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A Molecular Epidemiological Analysis of the First Toxin-Producing *Shigella dysentery* Type 1 Strain Isolated in Taiwan

Abstract

It was the first *Shigella dysentery* type 1 case detected in Taiwan. The case was a Japanese male visitor who entered Taiwan on 26 November, and was admitted to hospital for abdominal pain, diarrhea, fever and bloody stool. The case was reported and specimen sent to the Center for Disease Control of the Department of Health (CDC) as suspected typhoid case. By isolation, the CDC confirmed the case as *Shigella dysentery* type 1 infection (coded TA-1). Preventive measures were immediately taken. Conditions of the case are stable and no further infections have been reported. By PCR and RPLA, the strain was proved to be shiga-toxin-producing. Further comparison with the same serotype strain preserved by Japan in 1966 (coded #450) found that though both of them carried shiga-toxins, they were significantly different in drug-resistance graph and pulsed field gel electrophoresis graph patterns. It was speculated that the two strains were not molecularly related. A further analysis of the gene sequences of the shiga-like toxin (SLT) and the Gyrase A of TA-1 and #450 strains showed that the SLT gene sequences (475bp) of both strains were the same; whereas the Gyrase A gene sequences (648bp) of TA-1 showed point mutation at 185 and 197, from ser^{185} (TCG)→lys (TTG), and Asp^{197}(GAC)→Gly (GGC). This was due to the gene mutation of resistance to ciprofloxacin.

Introduction

In 1898, Dr Shiga of Japan first detected *Shigella*. It was a gram-negative bacillus of no mobility. It was later proved to be pathogenic. It is one of the *Enterobacteriaceae* family, and is of the same characteristics and closely related to *E. coli*. The genus *Shigella* is comprised of four serogroups. Group A, *Shigella dysenteriae*, has 1-13 serotypes. Of which, type 1 can produce heat-unstable enterotoxin to induce serious infection.

The major pathogenic agent of *Shigella* is the shiga-toxin. It is transcoded and translated from the shiga-like toxins (SLT) produced at the stx chromosome. Enterotoxins produced by groups B, C and D *Shigella* though infect intestine membranes, do not enter blood streams. Shiga toxins will, however, damage cell vessels of the lower parts of intestine membranes to enter the circulatory system. Studies have shown that the toxin is associated with hemolytic uremic syndrome (HUS), giant colonitis and acute renal failure[1,2]. The mechanism though is unknown, it is probably associated with the early cytotoxicity and keratocytes linkage[3].

Shigellosis is a Category II Group B notifiable communicable disease in Taiwan. Cases must be reported and isolated. No cases of *Shigella dysentery* type 1 infection have been reported in Taiwan since 1950. This was the first imported case in 50 years, and therefore, received serious attention from the health and disease control authorities. In addition to the isolation treatment by the medical care institution and the disease control measures taken by the disease control authorities, the CDC conducted further analysis of the shiga-toxin producing and molecular characteristics of the strain.

Materials and Method

Symptoms of Patient and Sources of Specimen

The case, a 25-year-old Japanese male, was admitted to hospital on 27 November for abdominal pain, diarrhea and bloody stool. The patient visited India a month before. The case was reported as a suspected typhoid case and specimen was sent to the CDC for laboratory testing. The CDC later confirmed that the case was a *Shigella dysentery* type 1 infection. Authorities concerned were immediately notified to take necessary action.

Isolation and Assessment of Strain

Fecal specimen of the patient was cultured with SS agar, XLD and MacConkey agar. Suspected colonies were picked up and inoculated on TSI, Lys and SIM media. The results were K/A, Lys(-), without H_2S and gas, urea(-), and no mobility. They corresponded to the characteristics of *Shigella*. *Shigella*-specific antiserum was then applied to determine its serotype. VITEK system was used to further confirm that it was *Shigella dysentery* type 1 (coded TA-1). The *Shigella dysentery* type 1 strain preserved in 1966 (coded #450, 59536WEW1) was then incubated and activated for comparative analysis.

Determination of Shiga-Toxin

VET-RPLA assessment kit (Seiken) was used to determine if the strain was Shiga-toxin producing. SLT-I primer (20bp) was used for the polymerase testing of the genes of the shiga-like toxins to produce products of 475bp and to determine the existence of toxin genes.

1) RPLA (reversed passive latex agglutination)

The VET-RPLA assessment kit (Denka Seiken Co., Ltd.) for heat labile enterotoxin of *E. coli* was used to determine by the standard operational

procedures of the kit the Shiga-toxin reactions of the TA-1 and #450 strains.

2) PCR (polymerase chain reaction)

(1) selection of primers

Primer: LT-IF: 5'-CAG TTA ATG TGG TGG CGA AG-3'

Primer: LT-IIF: 5'-CTT CGG TAT CCT ATT CCC GG-3'

LT-IIR: 5'-CGC TGC AGC TGT ATT ACT TTC-3'

Producing products of 862bp.

Primer: GA-IF: 5'-TAC ACC GGT CAA CAT TGA GG-3'

GA-IR: 5'TTA ATG ATT GCC GCC GTC GG-3'

Producing products of 648bp.

(2) Conditions of reaction: PCR testing was conducted with three primers, LT-I, LT-II and GA-1 under 94℃, for 2 minutes and 30 seconds, 60℃ for one minute, 72℃ for two minutes for one cycle. 29 more cycles of 94℃ for one minute, 60℃ for one minute, 72℃ for two minutes and finally 72℃ for 10 minutes were conducted.

(3) Determination of PCR products: 7λ of reaction product was taken for 1.2% agarose analysis under 100V, 1% TAE buffer for electrophoresis for 25 minutes. 100bp DNA markers were used to contrast the sizes of the products.

Analysis of the Drug Resistance of the Strain

1) Disk diffusion test

12 disks including ampicillin (AM; 30 μg), trimethoprim-sulfamethoxazole (SXT; 1.25 μg), chloramphenixol (C; 30 μg), streptomycin (S; 30 μg), tetracycline (Y; 30 μg), and other antibiotics such as ciprofloxacin (CIP; 10 μg), amicikin (AN; 30 μg), cephalothin (CF; 30 μg), cefamandole (MA; 30 μg), ceftiriaxone (CRO; 30 μg), gentamicin (GM; 30 μg), and nalidixic acid (NA;

30 μg) were selected for use. Bacterial fluids were placed in the standard McFarland 0.5 barium sulfate buffer of the TSB, evenly applied on the Mueller-Hinton medium and cultured under 37℃ 18-20 hours to observe the size of the inhibition circles. BBL was used for the reading of drug resistance.

2) MIC (minimum inhibitor concentration)

The drug sensitivity assessment kit (GNS-206) for the gram-negative bacilli of the VITEK system including 18 antibiotics such as ampicilliin, tetracycline, ciprodloxacin, ofloxacin, levefloxacin, and the second and third generations of cephalosporins was used. Strains cultured for 16-18 hours were mixed with saline solution to McFarland 0.2 barium sulfate buffer. The fluid was placed on the kit in VITEK to determine the minimum drug inhibitor concentration of strains.

PFGE (pulsed-field gel electrophoresis)

Section of chromosome DNA was performed by *Xba-1, Not-1* and *Sfi-1*. PFGE (DR-III, Bio-Rad) was used at 3.5-35 second intervals, 6v/cm for 24 hours. 1.2% SemKem Gold agarose and 0.5xTBE electro fluids were used for EtBr dyeing and photographing. DNA molecular graphs were analyzed by section.

Gene Sequencing of Shiga-like Toxin and Gyrase A

30 μL of gene products of SLT-1 and Gyrase A were determined with an ABI 377 auto sequencer for gene sequencing. Gene sequences were compared and analyzed.

Results

The Seiken's VET-RPLA assessment kit was used for testing shiga-toxin

of two *Shigella dysentery* type 1 strains (coded TA-1 and #450). By positive and negative controls, the two strains both showed toxin positive agglutination reactions. SLT-1 primer (20bp) was further used for PCR reaction to produce gene product of shiga-like toxin of 475bp size. No such products could be found in *Shigella* groups B and D, and Campylobacter strains. SLT-II primer (20bp) was used on ETEC control strains to produce product of 862bp. No such products were found in *Shigella dysentery* type 1 (see Figure 1).

The disk diffusion test showed that TA-1 strain demonstrated resistance patterns to AM, S, SXT, C, T, NA and CIP). As compared with #450, they were of susceptible patterns. Testing of the MIC minimum inhibitor concentration by VITEK drug sensitivity kit showed that the two strains demonstrated different responses to AM, NA, OFL (ofloxacin), LEV (levofloxacin) and CIP (TA-1 was resistant, and #450 was susceptible).

Restriction enzymes such as *Xba-1, Not-1* and *Sfi-1* were used for electrophoresis. All graphs showed that the differences of the DNA bands were >4 to clearly distinguish the sources of TA-1 and #450 strains (Figure 2).

By sequencing of shiga-like toxin genes (475bp) and Gyrase A genes (648bp) to compare the differences between the two strains, it was found that the SLT toxin gene sequences of TA-1 and #450 strains were the same. When Gyrase A gene sequences were compared, the Gyrase A genes of TA-1 showed mutation at 185 and 197, from Ser^{185} (TCG)→Lys (TTG) and Asp^{197} (GAC)→Gly (GGC). This finding corresponded to the mutation of anti-ciprofloxacin 9Table 2).

Discussion and Conclusion

Shigella dysentery type 1 infection is a Category II Group B notifiable communicable disease. Cases must be reported and isolated for treatment. No such cases have been reported in Taiwan since 1950. The present case entered Taiwan on 26 November, and was admitted to hospital on 27 November. Laboratory testing by the CDC confirmed the infection. It was speculated, by its incubation period of 3-5 days and the case's previous visit to India a month before, that this was an imported case of communicable disease.

The pathogenic agent of *Shigella dysentery* type 1 is the shiga-toxin. The toxin will induce hemorrhage by damaging the lower part of the blood vessels of the intestine membrane to enter the circulatory system to cause serious disorders. Studies have shown that shiga-toxin is closely associated with hemolytic uremic syndrome (HUS), giant colonitis and acute renal failure[4]. Fontaine et al. found in 1988[3] that by mutation at stx, *Shigella dysentery* though continued to attack cells of the lower part of the intestines, would not damage blood vessels. In 1989, Lopez and Diaz et al.[2] pointed out that the epidemiological data of HUS in children were closely associated with shiga-toxins. In 1991, Tesh et al.[1] also confirmed that HUS and acute renal failure were associated with shiga-toxins.

The present case was the first imported *Shigella dysentery* Group A infection. In addition to the isolation treatment by medical care institutions and preventive measures taken by disease control authorities to study causes of infection and trace its sources to prevent further transmission, it was considered necessary to study whether the strain concerned could produce

shiga-toxin and to analyze its molecular characteristics. For comparison, the strain coded #450, the only preserved and dried *Shigella dysentery* type 1 strain by the CDC obtained from Japan in June 1966, was used for analysis.

The VT1 (SLT-I) and VT2 (SLT-II) used in the study by Pollard et al. in 1990 were used as primers in the present study for the polymerase chain reaction of shiga-like toxin. The agarose gel analysis of the control positive EHEC strain produced a product of 475bp, confirming that the imported case carried shiga toxin genes. In contrast, some common entero-bacilli such as dysentery B and D, and *Salmonella* would have no such products. For further comparison, #450 strain of the CDC was used for PCR. It also carried shiga-like toxin genes.

TA-1 and #450 strains were isolated at different times and places. TA-1 could have originated from the Southeast Asia region of 2002. #450 strain came from Japan in 1966. Though they were of the same type 1 serotype, they should have certain molecular differences. To further analyze their characteristics and molecular data, compared of between the drug sensitivity tests and pulsed-field gel electrophoresis.

Drug sensitivity tests showed that TA-1 strain demonstrated resistant pattern and #450 demonstrated susceptible pattern to disk drugs such as ampicillin, streptomycin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, nalidixic acid, and ciprofloxacin. The MIC (minimum inhibitor concentration) of both strains also showed different drug resistance patterns. TA-1 strain had higher MIC values to ampicillin, nalidixic acid, ciprofloxacin, ofloxacin (OFL) and levofloxacin (LEV) than the #450 strain. The #450 strain originated in 1966 showed effective responses to all drugs; whereas the TA-1 strain, after decades of evolution and drug resistance,

showed multiple drug resistance.

Pulsed-field gel eletrophoresis was used primarily to isolate DNA molecules of large section by constantly modifying electric fields. It is a common study method used in epidemiological investigations. The advantages are that it is stable and highly reproducible; it, however, is time-consuming and elaborated. Some major factors to the success of PFGE are the completeness of strains, selection of inhibition enzymes, directions of pulsed-fields and time for the transformation of electric fields. The present study used the three inhibition enzymes often used by previous researchers[7,8], *Xba-1* (5'-T ↓ CTAGA-3'), *Not-1* (5'GC ↓ GGCCGC-3'), and *Sfi-1* (5'GGCCNNNN ↓ NGGCC-3'). Conditions for electrophoresis were 120 degrees for electric field directions ($+60^0$, -60^0), at 5-35 second intervals for 24 hours for the analysis of TA-1 and #450 strains. Pulsed graphs showed that TA-1 and #450 strains were significantly different, and their differences were >4 bands. These two strains were of different molecular types and not related. Reasons for the significant differences were probably mutation of drug resistance genes or reorganization of genes to make inhibition enzymes unable to recognize them.

TA-1 and #450 though were isolated at different times and places, they both could produce shiga-like toxins. By analyzing their toxin gene sequences, it was found that their gene sequences were the same. It was speculated that pathogenic toxins should carry the same gene sequence. TA-1 was found resistant to ciprofloxacin of the quinolones group. Recent study also showed its resistance mechanism to fluoroquinolones group. This was due to the point mutation of Gyrase A, from Ser^{185} (TCG)→Lys (TTG) and Asp^{197} (GAC)→Gly (GGC). This corresponded to the mutation gene product of ciprofloxacin-resistant drug.

Recommendations

The increasing number of tourists to particularly the neighboring southeast Asian countries in the recent years, and also the increasing number of alien laborers and stowaways entering Taiwan have posed some major challenges to the disease control of Taiwan. The present case was the first imported *Shigella dysentery* type 1 infection. By immediate assessment and disease control measures, the infection was effectively controlled. The fact that the case was reported as a suspected typhoid showed that physicians were relatively ignorant of the clinical symptoms of some specific communicable diseases. Improvement should be made. The laboratory though made prompt and accurate testing in time, more should be done to set up molecular serotyping techniques and data banks for *Shigella dysentery* strains for the comparative analysis of future cases.

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Table 1. Disk Diffusion Test and MIC

Methods / Antibiotics		Disk diffusion					VITEK (MIC)			
		Disk Concentration	Diameter (mm)		Strain		MIC Concentration		Strain	
		μg	R	S	TA-1	# 450	R	S	TA-1	#450
Penicillins										
A	Ampicillin	10μg	≦13	≧17	R (-)	S (18)	≧32	≦0.25	R (≧32)	S (≦0.5)
Tetracyclines										
T	Tetracycline	30μg	≦15	≧19	R(-)	S (26)	≧16	≦1	R (≧16)	S (≦1)
Quinolones										
NA	Nalidixic Acid	30μg	≦13	≧19	R(-)	S (20)	≧32	≦16	R (≧32)	S (≦16)
CIP	Ciprofloxacin	5μg	≦15	≧21	10	S (35)	≧4	≦0.5	R (≧4)	S (≦0.5)
LEV	Levofloxacin	5μg	ND	ND	ND	ND	≧8	≦1	R (≧8)	S (≦1)
OFL	Ofloxacin	5μg	ND	ND	ND	ND	≧8	≦1	R (≧8)	S (≦1)
Cephalosporins										
CRO	Ceftriaxone	30μg	≦13	≧21	S (26)	S (32)	≧64	≦8	S (≦8)	S (≦8)
Others										
SxT	Trimethoprim/ sulfamethoxazole	1.25/ 23.75μg	≦10	≧16	R (-)	S (25)	≧320	≦10	R (≧320)	S (≦10)
C	Chloramphenicol	30μg	≦12	≧18	R (-)	S (30)	≧16	≦1	R (≧16)	S (≦1)

1. Disk diffusion: Bacterial fluid under McFarland 0.5 of TSB was placed on Mueller-Hinton medium under 37℃ for culturing for 18-20 hours to observe the size of the inhibition cycles.
2. MIC: Fresh colonies was mixed with saline solution and placed in McFarland 0.2 barium sulfate buffer, added VITEK drug sensitivity assessment card (GNS-206) for 16-18 hours.
3. ND: no testing.

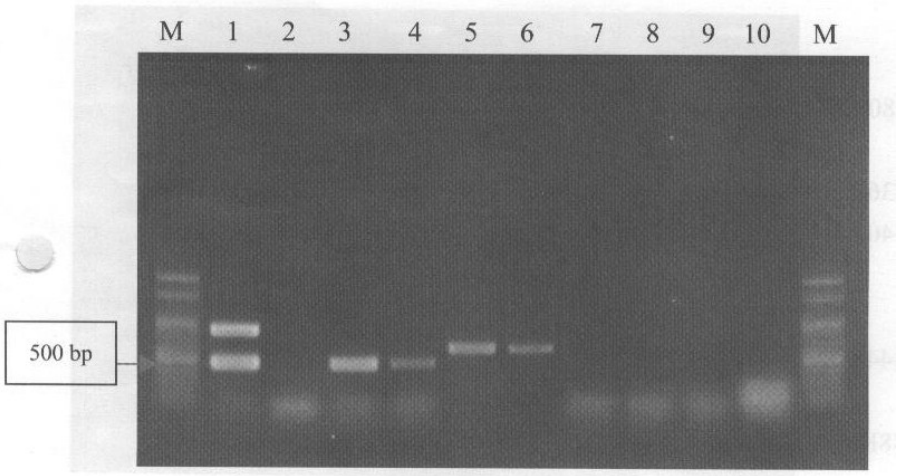

Figure 1. PCR of Shiga-Toxin (1) M: 100bp marker; lane 1 is positive controls; lane 2 is negative controls; lane 3 is TA-1 strain, using SLT-1 primer; lane 4 is #450 strain, using SLY-1 primer; lane 5 is TA-1, using GA-1 primer; lane 6 is #450, using GA-1 primer; lane 7 is *Shigella sonnei*; lane 8 is *Salmonella typhimurium*; lane 9 is *Camylobacter*. (2) 1% SemKem LE agarose, 100V, 30 minutes of electrophoresis.

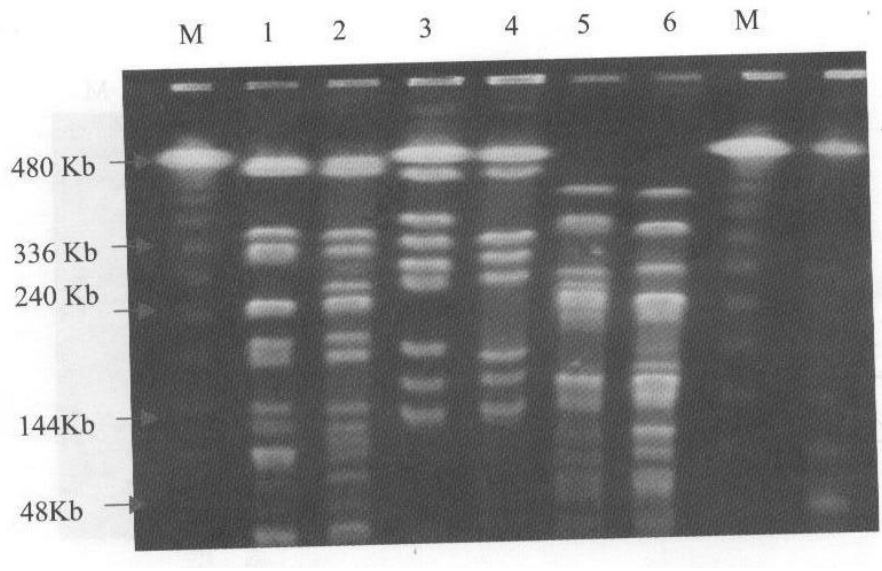

Figure 2. Pulsed-Field Gel Electrophoresis (1) M: is λ-Ladder 48kb marker; lane 2 is TA-1 strain bisected by *Xba-1*; lane 3 is #450 bisected by *Xba-1*; lane 4 is TA-1 bisected by *Not-1*; lane 5 is #450 bisected by *Not-1*; lane 6 is TA-1 bisected by *Sfi-1*; lane 7 is #450 bisected by *Sfi-1*; (2) 1.2% SemKem Gold agarose at 120 degrees ($+60^0$, -60^0). At 3-35 second intervals for 24 hours.

Table 2. Nuclotides mutation of Cipfloxacin-Resistant TA-1 Strain

	185	197
（No.＃450）	5'－－－－－－TCG－－－－－－－－GAC－－－－－－－－－－3'	
（No.TA-1）	5'－－－－－－TTG－－－－－－－－GGC－－－－－－－－－－3'	

Ser ^{185}（TCG）→Leu（TTG）

Asp ^{197}（GAC）→Gly（GGC）