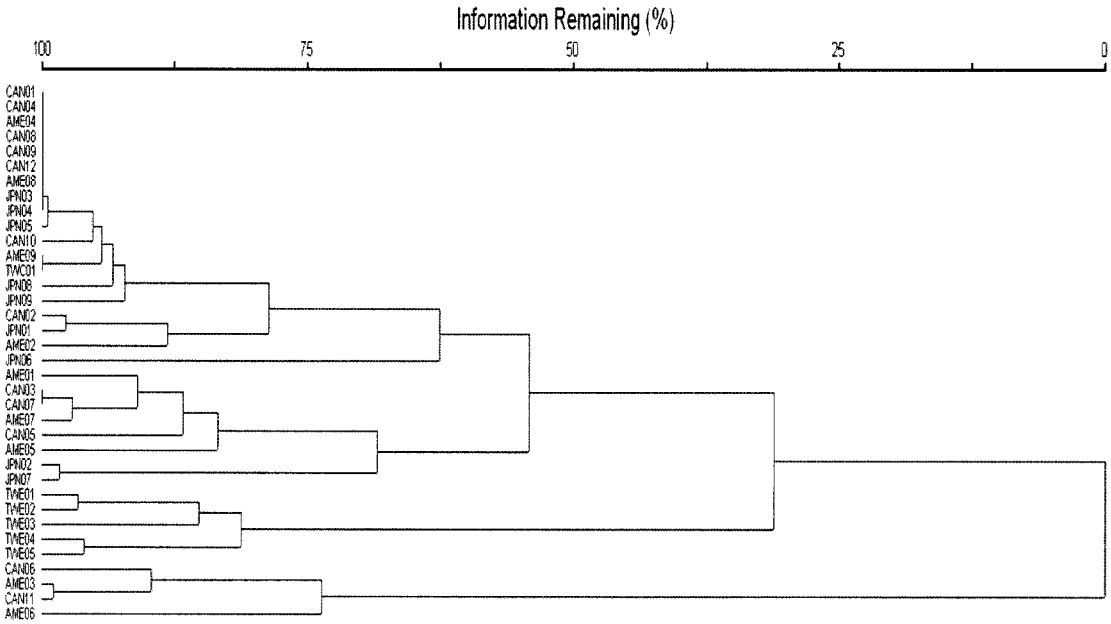

Fig. 4. The percentage of relationships for *E. coli* O157:H7 in this study by using the AFLP method.

Table 3. The results of minimum inhibitory concentration in *E. coli* O157:H7 isolated in Taiwan

	Strains					
Antibiotics	TWE01	TWE02	TWE03	TWC01	TWE04	TWE05
Ampicillin	2[a)]	4	4	3	>256	4
Tetracycline	2	3	2	3	>256	3
Chloramphenicol	3	3	3	3	>256	3
Sulfamethoxazole	8	16	24	6	>256	8
Ceftriaxone	0.064	0.094	0.064	0.064	0.064	0.064
Ceftazidine	0.25	0.25	0.25	0.25	0.25	0.25
Cefepime	0.064	0.064	0.047	0.047	0.047	0.047
Nalidixic acid	3	4	4	4	4	4
Ciprofloxacin	0.023	0.023	0.023	0.023	0.023	0.023
Sparfloxacin	0.016	0.023	0.023	0.023	0.023	0.012

[a)] Minimum inhibitory concentration (μg/ml).

the antimicrobial resistance of the isolated strains. All the environmental and clinical isolates from Taiwan tested were found sensitive to all of the 10 antibiotics except one strain isolated from Taoyuan, TWE04 (Table 3). We found that this strain was isolated from a herd of cattle that had been fed with these antibiotics when we collected the samples to isolate this strain. This information implied that this strain was resistant to these antibiotics that were associated with the therapy for mastitis.

Discussion

In this study, we used three molecular subtyping methods to distinguish the strains isolated from different areas. Since the random amplified length polymorphic DNA (RAPD) method failed in discriminating the test strains (data not shown), we employed PFGE and AFLP. The results showed that all strains isolated from the dairy herds in Taiwan are closely related. TWE02 and TWE03, which were isolated in eastern Taiwan, were indistinguishable by PFGE. Although these 2 strains had the same PFGE pattern, they were isolated from different herds and their toxin producing abilities were different from each other, and they also had different AFLP patterns. Consequently, we believe that these 2 strains are not identical, but that they are genetically similar. In the geographic distribution analysis, we found that these local environmental isolates had a better connection with the regions they came from. Strains TWE02, TWE03, and TWE05 were all isolated from eastern Taiwan, and these strains had more than 80% similarity among them. TWE01, which was collected from southern Taiwan, also had about 80% similarity

with the strains from eastern and northern Taiwan. The isolates from the environment of Taiwan were highly related to one another, and these included TWE04, which was isolated from Taoyuan in northern Taiwan. The similarity among them was on an average somewhere between 75–85%. However, TWE04 had a unique characteristic in that it is resistant to more than four antibiotics. Schroeder et al. (27) indicated that approximately 50% of the 534 isolates from animals were resistant to sulfamethoxazole, tetracycline, or streptomycin. They also found and suggested that antimicrobial resistance was widespread among *E. coli* inhabiting humans and animals (27). Except for TWE04, Taiwanese environmental isolates were sensitive to most of the antibiotics such as ampicillin, tetracycline, ceftriaxone, or cefepime. Based on information obtained from the dairy herd owner, we discovered that the herd, from which the antibiotic-resistant strain TWE04 was isolated, had been medicated to treat a dairy cattle disease, prior to our collecting the feces samples as well as the environmental samples. It is reasonable to conjecture that the high rates of resistance were related to the antibiotic treatment of the cattle. Since it has a high probability of producing an antimicrobial-resistant strains, the use of antibiotics in clinical and agriculture medicine apparently becomes an important issue. Samples need to be selected at the right moment, and its use should be avoided if possible. The genotype of TWC01, the clinical strain of the first infectious case by *E. coli* O157:H7 in Taiwan, was not similar to the other environmental strains isolated from Taiwan. However, the results of the PFGE and AFLP showed that this strain was of the same cluster and similar to the strains isolated from Canada and the United States. Because the patient was a 6-year-old boy who had just returned from the United States this clinical isolate was compared with the databank in the Centers for Disease Control and Prevention, U.S.A., in a previously published paper, and it showed that this strain had a 100% similarity with an outbreak strain which was isolated from the U.S.A. (32). In conclusion, this clinical strain is distantly related to the Taiwanese strains, and is much closer to the North American strains instead. The results suggest that this clinical infection was not caused by the strains existing in Taiwanese environment.

Japan, a country in close proximity to Taiwan, had a number of serious infections of *E. coli* O157:H7 in the summer of 1996. The *E. coli* O157:H7 infections were prevalent in Canada and the U.S.A. and they have a high level of international trade with us. Taiwan has a similar level of international trade with Japan. It is for these reasons that we must do our best to monitor the infection of *E. coli* O157:H7 by comparing the molecular fingerprinting of the outbreaks happening in Taiwan with strains isolated in these countries. The results of the analysis of the fingerprinting patterns showed that the geographical distribution of the isolates did have a relationship with the genotypes. Therefore we could use these genotypes as a basis to explain the sources of infections. Davis et al. indicated that the *E. coli* O157:H7 strains could be transferred over global distances with considerable frequency (12).

PFGE is one of the best molecular typing methods for analyzing the differences between closely related organisms. In epidemiological studies, this technique is applied frequently to investigate the epidemiology of outbreaks. In 1996, at the Centers for Disease Control and Prevention (CDC), U.S.A., they constructed the "PulseNet" due to a large outbreak of food-borne illness caused by the bacterium, *E. coli* O157:H7 (28). This outbreak occurred in the western United States, and the scientists at CDC performed PFGE to characterize clinical and food isolates of *E. coli* O157:H7, and they demonstrated its effectiveness in the investigation of an outbreak. About 22 countries or areas in the region where the coordination work to control and prevent food-borne outbreaks was to be carried out joined the "PulseNet" (6, 28). The amplification fragment length polymorphisms (AFLPs) are polymerase chain reaction-based markers for rapid screening of genetic diversity (22). This technique is a relatively cheap, easy, fast, and reliable method to generate hundreds of informative genetic markers (26) and has been used for subtyping the *E. coli* and *E. coli* O157:H7 (4, 14, 33). However, these molecular subtyping methods have some disadvantages, such as lengthy and complicated procedures. It is sometimes difficult to resolve the very closely related strains by PFGE and considerable polymorphism is one of the shortcomings of AFLP. The results of these subtyping methods have always shown some discrepancies and we confirmed this in our study (Figs. 3 and 4). The isolates should not be classified as epidemiologically related or as an unrelated group on the basis of one typing method. That is specifically why we used these two molecular subtyping methods to investigate the relationships between the strains isolated from Taiwan and foreign countries.

Evidence from epidemiological and molecular studies of bovine *E. coli* O157:H7 suggests that strains are frequently transmitted across wide geographic distances (12). Global traffic is quite convenient these days, and there is more and more contact among nations across the world. At the same time, agricultural, human, and food-borne diseases are also spread more easily and quickly, and it is becoming difficult to prevent and con-

trol them. Therefore, establishing molecular subtyping methods becomes a key point in controlling infections and diseases. In Taiwan, there is not any one department which has the all-inclusive capabilities to analyze food-borne pathogens for molecular subtyping. In addition, no one molecular subtyping method has been agreed upon as the official method to investigate food-borne or infectious diseases. In Taiwan, the present study is the first one to evaluate the regular methods of molecular subtyping for *E. coli* O157:H7 infection, and to compare the three kinds of subtyping methods including the random amplified polymorphic DNA method. So far, no international organization has set up an information network though it is urgently needed. At present, it remains difficult to quickly trace the source of any food-borne pathogen, especially if the infection comes from a different country.

According to the official records of the Taiwanese government, there had been no infection by *E. coli* O157:H7 in Taiwan until 2001. The infection in 2001 is a case which originated from a foreign area. However, our surveys show that the *E. coli* O157:H7 strains are actually present in the environment of Taiwan. These strains were found mainly in the eastern and southern parts of Taiwan, far away from large population centers. Travel into these areas is not convenient, and the areas have always been sparsely populated. Moreover, people in Taiwan are not interested in raw or semi-raw foods. From this we concluded that the eating habits of people, and the distribution of the pathogen were important factors. A risk for developing a serious infection by *E. coli* O157:H7 in Taiwan may exist, and the establishment of a global network database and joining the network are important tasks if we wish to control agricultural and food-borne pathogens and reduce victims and economic losses due to the infection and the spread of the pathogen. In conclusion, this study was the first to apply three kinds of molecular subtyping methods in the analysis of *E. coli* O157:H7 strains isolated from Taiwan, and to compare them with foreign strains. The results showed that the isolates from the U.S.A., Canada, Japan, and Taiwan all have molecular fingerprinting patterns unique to each geographical location. The environmental strains isolated from Taiwan were related to each other, and their similarity was about 75–80%. The first clinical isolate was similar to the strains from North America. We were able to confirm that this clinical infection was not related to the local strains. The establishment of standard molecular subtyping methods, and the creation of a global database is an important issue to prevent future infections and diseases.

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