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Original Article

Molecular epidemiology of the emerging ceftriaxone resistant non-typhoidal *Salmonella* in southern Taiwan

Chi-Yu Chen [a,b,c], Ping-Hsuan Hsieh [d,e], Chung-Yu Chang [e,f], Shan-Tzu Yang [b], Yen-Hsu Chen [e,g,h], Ko Chang [a,b,*], Po-Liang Lu [a,g,i]

[a] *Division of Infectious Diseases, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Department of Internal Medicine, Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Department of Emergency Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*
[d] *Department of Laboratory Medicine, E-DA Hospital, Kaohsiung, Taiwan*
[e] *Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[f] *Department of Microbiology and Immunology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[g] *School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[h] *Department of Internal Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*
[i] *Division of Clinical Microbiology, Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*

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Abstract *Background/Purpose:* : The increasing trend of ceftriaxone resistant non-typhoidal *Salmonella* (NTS) worldwide is of serious concern, however, data is lacked in southern Taiwan. *Methods: Salmonella* isolates were collected at a regional hospital in Kaohsiung during 2004–2013. Ceftriaxone resistant NTS isolates were further characterized for beta-lactamases, typed by pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and their plasmids were analyzed by PCR replicon typing and plasmid mutilocus sequence typing.

Abbreviations: NTS, non-typhoidal *Salmonella*; ESBLs, extended-spectrum-β-lactamases; PFGE, Pulsed-field gel electrophoresis; MLST, multilocus sequence type.

* Corresponding author. Division of Infectious Disease, Departments of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, No. 100 Tzyou 1st Road, Kaohsiung 807, Taiwan. Fax: +886 7 3214227.
E-mail address: johnsonckk@yahoo.com.tw (K. Chang).

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Results: Among 528 NTS isolates, the most common serogroup is serogroup B (44.9%), followed by serogroup D, and serogroup C. Eleven (2.1%) isolates were resistant to ceftriaxone and were distributed in three peak periods (2010, 2011, and 2013). PFGE and MLST revealed the ten serogroup B isolates were of two clones. Beta-lactamase genes were detected in 10 of the 11 isolates, including CMY-2 (5 isolates), TEM-1 (2), CTX-M-14 (1), and 2 isolates carried both TEM-1 and CMY-2. Plasmid incompatibility types were identified in 9 (81.8%) isolates; three were IncI1, three was IncHI2, one was IncFIB and two had both replicons of IncI1 and IncHI2. The only ESBL gene $bla_{CTM\text{-}X\text{-}14}$ was found in an isolate with plasmid belonged to IncHI2, which has not been reported in NTS in Taiwan before. Most MLST types and plasmid MLST types of NTS isolates in this study are different from those in northern Taiwan.
Conclusion: Though clonal spread of ceftriaxone resistant NTS was suggested by PFGE and MLST, plasmid characterization and beta-lactamase detection revealed their plasmid types and beta-lactamase types were different.

Introduction

Salmonellosis is a food-borne disease worldwide. Non-typhoidal *Salmonella* (NTS) causes a variety of diseases in the both developed and developing countries. Recently, salmonellosis was the most common pathogens isolates from children in Taiwan.[1,2] The fluoroquinolones and extended cephalosporins are the choices for treating salmonellosis, whereas ceftriaxone is the main therapeutic drug for children.[3] However, increasing ceftriaxone resistance has become a serious public health problem in recent years.[4] The mechanisms of resistance to ceftriaxone have been identified to be plasmid mediated *ampC* or ESBL genes: $bla_{CMY\text{-}2}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{SHV\text{-}2a}$, and $bla_{SHV\text{-}12}$ genes in northern Taiwan.[5] Previous studies have identified that $bla_{CMY\text{-}2}$ gene is located on a self-transfer plasmid, incompatibility family IncI1 and Inc A/C in northern Taiwan and USA.[4,6]

NTS can cause infection or colonization in both human and livestock animal. An epidemiologic study in Taiwan revealed NTS isolates from pigs and human with a common PFGE pattern had either identical or very similar antibiotics resistance patterns.[7] However, there is no data about ceftriaxone resistant NTS in that study. We aimed to investigate the trend of ceftriaxone-resistant NTS from 2004 to 2013 and its molecular epidemiology in southern Taiwan where is the main area of livestock farms in Taiwan.

Material and methods

Bacterial isolates

NTS isolates were collected from patients who visited Kaohsiung Municipal Hsiao-Kang Hospital, a 496-bed hospital in southern Taiwan, from January 2004 to December 2013. Approval for the study was obtained from the Institutional Research Board Committee of Kaohsiung Medical University Hospital (KMUHIRB-20130158). *Salmonella* serotypes were determined by the slide agglutination method to identify the somatic O antigen and flagellar H antigen with the use of Antiserum (Difco™ antisera, USA) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for ampicillin, ertapenem, ceftriaxone, cefepime, tigecycline, and trimethoprim/sulfamethoxazole was performed using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), 2013. *Escherichia coli* ATCC 25922 was used as the quality control strain. Furthermore, MICs were performed by Vitek 2 (bioMérieux) and the results of ceftriaxone resistant isolates were interpreted according to the criteria recommended by the CLSI guidelines of 2013 (MIC $\geq$ 4 mg/L). Eleven ceftriaxone resistant isolates NTS were enrolled for further serovar typing, plasmid replicon typing, plasmid multilocus sequence typing (pMLST) and phylogenetic analysis.

Genomic DNA preparation

The genomic DNA preparation were according to Schlesinger methods.[8] Eleven of ceftriaxone resistant NTS isolates stocks were sub-cultured on an Eosin methylene blue (EMB) and blood agar (BAP) bi plate and a single isolated colony was inoculated into a 100 μl double distilled water and then boiled at 95 °C, and then centrifuge 12000 g for 15 min. The supernatant was collected as DNA template and cell debris were discarded.

Serovar typing of isolated ceftriaxone resistant NTS

Multiplex PCR methods consisting of two five-plex PCR reactions and one two-plex PCR reaction were designed for differentiating between the most common salmonella enterica subgroups as previously described.[9] The multiplex PCR assays were performed by a final volume of 34 μl containing 5 μl of DNA template, 4.8 μl 10X reaction buffer, 3 μl dNTPs (2.5 mM of each dNTP), 1 ul (50 ng) of each primer, 5.0 units *Taq* polymerase (TaKaRa, Shiga, Japan)

and de-ionized water to make up the volume. The PCR reaction were performed in a DNA thermal cycler (Applied Biosystems Aluminum 96-Well GemeAmp® PCR System 9700, Thermo Fisher Scientific, USA) under the following conditions: 1 cycle of 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. All PCR products were separated by electrophoresis in 3.5% agarose gel and then visualized under UV light.

Detection of genes for AmpC and ESBL genes

The plasmid-borne *ampC*-like genes (encoding CMY or DHA)[10–14] were detected by PCR amplification.[15–17] The reaction was performed in a total volume of 50 μl. The amplification conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 52–55 °C for 1 min and 72 °C for 1 min, with a final stage at 72 °C for 10 min to complete the synthesis of DNA. The amplicons were sequenced and the entire sequence of each gene was compared with sequences in the GenBank nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed field gel electrophoresis (PFGE) and phylogenetic analysis

Genetic relatedness of isolates was determined by PFGE, which was performed according to the CDC PulseNet protocol.[18] The restriction enzyme *Xba*I (New England Biolabs Inc., MA, USA) was used at the temperature suggested by the manufacturer. Restriction fragments were separated by PFGE in a 1% agarose gel (Bio-Rad) in 0.5 × TBE buffer for 19 h at 200 V and 14 °C and with ramp times of 2.2–63.8 s using CHEF Mapper apparatus (Bio-Rad). The gels were then stained with ethidium bromide and photographed under UV light. The Dice coefficient was used to calculate similarities, and UPGMA was used for cluster analysis with GelCompar II software version 6.5 (Applied Maths).

Multilocus sequence typing (MLST) of NTS

MLST of NTS was carried out by amplification and sequencing of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) according to the protocols available on *Salmonella enterica* MLST Database at http://mlst.warwick.ac.uk/mlst/dbs/Senterica. Allelic profiles and sequence types (ST) were assigned according to the MLST scheme at the same website.

Plasmid replicon and multilocus sequence typing (pMLST)

The incompatibility groups of plasmids in ceftriaxone resistant NTS were determined by PCR-based replicon typing (PBRT).[19,20] The replicon sequence typing (RST) for IncF plasmid was performed according to the scheme proposed by Villa et al.[21,22] Plasmid multilocus sequence typing for IncI1 and IncHI2 plasmids were performed as previously described.[22,23] Alleles and plasmid sequence types (ST) were analyzed at plasmid MLST website (https://pubmlst.org/plasmid/).

Conjugation and transfer of cefotaxime resistance determinants

The donor isolates were grown with shaking at 37 °C overnight in LB medium containing 2 μg/ml of cefotaxime. The recipient cell was *E.coli* J53. The 200 μl of donor isolates and 200 μl recipient were added in 20 ml LB broth. After centrifuge 8000 rpm for 7 min, we remove the supernatant and re-suspend the pellet using the remaining liquid in tube and dropped the concentrated bacterial suspensions onto LB agar and incubated at 37 °C overnight. The single colony was scraped from LB agar and refresh in BHI broth containing 100 ug/ml sodium azide and 2 ug/ml cefotaxime. After shake for 2 or 4 h, plating 100 ul BHI broth onto LB agar containing 100 ug/ml sodium azide and 4 ug/ml cefotaxime. Plasmids of transconjugants were checked for replicon typing and bla_{CMY-2} gene checked by PCR.

Analysis of the flanking regions of bla_{CMY-2}

To identify the flanking regions of bla_{CMY-2}, the genetic structure of bla_{CMY-2} gene was detected with multiplex PCR methods.[24]

Results

A total of 528 NTS isolates were collected from January 2004 to December 2013 in Kaohsiung Municipal Hsiao-Kang Hospital. The distributions of *Salmonella* serogroups were as follows: two isolates belonged to serogroup A; 237 (44.9%) isolates were serogroup B; 129 were serogroup C; 142 were serogroup D; 6 were serogroup E; and 12 belonged to other *Salmonella* serogroups. During the study period, we found that the prevalence of salmonellosis has decreased annually since 2009 (Fig. 1). Serogroup B was the most predominant serogroup, followed by serogroup C in our early study period. Serogroup D has become the second common serogroup since 2009. Eleven ceftriaxone-resistant NTS isolates from stool, including ten in serogroup B and one in serogroup C, were identified during the study period (Table 1). The further serovar typing revealed 1 serovar Thompsom (serogroup C) and 10 serovar Typhimurium (serogroup B) (Table 1). Serovar Thompsom was isolated in 2005 and all the others were isolated in 2008–2013. The ceftriaxone resistant NTS isolates were from 11 individual patients, including 4 patients younger than 2 years, 1 patient was 83 years old male. Seven patients were females. All of them were present in gastroenteritis and totally survived.

All eleven NTS isolates were resistant to ampicillin. Three (27.3%) were resistant to ciprofloxacin. Although the isolate number of salmonellosis decreased annually, the resistance rate of NTS to ceftriaxone has increased from 1.23% of 2005 to 8.33% of 2013. During the study period, three peaks in ceftriaxone resistance rates were noted (Fig. 2). Among ceftriaxone resistant NTS isolates, bla_{CMY-2} gene was the most prevalent beta-lactamase gene (7 isolates) followed by bla_{TEM-1} (4 isolates), and $bla_{CTM-X-14}$ (1

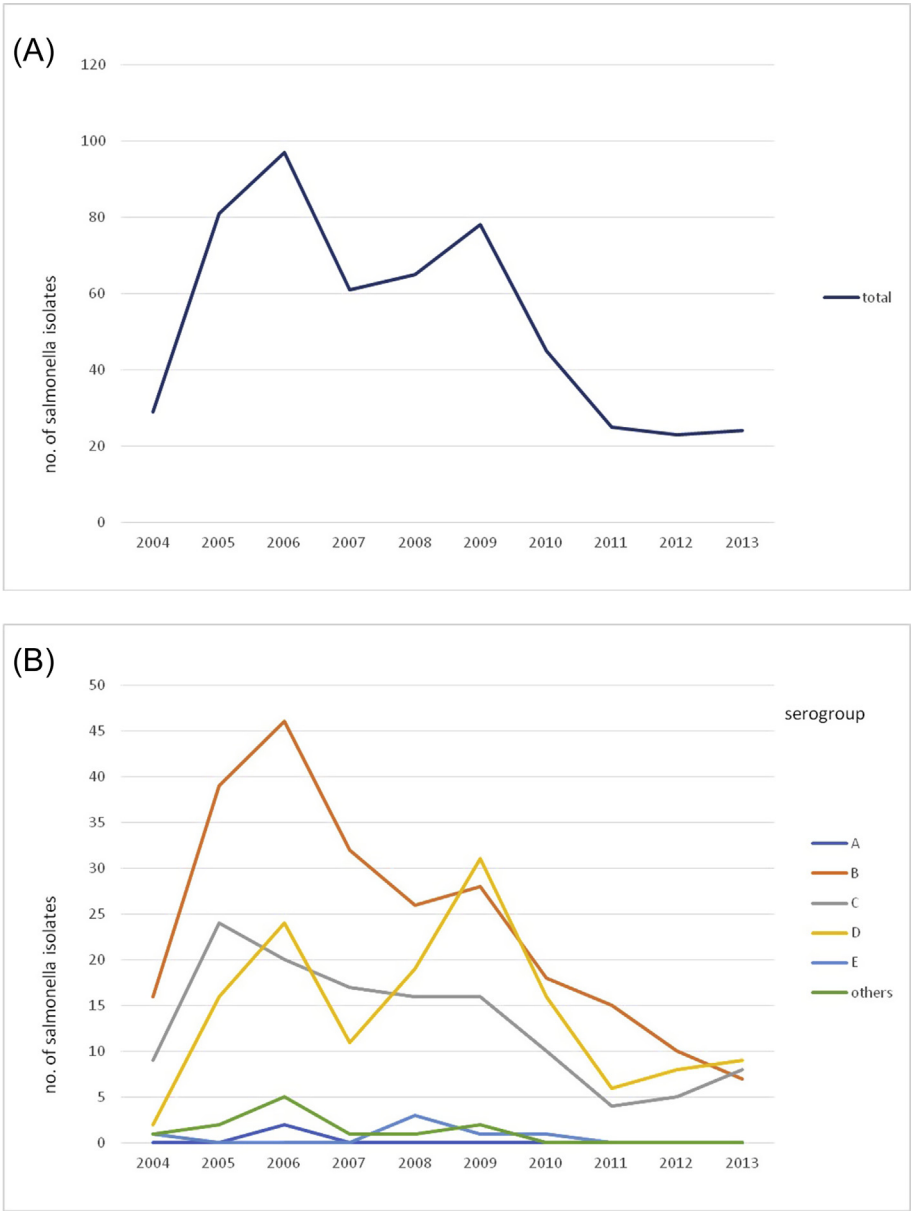

Figure 1. Annual isolates versus serogroups in Kaohsiung Municipal Hsiao-Kang Hospital from 2004 to 2013. (A) NTS isolate number in each year. (B) Isolate number of NTS serogroup.

isolate). Two isolates carried both bla_{TEM-1} and bla_{CMY-2} (Table 1).

PFGE and MLST reveal six isolates were of pulsotype I and their MLST were all ST36 type (Table 1, Fig. 3). Four isolates were of pulsotype II and their MLST type belonged to ST34 (Fig. 3). The only S. Thompson isolate (S22) was isolated in the 2005. Three isolates in 2010 were also resistant to ciprofloxacin. Three isolates (S38, S42, and S46) harboring IncI1 plasmid and 3 isolates (S36, S39 and S40) harboring IncHI2 plasmid. Two isolates harboring both IncI1 and IncHI2 plasmid (S41, S43) (Table 1). One isolate (S45) has IncFIB plasmid. Two isolates' plasmid replicon typing were non-typeable (Table 1). The pMLST of IncI1 plasmids reveal two ST96 (S38 and S41), one ST56 (S42) and one ST72 (S46). A new sequence type (ST245) of IncI1 plasmid in S43 was first identified. One IncI1 and all IncHI2 plasmids revealed non-typeable because of no PCR product for at least one of the alleles in the pMLST (Table 1). A comparison of pMLST sequence types of IncI1 and IncHI2 plasmids in the study with previously reported in northern Taiwan as shown in Table 2. Except that pMLST ST56 was found in both northern and southern Taiwan, the other pMLST types of ceftriaxone resistant NTS were different between isolates from northern and southern Taiwan.

Two isolates (S41 and S43) had plasmids with both IncI1 and IncHI2 amplicons. Conjugation experiments revealed only IncI1 of S41 is transferable (Fig. 4). The successful transconjugant from S41 was positive for PCR of bla_{CMY-2} gene and amplicon of plasmid IncI1, indicating bla_{CMY-2} gene was in the plasmid IncI1 of S41. No transconjugant was obtained for isolate S43. The flanking regions of bla_{CMY-2} containing specific *tnpA*-bla_{CMY-2}-*blc*-*sugE* structure were identified in five

Table 1 Characteristics of the ceftriaxone resistant non-typhoidal *Salmonella* isolates from 11 patients at a hospital in southern Taiwan.

Serovar/Serogroup	Strain No./ Year of isolation	Susceptibility to AM/CIP/C/ TMP-SMX[a]	Pulsotype *XbaI*	Strain ST	Plasmid replicon type (Plasmid ST[b] or FAB formula[c])[d] and insertion sequence	Beta-lactamase genes[e]
Thompson/C	S22/2005	R/S/R/R	III	ST292	non-typeable	not detected
Typhimurium/B	S36/2008	R/S/R/R	I	ST36	IncHI2 (non-typeable)	$bla_{CTX-M-14}$
Typhimurium/B	S38/2010	R/S/R/R	I	ST36	IncI1 (ST96)	**bla_{CMY-2}**
Typhimurium/B	S39/2010	R/R/R/R	I	ST36	IncHI2 (non-typeable)	**bla_{CMY-2}**
Typhimurium/B	S40/2010	R/R/R/R	I	ST36	IncHI2 (non-typeable)	**bla_{CMY-2}**
Typhimurium/B	S41/2010	R/R/R/R	I	ST36	IncI1 (ST96) IncHI2 (non-typeable)	**bla_{CMY-2}**
Typhimurium/B	S42/2011	R/S/S/S	II	ST34	IncI1 (ST56)	**bla_{CMY-2}**/bla_{TEM-1}
Typhimurium/B	S43/2011	R/S/R/R	I	ST36	IncI1 (ST245)[f] IncHI2 (non-typeable)	bla_{CMY-2}
Typhimurium/B	S44/2011	R/S/S/R	II	ST34	non-typeable	bla_{TEM-1}
Typhimurium/B	S45/2013	R/S/R/R	II	ST34	IncFIB (F46:A-:B20)	bla_{TEM-1}
Typhimurium/B	S46/2013	R/S/S/S	II	ST34	IncI1 (ST72)	bla_{CMY-2}/bla_{TEM-1}

[a] AM, Ampicillin; CIP, ciprofloxacin; C, chloramphenicol; TMP-SMX, Trimethoprim/sulfamethoxazole.
[b] Plasmid ST, plasmid sequence types were performed for the IncI1 and IncHI2 plasmids and were determined by plasmid MLST.
[c] The FAB formula was performed for the IncFIB plasmid and was determined by replicon sequence typing (RST).
[d] Non-typeable, no PCR amplification product.
[e] The flanking regions of bla_{CMY-2} carrying *tnpA*-bla_{CMY-2}-*blc*-*sugE* structure (Tn*6092*) are in bold.
[f] ST245, a new plasmid sequence type found in this study.

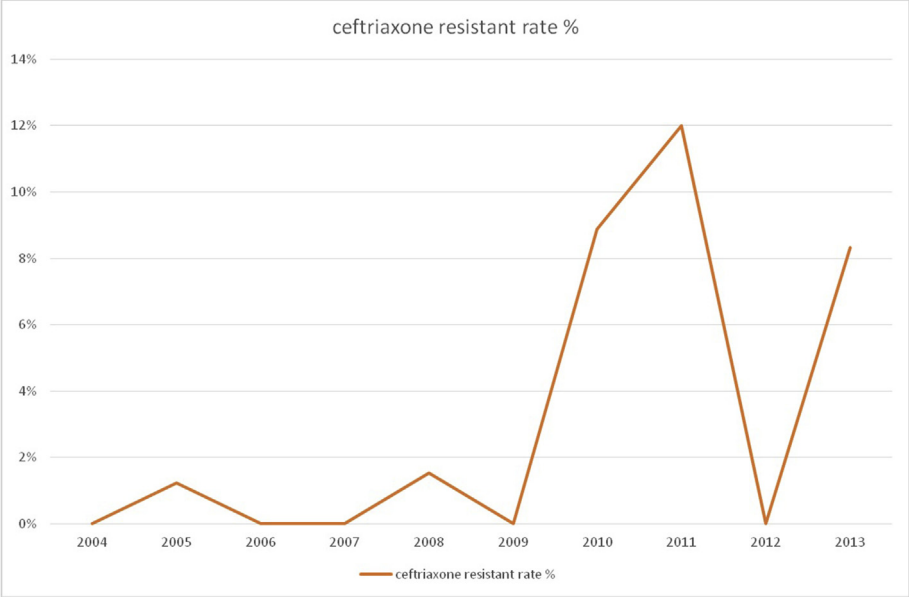

Figure 2. Distribution of resistance rates to ceftriaxone in Kaohsiung Municipal Hsiao-Kang Hospital from 2004 to 2013.

isolates (S38, S39, S40, S41 and S42) (Table 1). Two other CMY-2 carrying isolates did not have the PCR products for *tnpA*, bla_{CMY-2} and *sugE*.

Discussion

Although the overall incidence of salmonellosis had gradually decreased, the rate of ceftriaxone-resistant NTS increased in southern Taiwan. The similar trend has also been found in northern Taiwan.[4] In our study, serogroup B was the most prevalent salmonellosis in southern Taiwan and was also the most common pathology of gastroenteritis amount children in northern Taiwan.[2] It is different from the epidemiology in northern Taiwan where serogroup D was the most predominant serogroup.[4] Most of the ceftriaxone-resistant isolates were of serovar Typhimurium, which belonged to serogroup B. The remaining one

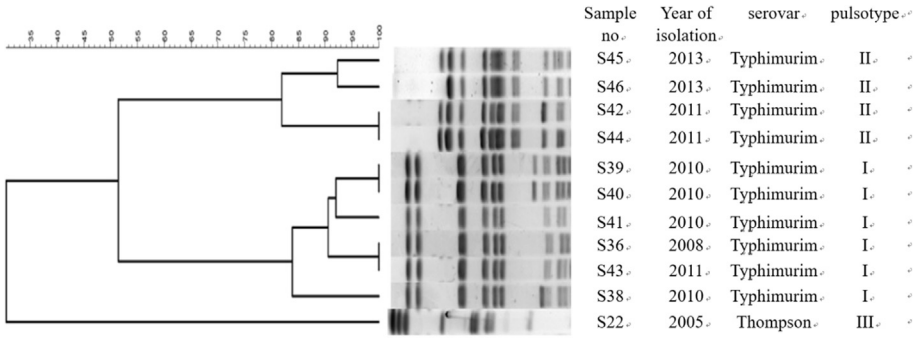

Figure 3. Dendrograms of *Xbal* PFGE analysis of clinical isolates of *Salmonella enterica* generated by GelCompar II software version 6.5 (Applied Maths).

Table 2 Comparison of pMLST of IncI1 and IncHI2 plasmids derived from this study and the previously reported in Taiwan.

Salmonella serovar	Area of isolated	Year of isolation	pMLST ST	Tn*6092* Carrying plasmid	References
Choleraesuis	Taoyuan	2007	51	IncI1	Ref. Su et al.[4],[b]
Choleraesuis	Taoyuan	2008	52	IncI1	Ref. Su et al.[4],[b]
Typhimurium	Taoyuan	2010	53	IncI1	Ref. Su et al.[4],[b]
Typhimurium	Taoyuan	2010	54	IncI1	Ref. Su et al.[4],[b]
Typhimurium	Taoyuan	2010	55	IncI1	Ref. Su et al.[4],[b]
Typhimurium	Taoyuan	2010	54	IncI1	Ref. Su et al.[4],[b]
Agona	Taoyuan	2010	56	IncI1	Ref. Su et al.[4],[b]
Enteritis	Taoyuan	2010	56	IncI1	Ref. Su et al.[4],[b]
Typhimurium	Kaohsiung	2010	96	IncI1	This study
Typhimurium	Kaoshiung	2010	96	IncI1	This study
Typhimurium	Kaoshiung	2010	non-typeable	IncHI2	This study
Typhimurium	Kaoshiung	2010	non-typeable	IncHI2	This study
Typhimurium	Kaoshiung	2011	56	IncI1	This study
Typhimurium	Kaoshiung	2011	245[a]		This study
Oranienburg	Taoyuan	2011	53		Ref. Yang et al.[31],[c]
Typhimurium	Kaohsiung	2013	72		This study

[a] A new plasmid sequence type found in this study.
[b] Reference Su et al.[4]
[c] Reference Yang et al.[31]

serogroup C isolate was of serovar Thomposon. In northern Taiwan, ceftriaxone resistant NTS were found in serovar Choleraesuis, serovar Typhimurium, serovar Agona, and serovar Enteritidis. The beta-lactamase genes identified in our study were bla_{CMY-2}, bla_{TEM-1}, and $bla_{CTX-M-14}$. In northern Taiwan, the related resistant genes have been reported to be bla_{SHV-2a}, bla_{SHV-12}, $bla_{CTX-M-3}$ and bla_{CMY-2}.[4,5] The major MLST types and plasmid MLST types are also different between southern and northern Taiwan.

PFGE and MLST revealed the ten serogroup B isolates were of two clones. Though clonal spread of ceftriaxone resistant NTS in the study was suggested by PFGE and MLST, further plasmid characterization and beta-lactamase detection revealed their plasmid types and beta-lactamase types were different.

Su, Teng et al. has described a self transferable bla_{CMY-2}-harboring IncI1 plasmid with IS*Ecpl*-bla_{CMY-2}-*blc-sugE* structure (Tn*6092*) was responsible for contribution of ceftriaxone resistant NTS in the northern Taiwan.[4] Although most of CMY-2 containing isolates in the study have the genetic structure of Tn*6092*. We found two among seven CMY-2 containing isolates do not have that genetic structure. In USA, bla_{CMY}-harboring plasmid was identified in IncI1 from poultry source and Inc A/C from cattle products.[6] IncN plasmid harbor $bla_{CTX-M-14}$ ESBL was identified in China. Recently, a novel plasmid, IncHI2, harboring $bla_{CTX-M-14}$, bla_{OXA-1}, and bla_{TEM-1} resistant genes was isolated in Kenya.[25,26] IncHI2 has also been identified in China and European countries and is well-known for its ability to spread $bla_{CTX-M-14}$ and oqxAB resistant genes in serovar Typhimurium in food animals in China.[27,28] The IncHI2 plasmid has also been reported related to multiple resistant *Enterobacter cloacae* in Taiwan.[29] The presence of the multidrug-resistant plasmid IncHI2 may facilitate clonal spreading under antibiotics used in food-producing animals.[28] Our data show that both IncI1 and IncHI2 plasmids involved in ceftriaxone resistant NTS in southern Taiwan. Furthermore, this is the first report of IncHI2 plasmid in

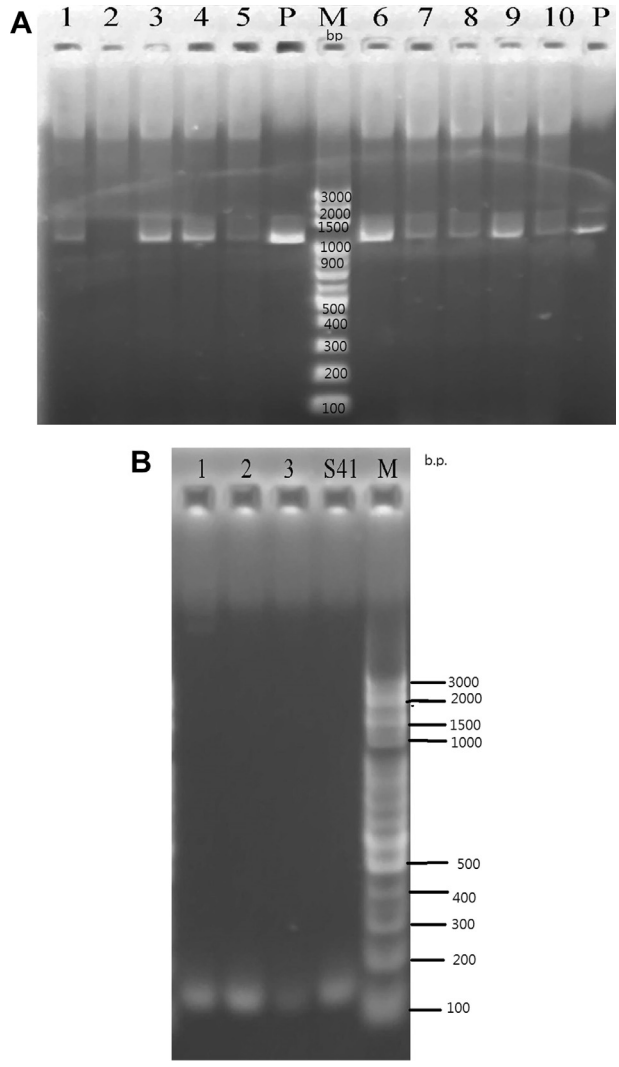

Figure 4. (A) Amplicons of bla_{CMY-2} in transconjugants. Lanes 1 to 5, tranconjugants of S41; lanes 6 to 10, transconjugants of S43; P, positive control; M, size marker. (B) Amplicons of IncI1 replicon in transconjugants. Lanes 1 to 3, tranconjugants of S41; S41, as the positive control; M, size marker.

association with $bla_{CTX-M-14}$ in serovar Typhimurium in Taiwan that such plasmid has been spread in serovar Typhimurium isolates in China and European countries.

The limitations of this study are the followings. First, the PCR used for replicon typing may lead to a false-negative finding due to the high degree of plasticity of *Enterobacteriaceae* plasmids.[30] Second, information on the diet of the patients or livestock contact histories obtained from the medical records was not detailed. Therefore, an epidemiological investigation was difficultly undertaken to determine the origin of the salmonellosis. Further study is needed to elucidate the transmission of ceftriaxone-resistant salmonellosis.

In summary, Serogroup B is the major NTS in southern Taiwan and ceftriaxone resistant NTS isolates were mainly of in serovar Typhimurium of serogroup B. The bacterial characteristics of ceftriaxone resistant NTS isolates in southern Taiwan is different from those in northern Taiwan. Though clonal spread of ceftriaxone resistant NTS in southern Taiwan was suggested by PFGE and MLST typing, plasmid characterization and beta-lactamase detection revealed their plasmid types and beta-lactamase types were different. The appearance of IncHI2 plasmid with $bla_{CTX-M-14}$ in Taiwan warrants for further monitor.

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