FEMS

Immunology and Medical Microbiology

ELSEVIER

FEMS Immunology and Medical Microbiology 29 (2000) 221–225

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Application of randomly amplified polymorphic DNA (RAPD) analysis for typing Avian *Salmonella enterica* subsp. *enterica*

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Received 29 December 1999; received in revised form 8 August 2000; accepted 13 August 2000

Abstract

Randomly amplified polymorphic DNA (RAPD) analysis was performed for the molecular genetic typing of 30 *Salmonella enterica* subsp. *enterica* strains isolated from chickens and ducks in Thailand. Six different primers were tested for their discriminatory ability. While some of the primers could only differentiate between the different serovars, the use of multiple primers showed that the RAPD method could also subdivide within a given serovar. The Ready-To-Go RAPD analysis beads used, resulted in reproducible and stable banding patterns. As the RAPD technique is simple, rapid and rather cheap, we suggest that it may be a valuable new tool for studying the molecular genetic epidemiology of *S. enterica* ssp. *enterica*, both inter- and intra-serovars.

Keywords: *Salmonella enterica* subsp. *enterica*; Avian; Randomly amplified polymorphic DNA

1. Introduction

Avian salmonellosis causes a large group of acute or chronic diseases in fowl and is caused by *Salmonella enterica* subsp. *enterica*. In chickens enteric disease caused by *S. enterica* ssp. *enterica* is an important cause of mortality and morbidity. Hence, avian salmonellosis is of large economic concern in all phases of the poultry industry from production to marketing. Salmonella have few host barriers and may infect almost any animal including man. Therefore salmonellosis is also of major importance for the public health sector. Human epidemic outbreaks are often related to infected poultry and poultry products. The widespread occurrence of avian salmonellosis ranks the disease as one of the most important of egg-borne bacterial zoonoses.

National control and eradication programs have been beset with numerous obstacles [1]. Therefore, there is a great need for new cheap, fast and simple highly discriminatory (i.e. differentiation within the spp. level) epidemiological tools for building more efficient *Salmonella* control programs.

Traditionally, *S. enterica* ssp. *enterica* are classified according to growth requirements, colony morphology and biochemical characteristics. Further differentiation is obtained by using additional classical typing methods, such as serotyping, extended biotyping [2–4] or phage typing or, alternatively, by applying nucleic acid-based typing methods, such as ribotyping [5], plasmid profile [6–9] and IS*200* profile [5]. However, sensitive techniques do exist that can genetically differentiate and type bacterial strains into distinct clones. These techniques include pulsed-field gel electrophoresis (PFGE) [5], AFLP-fingerprinting [10] and randomly amplified polymorphic DNA (RAPD) techniques [11–13]. Here we report on some primary results using the RAPD technique to elucidate its potential value as a molecular genetic epidemiological tool to study avian salmonellosis.

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0928-8244/00/$20.00
PII: S0928-8244(00)00208-X

2. Materials and methods

2.1. Isolation and identification of avian Salmonella

S. enterica ssp. *enterica* isolates were obtained from the WHO National Salmonella and Shigella Center, Ministry of Public Health, Thailand and the Department of Veterinary Microbiology, Faculty of Veterinary Sciences, Chulalongkorn University, Thailand. All strains were identified by serotyping and eight strains of *S. enteritidis* were all of phage type 4. The identified strains of *S. enterica* ssp. *enterica* were isolated from chickens and ducks that were raised in an intensive farming area in the central plain of Thailand.

2.2. Chromosomal DNA preparation

A total of 30 strains of *S. enterica* ssp. *enterica* were cultured on 5% horse blood agar. Five bacterial colonies, approximately 2.5×10^8 cfu, were transferred to conical tubes containing 0.5 ml of sterile deionized water, vortexed for 10 s, sonicated for 5 min, and then centrifuged at 13 000 rpm for 5 min at 4°C. The DNA in the supernatant was purified by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then by chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 1/10 volume of 3 M NaCl and two volumes of 95% cold ethanol and dried at room temperature before being dissolved in 100 μl sterile deionized water. The concentration and purity of DNA was determined by the optical density at 260 and 280 nm using a Gene Quant Calculator (Pharmacia Biotech).

2.3. Calculation of discrimination index

All the DNA samples from the different *Salmonella* isolates were amplified using six different RAPD primers (Amersham Pharmacia Biotech, Sweden) (Table 1). The discrimination index was calculated using Simpson's index of diversity [14] (Table 1) given by:

$$D = 1-\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j-1)$$

where D is the numerical index of discrimination, N is the total number of strains, s is the total number of types described, and n_j is the number of strains belonging to the j type.

2.4. RAPD-polymerase chain reaction (PCR) conditions

The RAPD kit (Amersham Pharmacia Biotech, Sweden) used in this study is provided by the manufacturer as room-temperature-stable dried beads that contain thermostable polymerase (Ampli*Taq* DNA polymerase and Stoffel fragment), dNTP (0.4 mM each dNTP in a reaction volume), bovine serum albumin (2.5 μg) and buffer (3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris, (pH 8.3) in a reaction).

Briefly, 10 μl of 5 μM RAPD analysis primer, 8 μl of template DNA (10 ng μl^{-1}) and 7 μl of sterile distilled water were added to the reaction beads. The total volume of the reaction mixture was thus 25 μl. The reaction mixture was heated in a Gene Amp PCR system 2400 thermocycler (Perkin Elmer) for 45 cycles of: 30 s at 94°C; 15 s at 36°C and 30 s at 72°C. RAPD products were visualized by means of ethidium bromide staining after electrophoresis in a 2% agarose gel. The RAPD electrophoresis bands were photographed and recorded in a Tiff file. For reproducibility, PCR was done at least twice on all the samples.

2.5. RAPD data analysis

The RAPD patterns of individual strains were scored based on band presence or absence. The index of similarity (F) between samples was calculated using [15]:

$$F_{xy} = 2n_{xy}/(n_x + n_y)$$

where n_{xy} is the number of RAPD bands shared by the two samples and n_x and n_y are the number of RAPD bands in each sample. The genetic distance (d) was calculated using the formula of Hillis and Moritz [16]:

$$d = 1-F$$

3. Results and discussion

The sequences of the RAPD primers used in this study

Table 1
Randomly amplified polymorphic DNA primers used in this study

Primer	Sequence (5′ to 3′)	Discrimination index
RAPD analysis primer 1	5′-d(GGTGCGGGAA)-3′	0.865
RAPD analysis primer 2	5′-d(GTTTCGCTCC)-3′	0.992
RAPD analysis primer 3	5′-d(GTAGACCCGT)-3′	0.973
RAPD analysis primer 4	5′-d(AAGAGCCCGT)-3′	0.837
RAPD analysis primer 5	5′-d(AACGCGCAAC)-3′	0.911
RAPD analysis primer 6	5′-d(CCCGTCAGCA)-3′	0.848
All primers	all sequences	1.00

are shown in Table 1. All RAPD analyses were run at least in duplicate and there was no loss in numbers or shift in the position of bands when the RAPD analyses were repeated. No amplification products were seen in reactions performed in the absence of primer or in the absence of a DNA template.

We found that most of the different serovars produced different RAPD types using only one primer. For instance all eight *S. enteritidis* (all of phage type 4) showed identical patterns using primer #4 and this pattern was distinct from the pattern with primer #4 of all other tested strains (Table 2). Hence, this primer appears to be specific on an inter-serovar level. Of these same eight strains, five showed an identical pattern with primer #1 while the remaining three strains showed individual patterns with this primer. Using all six primers the RAPD could differentiate between all of the *S. enteritidis* isolates (Table 2).

Similarly, the *Salmonella blockley*, the *Salmonella hadar* and the *Salmonella montevideo* isolates showed serovar specific patterns with primer #5, #1 and #5, respectively. The more primers used, the higher the number of RAPD types that could be discerned. In all instances, however,

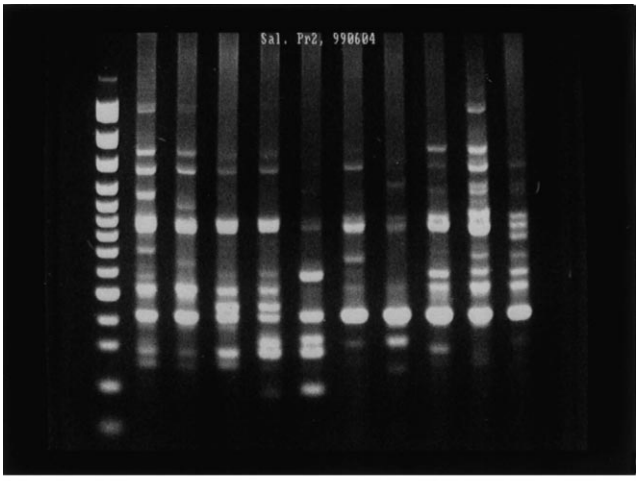

Fig. 1. Ten RAPD profiles of avian *Salmonella* from Thailand run using primer number 2 (5′-d(GTTTCGCTCC)-3′). The species were: lane 1, *S. hadar* (strain No. 11); lane 2, *S. hadar* (strain No. 12); lane 3, *S. hadar* (strain No. 13); lane 4, *S. hadar* (strain No. 14); lane 5, *S. hadar* (strain No. 15); lane 6, *S. typhimurium* (strain No. 16); lane 7, *S. typhimurium* (strain No. 17); lane 8, *S. typhimurium* (strain No. 18); lane 9, *S. saintpaul* (strain No. 19); lane 10, *S. saintpaul* (strain No. 20), lane M = marker ladder (100 bp; MBI Fermentas).

Table 2
Characteristics of the avian *S. enterica* ssp. *enterica* strains analyzed

Strain number and host	Species	RAPD profiles[a]						
		primer 1	primer 2	primer 3	primer 4	primer 5	primer 6	cumulative RAPD profiles based on six primers
1 (chicken)	*S. enteritidis*	A1	B1	C1	D1	E1	F1	1
2 (chicken)	*S. enteritidis*	A1	B2	C2	D1	E2	F2	2
3 (chicken)	*S. enteritidis*	A1	B1	C3	D1	E2	F1	3
4 (chicken)	*S. enteritidis*	A1	B1	C3	D1	E2	F1	4
5 (chicken)	*S. enteritidis*	A1	B3	C4	D1	E2	F1	5
6 (chicken)	*S. enteritidis*	A2	B4	C5	D1	E3	F1	6
7 (chicken)	*S. enteritidis*	A3	B5	C6	D1	E3	F1	7
8 (chicken)	*S. enteritidis*	A4	B6	C7	D1	E2	F1	8
9 (duck)	*S. blockley*	A5	B7	C8	D2	E4	F3	9
10 (chicken)	*S. blockley*	A6	B8	C9	D3	E4	F4	10
11 (chicken)	*S. hadar*	A7	B9	C10	D4	E5	F5	11
12 (chicken)	*S. hadar*	A7	B10	C10	D4	E6	F6	12
13 (chicken)	*S. hadar*	A7	B11	C10	D5	E7	F7	13
14 (chicken)	*S. hadar*	A7	B12	C10	D5	E7	F8	14
15 (duck)	*S. hadar*	A7	B13	C11	D6	E8	F8	15
16 (chicken)	*S. typhimurium*	A8	B14	C12	D7	E9	F9	16
17 (duck)	*S. typhimurium*	A8	B15	C13	D8	E10	F9	17
18 (duck)	*S. typhimurium*	A9	B16	C14	D9	E11	F10	18
19 (duck)	*S. saintpaul*	A10	B17	C14	D10	E12	F11	19
20 (duck)	*S. saintpaul*	A9	B18	C15	D11	E11	F12	20
21 (duck)	*S. montevideo*	A11	B19	C16	D12	E13	F13	21
22 (chicken)	*S. montevideo*	A12	B20	C17	D13	E13	F14	22
23 (chicken)	*S. montevideo*	A12	B21	C18	D13	E13	F14	23
24 (duck)	*S. serftenberg*	A13	B22	C19	D14	E14	F15	24
25 (duck)	*S. chester*	A14	B23	C20	D15	E15	F16	25
26 (duck)	*S. amsterdam*	A15	B24	C21	D16	E16	F15	26
27 (chicken)	*S. muenchen*	A16	B25	C22	D17	E17	F17	27
28 (chicken)	*S. havana*	A17	B26	C23	D18	E18	F18	28
29 (chicken)	*S. anatum*	A18	B27	C24	D19	E19	F19	29
30 (chicken)	*S. agona*	A19	B28	C25	D20	E20	F19	30

A1–A19, B1–B28, C1–C25, D1–D20, E1–E20 and F1–F19 represent the different RAPD fragment patterns with respect to each primer.
[a] Letters A–F represent the RAPD fragment product shown as a single band on the gel.

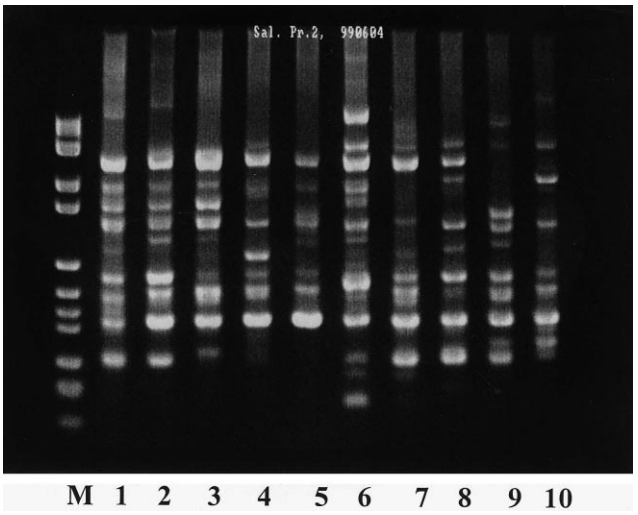

Fig. 2. Ten RAPD profiles of avian *Salmonella* from Thailand run using primer number 2 (5′-d(GTTTCGCTCC)-3′). The species were: lane 1, *S. montevideo* (strain No. 21); lane 2, *S. montevideo* (strain No. 22); lane 3, *S. montevideo* (strain No. 23); lane 4, *S. serftenberg* (strain No. 24); lane 5, *S. chester* (strain No. 25); lane 6, *S. amsterdam* (strain No. 26); lane 7, *S. muenchen* (strain No. 27); lane 8, *S. havana* (strain No. 28); lane 9, *S. anatum* (strain No. 29); lane 10, *S. agona* (strain No. 30); lane M = marker ladder VI (Boehringer-Mannheim, Mannheim, Germany).

each of the subspecies could be differentiated further into individual RAPD patterns when several primers were used in the analysis. Hence, using the combination of all six primers resulted in 30 individual and distinct RAPD patterns (Table 2).

The method of Hillis and Moritz was used to determine the genetic distances of the strains and, as expected, revealed close genetic relatedness, showing the genetic homogeneity of *S. enterica* ssp. *enterica* (Figs. 1 and 2).

The discriminatory capacity, calculated as Simpson's index of diversity, subdivided eight strains of *S. enteritidis* into six RAPD types, two strains of *S. blockley* into two RAPD types, five strains of *S. hadar* into five RAPD types, three strains of *S. typhimurium* into three RAPD types, two strains of *S. saintpaul* into two RAPD types and three strains of *S. montevideo* into three RAPD types (Table 2).

One of the major advantages of RAPD-PCR is that it can be used without previous knowledge of the nucleotide sequence of the target DNA and it is also cheap, reproducible and fast. Thus, RAPD results can be generated within 4 h in contrast with other techniques, such as Southern blot analysis which requires up to 4 days.

From this limited study it is evident that the RAPD technique seems promising not only for inter-serovar differentiation of *S. enterica* ssp. *enterica*, but dependent on the number of primers used, it is also feasible for 'fine-tuned' identification of clones and even sub-clones within a given *S. enterica* ssp. *enterica*. It is suggested that the RAPD method might be of value for screening and monitoring of the epidemiology of *Salmonella* infections in control programs both for man and animals. However, further large scale *Salmonella* typing studies along with RAPD analysis are obviously needed before the value of the RAPD technique can be fully assessed.

Acknowledgements

We thank Dr. I. Kramomthong (Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok) for providing *S. enterica* ssp. *enterica* strains, the STINT program funded by the Swedish government for financial support, Dr. K. Krovacek and L. Eriksson for valuable help in the laboratory and the expert help of secretary K. Larsson, all at the Department of Bacteriology, SLU.

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