Journal of Food and Drug Analysis

Volume 6 | Issue 3 Article 9

1998

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EJ087199800573

Journal of Food and Drug Analysis
1998. 6(3) : 573-578

藥物食品分析
第六卷 第三期

Molecular Subtyping of *Salmonella enterica* Serovar Paratyphi A from Southeast Asia

TZU-MING PAN*, CHEIN-SHENG LIN, TIEN-KWEI WANG, JIN-LAI TSAI, SU-ING HO AND CHIH-LUNG LEE

Bacteriology Division, National Institute of Preventive Medicine, Taipei, Taiwan 11513, R.O. C.

ABSTRACT

From 1987 to 1996, forty-two isolates of *Salmonella paratyphi* A were collected from Taiwan, China and other Southeast Asian countries. Pulsed-field gel electrophoresis (PFGE) was used to differentiate the genomic DNA of these isolates with four different restriction enzymes, *Xba* I, *Not* I, *Spe* I and *Avr* II. Based on the PFGE profiles obtained, *Avr* II allowed the most discriminatory results among these restriction endonucleases. By *Avr* II digestions, 42 isolates of *S. paratyphi* A were differentiated into three clones. The predominant clone accounted for 85.7% of the tested strains (36 in 42 isolates) and was found in many countries of Southeast Asia. Thus, *S. paratyphi* A appears to have limited genetic diversity in evolutionary process.

***Key words:* *S. paratyphi* A, pulsed-field gel electrophoresis, restriction endonucleases, Southeast Asia.**

INTRODUCTION

Salmonellae are ubiquitous human and animal pathogens. More than 2,300 serovars in *Salmonella* have been identified on the basis of variation in the somatic lipopolysaccharide (O) and flagella (H) antigens[1]. Only a few serovars of *Salmonella* such as *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. sendai* and *S. typhi* are exclusively or primarily restricted to grow in human host[2]. Typhoid fever, which is caused by *S. typhi*, has attracted medical attention for a long time. Paratyphoid fever caused by *S. paratyphi* A, B or C resembles typhoid fever in terms of its clinical feature. However, it is a milder illness and has attracted less attention from public health workers.

Infections caused by *S. paratyphi* A were rarely reported in Taiwan in the years between 1987 and 1993[3]. During this period, only eight cases were confirmed by this institute. However, thirty-four cases were confirmed from 1994 to 1996, and most of these cases were traced to trips to Southeast Asia [4]. Therefore, a need for molecular characteristics of these isolates has arisen.

Currently, pulsed-field gel electrophoresis (PFGE) is considered to have the most discriminatory power in bacterial subtyping[5,6]. In this study,

Correspondence to: Tzu-Ming Pan

Accepted for Publication: Aug. 14, 1998

S. paratyphi A isolates from Southeast Asia were differentiated by PFGE with a suitable restriction endonuclease. The genetic diversity of *S. paratyphi* A in Southeast Asia area was analyzed.

MATERIALS AND METHODS

I. *Bacterial Strains*

Forty two clinical isolates were obtained from blood culture or stool samples sent to *Salmonella* Reference unit, National Institute of Preventive Medicine, Taiwan, during the years from 1987 to 1996. (Table 1). Methods for cultivation, isolation and identification of *S. paratyphi* A were described previously[7,8]. Briefly, SS agar (Difco Laboratories, Michigan, USA) was used for primary isolation. Suspected colonies were tested for glucose utilization by Triple Sugar Iron agar (Difco Lab.) and lysine decarboxylation by Lysine Iron agar (Difco Lab.). After these preliminary tests, biochemical tests were performed with the API 20E system (bioMerieux Co., Marcy-I'Etiole, France). Finally, serotyping for O antigens and H antigens of *S. paratyphi* was perfomed according to the manufacturer's instructions (Difco Lab.).

II. *Pulsed-field Gel Electrophoresis (PFGE) Profile*

PFGE profiles of *S. paratyphi* A were obtained by the methods described by Barrett et al.[9] with slight modifications. Briefly, isolated colonies of *S. paratyphi* A were inoculated into 3 ml of tryptic soy broth, TSB (Difco Lab.) for 5 hrs at 37℃ with shaking. Bacterial cells were harvested by centrifugation and were adjusted to 1.2 at OD_{610}. A portion of the bacterial suspension was then mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad, Richmond, Calif.). The mixture was dispensed into a plug mold (Bio-Rad), and allowed to solidify. For bacterial lysis, the resulting plugs were then placed in a mixture of 50 mM Tris-buffer (pH 8.0), 50 mM EDTA (pH 8.0), 1 % sodium lauryl sarcosine, and 1 mg per ml of proteinase K and incubated overnight at 53℃ under gentle shaking. The plugs were washed twice with TE buffer (10 mM Tris buffer, pH 8.0, 10 mM EDTA) containing 1 mM phenylmethyl- sulfonyl fluoride (Sigma, St. Louis, Mo.), and washed twice with TE buffer for 1 hr at 4℃. A slice of each plug (2.5 mm) was cut and incubated for 4 hrs with 20 units of the following restriction enzymes: *Xba*I, *Not*I, *Spe*I and *Avr*II. The manufacturer's recommendation for specific buffers and reaction conditions was followed. The slices were then loaded into the wells of a 1.2 % pulsed-field certified agarose (Bio-Rad) plate in 0.5X TBE buffer. Electrophoresis was performed with a contour-clamped homogeneous electric field apparatus (CHEF-DRIII, Bio-Rad) at 14℃ with 200 V. The pulse time for *Xba*I digestions was ramped from 1 to 60 s for 25 hrs. For *Spe*I digestions, the pulse time was ramped from 2 to 40 s for 25 hrs. For *Not*I digestion, the pulse time was ramped from 2 to 5 s during the first 11 hrs and from 9 to 12 s for the following 11 hrs. For *Avr*II digestion, the pulse time was ramped from 7 to 12 s during the first 11 hrs and from 20 to 65 s for the following 12 hrs. A lambda ladder (Boehringer Mannheim, Germany) was used as the molecular size marker. The gels were stained with ethidium bromide (0.5 μg/ml) for 30 min and destained in distilled water for 3 hrs. DNA bands were visualized and photographed under UV light. Criteria for interpreting PFGE patterns were based on a number of fragment differences compared with those from outbreak[10].

RESULTS AND DISCUSSION

At first, four restriction endonucleases, *Xba*I, *Not*I, *Spe*I, and *Avr*II were used for subspecies typing of forty-two *S. paratyphi* A isolates collected in Taiwan. In our study with *Xba*I and *Not*I digestions, one PFGE pattern was predominant and accounted for 40 (95.2%) of the isolates, while the remaining two isolates had unique PFGE patterns. Therefore, the discrimination power of *Xba*I and *Not*I digestion was not satisfactory for this study. The discrimination power of *Spe*I, and *Avr*II digestions was better than that of *Xba*I and *Not*I. *Avr*II provided more discernible

Table 1. PFGE patterns for isolates of *S. paratyphi* A collected from Southeast Asia

No. Isolates	PFGE patterns (by *Avr*II)	Date of isolation (yr\mo\day)	Origin
UK01	A	'87\08\05	Unknown
THA01	B	'91\07\06	Thailand
TAI01	B	'91\10\29	Taiwan
IND07	A	'96\05\06	Indonesia
UK02	A	'92\04\17	Unknown
TAI02	A	'93\01\06	Taiwan
IND01	A	'93\03\13	Indonesia
VIE01	A	'93\04\04	Vietnam
TAI03	A	'93\08\26	Taiwan
TAI04	A	'93\08\30	Taiwan
IND02	A	'93\09\12	Indonesia
TAI05	A	'94\06\14	Taiwan
CHI03	A1	'94\08\05	China
SIN01	A	'94\10\08	Singapore
BUR01	A	'94\10\17	Burma
IND03	A	'94\10\21	Indonesia
CAM01	A	'94\12\13	Cambodia
UK03	A	'95\06\08	Unknown
IND04	A1	'95\07\22	Indonesia
IND05	A	'95\12\14	Indonesia
IND06	A	'96\03\13	Indonesia
IND07	A	'96\05\06	Indonesia
THA02	A	'96\05\12	Thailand
THA03 [a]	A	'96\05\21	Thailand
THA04 [a]	A	'96\05\23	Thailand
THA05 [a]	A	'96\05\25	Thailand
THA06 [a]	A	'96\05\25	Thailand
THA07 [a]	A	'96\05\25	Thailand
TAI06	A1	'96\07\24	Taiwan
IND08	A	'96\08\10	Indonesia
THA08	A	'96\08\21	Thailand
CHI01	A	'96\08\21	China
THA09 [b]	A	'96\08\28	Thailand
THA10 [b]	A	'96\08\28	Thailand
THA11 [b]	A	'96\08\31	Thailand
THA12 [b]	A	'96\09\03	Thailand
THA13 [b]	A	'96\09\05	Thailand
THA14 [b]	A	'96\09\05	Thailand
CHI02	B	'96\09\17	China
IND09	A	'96\10\15	Indonesia
TAI07	A	'96\12\09	Taiwan
TAI08	A	'96\12\13	Taiwan
TAI09	A	'96\12\13	Taiwan

[a] : Strains from same trip.

[b] : Strains from same trip.

PFGE profiles than *Spe*I. Thus, results of *Avr*II digestion were used for analyses. In *Avr*II digestion, 11 to 14 bands were observed in PFGE profiles and three PFGE patterns A, A1, and B were obtained (Fig.1). Among them, pattern A was predominant and accounted for 85.7% (36 of 42) of the isolates. This clone was found in isolates from many countries of Southeast Asia (Table 2). PFGE pattern A1 was very similar to pattern A and accounted for 7.1% (3 of 42) of the isolates. The three (7.1%) isolates of PFGE pattern B were from China, Taiwan, and Thailand, respectively.

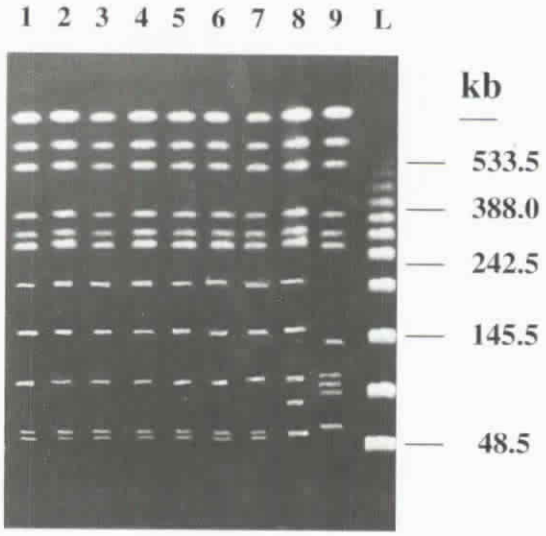

Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns of *Salmonella paratyphi* A isolates with *Avr*II digestions. These isolates originated from different areas of Southeast Asia (see Table 1). Lanes: 1, UK01; 2, IND07; 3, TAI02; 4, VIE01; 5, SIN01; 6, BUR01; 7, CAM01. Lanes 1 to 7 are PFGE pattern A. Lane 8, CHI03 (PFGE pattern A1). Lane 9, THA01 (PFGE pattern B). Lane L: lamda DNA marker, starting from bottom at 48.5kb.

Since paratyphoid fever is a mild disease, it has attracted less attention from public health workers and clinician physicians. Although three serovars of *Salmonella enterica*: Paratyphi A, Paratyphi B and Paratyphi C were identified, only *S. paratyphi* A has been occassionally isolated in Taiwan. In the years from 1987 to 1993, only eight cases caused by *S. paratyphi* A were officially confirmed. However, thirty-four sporadic cases of *S. paratyphi* A were confirmed by this institute in the years from 1994 to 1996. Among them, 29 (69%) cases were traced to trips to Southeast Asia .

Molecular subtypings on different serovars of *Salmonella* have been extensively studied in recent years[11-13]. However, reports on *S. paratyphi* were few. For *S. paratyphi* B, subtyping by the restriction fragment length polymorphism of ribosomal RNA has been reported by Ezquerra et al.[14] and a globally-distributed clone was found from human and environmental sources. However, subspecies typing of *S. paratyphi* A is rare, particularly in Asia[15].

Using multilocus enzyme electrophoresis (MEE), Selander et al.[16] differentiated 135 *S. paratyphi* A isolates from Africa and South America into six clones. It was shown that 85.9% (116 of 135) of their isolates belonged to a single clone, Pa1. Thus, molecular subtyping by MEE and PFGE appears to yield similar results. These findings indicate that *S. paratyphi* A has limited

Table 2. Subtyping of *S. paratyphi* A isolates by pulsed-field gel electrophoresis with restriction endonuclease *Avr*II

PFGE pattern	No. of isolates and geographic origin									Sum
	Burma	Cambodia	China	Indonesia	Singapore	Taiwan	Thailand	Vietnam	Unknown	
A	1	1	1	8	1	7	13	1	3	36
A1			1	1		1				3
B			1			1	1			3
Sum	1	1	3	9	1	9	14	1	3	42

genetic diversity in evolutionary process.

ACKNOWLEDGMENT

Chih-Lung Lee, one of the authors, thanks M. Lambert-Fair, T. Barrett, and B. Swaminathan of Centers for Disease Control and Prevention, U.S.A. for providing excellent PFGE training.

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東南亞地區副傷寒A型菌之分子流行病學分型

潘子明* 林建生 王添貴 蔡金來 何淑渼 李智隆

行政院衛生署預防醫學研究所細菌組

摘 要

1987至1996年間，本單位之沙門氏菌研究室共分離出本土與東南亞之副傷寒A型菌 (*Salmonella paratyphi* A) 計42株，其中大部分係境外移入，此與近年來國人出國頻繁有關。本土性之副傷寒案例以往罕見，但近年來已有明顯增加之警訊。爲確保國人健康及防疫追踪調查之需求，除傳統之血清分類法外，副傷寒A型菌之分子流行病學分型值得加以探討。本研究以脈場膠電泳法應用於此菌血清型之下的分型，經由四種不同限制酵素之切割，找出最具分型性之限制酵素*Avr*II，作爲日後例行檢驗之參考。以*Avr*II限制酵素之切割，可將來自本土及東南亞之42株副傷寒A型菌分成三種親族群 (clones)，但其中之一親族群佔85.7%，比率高且在東南亞分布頗廣。此乃表示副傷寒A型菌在演化過程中變異性少，故基因組 (genomic) DNA相似性很高。

關鍵詞：副傷寒A型菌，脈場膠電泳法，限制酵素，東南亞。