Kasetsart J. (Nat. Sci.) 44 : 79 - 83 (2010)

PCR-based Restriction Fragment Length Polymorphism for Subtyping of *Salmonella* from Chicken Isolates

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ABSTRACT

Genotypic diversity in two flagellin genes, *fliC* and *fljB*, encoding phase-1 and phase-2 flagellin of *Salmonella enterica*, offers a potential biomarker for *Salmonella* subtyping. Forty-seven *Salmonella* isolates of 20 different serovars derived from chicken samples in Thailand were studied using the *fliC*/*fljB* PCR-based RFLP assay. With two restriction endonucleases, *Mbo*I and *Hha*I, the *fliC* showed 11 and 9 patterns, while the *fljB* showed 6 and 7 patterns respectively. Though the PCR-based RFLP test cannot replace serotyping, the assay is based on the flagellin genes encoding proteins on the bacterial surface that are related to serotyping scheme. Overall, the assay was reproducible and successfully applied to simply screen *Salmonella* serovars as an alternative subtyping test for rapid traceability of *Salmonella* contamination in chicken production.

Key words: *Salmonella,* PCR-based RFLP, flagella, subtyping

INTRODUCTION

Salmonella enterica is considered one of the major pathogens in public health worldwide. There are more than 2,500 serovars of *Salmonella* that have been recognized on the basis of antigenic properties of the cell wall lipopolysaccharide (O antigen), the phase-1 flagellar protein (H1) and the phase-2 flagellar protein (H2) (Popoff, 2001). Only limited serovars of *Salmonella* are associated with poultry and consequently of concern to public health. For example, *Salmonella enterica* serovar Enteritidis frequently has been involved with consumption of contaminated raw or under-cooked eggs (Sarwari *et al*., 2001).

The classification of *Salmonella* isolates into specific serovars is essential for epidemiologic studies and tracing the source of outbreaks (Hong *et al*., 2003). However, production and quality control of the hundreds of antisera required for serotyping are laborious and time-consuming. To circumvent the problems, many genotyping techniques, including pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and multiplex PCR have been applied as alternative methods for *Salmonella* subtyping (Luk *et al*., 1993; Chansiripornchai *et al*., 2000; Gallegos-Robles *et al*., 2008).

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Received date : 20/05/09 Accepted date : 02/09/09

PCR-based RFLP is considered a rapid, cost-effective approach with good reproducibility for molecular typing in bacterial epidemiologic studies. Many researchers have focused on the flagellin genes for *Salmonella* subtyping because most of them possess the two structural genes (*fliC* and *fljB*) that contain a hypervariable central region and a conserved flanking DNA region. While the flanking region makes it possible to design the primer sets for amplification of any *Salmonella* flagellin genes, the hypervariable central region makes it possible to differentiate their serovars by RFLP technique. Furthermore, the flagellin genes encode proteins onto the *Salmonella* surface that are related to serotyping scheme.

The RFLP profiles of *fliC* (phase 1 flagellin) and *fljB* (phase 2 flagellin) genes, obtained by digestion of each PCR products with restriction endonucleases, have clearly reflected the antigenic variation in the phase 1 and 2 flagellar antigens. The technique was developed and validated for testing a number of serovars (Dauga *et al*., 1998; Hong *et al*., 2003). The objective of this study was to determine the reproducibility and discriminatory power of the *fliC*/*fljB* PCR-based RFLP assay for simple subtyping of the field strains of *Salmonella* that had been isolated from chicken in Thailand.

MATERIALS AND METHODS

Bacterial strains and DNA templates

Forty-seven *Salmonella* isolates derived from chicken specimens were sourced from the Kamphaengsaen Veterinary Diagnostic Unit, Faculty of Veterinary Medicine, Kasetsart University, Thailand. The isolates were tested for their serovars by the WHO National *Salmonella* and *Shigella* Center Laboratory, Thailand, following the Kauffman-White scheme. For each purified isolate, DNA was extracted using the boiling method as described by Radu *et al*. (2001) and used as templates in the PCR. *Salmonella* Typhimurium strain LT2 was used as a standard control strain.

PCR amplification

The nucleotide primers for *fliC* and *fljB* amplification have been described by Dauga *et al*. (1998). The PCR assay following Hong *et al*. (2003) was performed in a reaction volume of 35 µl with a PTC-200 thermocycler (MJ Research). Each PCR reaction had a final concentration of 1X buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.7 µM of each primer, 0.6 U of Taq polymerase (Fermentas), and 4 µl of DNA template. The PCR program parameters included an initial denaturation at 94°C for 5 min followed by (i) 94°C, 1 min, (ii) 55°C, 1 min, (iii) 72°C, 1 min for 35 cycles with an additional extension at 72°C for 7 min at the end of the amplification. Amplification products were detected by electrophoresis in 1.0 % (w/v) agarose gels stained with ethidium bromide (0.5 µg/ml) in 1X TAE buffer at 100 V for 20 min. A standard marker λ/*Hin*dIII+ϕX174 DNA/*Hae*III (FINNZYMES) was utilized for estimating the size of the PCR products.

RFLP analysis

The *fliC* and *fljB* PCR products were directly digested by the restriction endonucleases *Mbo*I and *Hha*I (Fermentas). Using a modification from Hong *et al*. (2003), each digestion was performed in a reaction volume of 20 µl consisting of 15 µl of PCR product, 2 µl of 10X buffer, 5 U enzymes, at 37°C for 2.5-3 h. The RFLP patterns of PCR amplicons were examined by 1.8% (w/v) agarose gel electophoresis, stained with ethidium bromide (0.5 µg/ml) in 1X TAE buffer at 100 V for 45 min. The 100 bp DNA ladder (FINNZYMES) was used as the molecular weight marker in the RFLP analysis.

RESULTS AND DISCUSSION

Twenty serovars of *Salmonella* DNA were amplified for detection of the *fliC* and *fljB* genes. As expected, a 1.5 kbp *fliC* fragment was successfully amplified from almost all the tested serovars, except a 1.24 kbp fragment from three serovars: *S*. Corvallis, *S*. Albany, and *S*. Emek. The smaller size (1.24 kbp) of the *fliC* fragment derived from these three serovars can presumably be attributed to a deletion of a 261 bp fragment in the flagellin C gene. This suggested that the deletion of the same size can be observed not only in the variant serovar Typhi (H1:j) as described in Dauga *et al*. (1998) but in other serovars as shown in this study. According to the *fljB* gene amplification, seven serovars of *Salmonella*: *S*. Corvallis, *S*. Enteritidis, *S*. Emek, *S*. Albany, *S*. I4,12:d:-, *S*. Rissen and *S*. Agona, yielded positive PCR fragments. This was in disagreement with previous studies that reported the absence of the *fliA, hin*, or *fljB* genes in some of these serovars (Dauga *et al*., 1998; Hong *et al*., 2003).

The current study followed the PCR-based RFLP assay as described in Hong *et al*. (2003) to test the reproducibility and discriminatory power of the assay when it was applied to testing field isolates. The RFLP patterns were designated by letters A and B indicating the restriction enzyme *Mbo*I, and M and N indicating the restriction enzyme *Hha*I, following the patterns designed by Hong, *et al*. (2003). The RFLP analysis with *Mbo*I revealed 11 and 6 discriminated restriction profiles for *fliC* and *fljB* respectively (Table 1 and Figure 1). There were 9 distinct profiles for *fliC* and 7 profiles for *fljB* obtained when *Hha*I endonuclease was used (Figure 2). The combination of these two

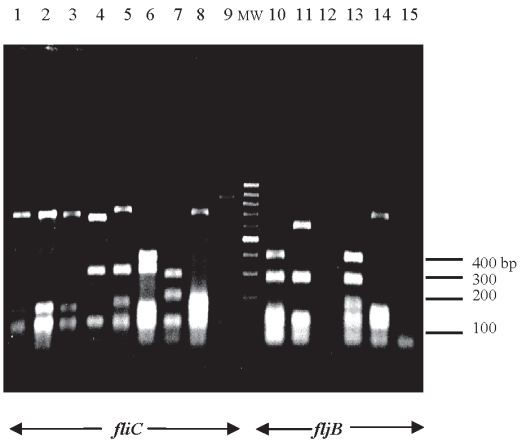

Figure 1 *Salmonella fliC* and *fljB* PCR-based RFLP profiles from different *Salmonella* serovars with restriction enzyme *Hha*I. MW, 100 bp ladder. (A) Lane 1, *S*. Emek. Lane 2, *S*. Albany. Lane 3 and 10, *S*. Give. Lane 4 and 11, *S*. Bovismorbificans. Lane 5, *S*. 1,4,12:d:-. Lane 6 and 12, *S*. Mbandaka. Lane 7 and 13, *S*. Newport. Lane 8 and 14, *S*. Livingstone. Lane 9 and 15, *S*. Blockley.

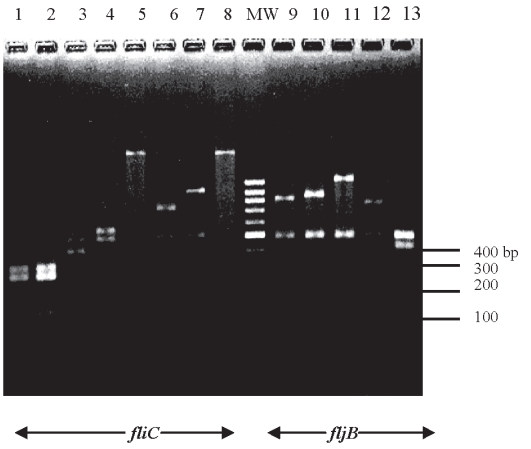

Figure 2 *Salmonella fliC* and *fljB* PCR-based RFLP profiles from different *Salmonella* serovars with restriction enzyme *Mbo*I. MW, 100 bp ladder. Lane 1, *S*. Emek. Lane 2, *S*. Albany. Lane 3 and 9, *S*. Give. Lane 4 and 10, *S*. Bovismorbificans. Lane 5, *S*. 1,4,12:d:-. Lane 6 and 11, *S*. Mbandaka. Lane 7 and 12, *S*. Newport. Lane 8 and 13, *S*. Livingstone.

restriction profiles could differentiate over 80% of the serovars from each other (Table 1). This combination, however, was not able to differentiate *S. enterica* serovars Hadar and Virchow, Emek and Albany. Each pair of serovars revealed the same RFLP patterns as shown in Table 1. Moreover, each isolate, among four isolates identified as serovar Schwarzengrund, had different RFLP patterns, with one pattern being the same as the pattern of *S.* Schwarzengrund that was described in Hong *et al*. (2003). This may be explained by Dauga *et al*. (1998), who considered that the high diversity of flagellin antigens makes it difficult to find a replacement technique for subtyping all *Salmonella* serovars. They also found that the flagella genes-RFLP was unable to discriminate among serovars that harbor *fliC* encoding d, i or r, i antigens. Theoretically, flagella genes-RFLP is based on the discrimination technique of serotyping, however RFLP patterns often split flagellar types in a different way to serotyping.

Even though the PCR-based RFLP analysis was unable to differentiate all the serovars, this assay technique was useful for simple subtyping and tracing the contamination of many common serovars found in Thailand. Remarkably, some extra RFLP patterns of several serovars could be added that had not been described in the previous references. The identical RFLP patterns of *Salmonella* serovars between the current study

Table 1 The PCR-based RFLP profiles of *Salmonella fliC* and *fljB* genes with *Mbo*I and *Hha*I.

S. enterica serovar	No. of isolate	*fliC* RFLP (*Mbo*I)	*fljB* RFLP (*Mbo*I)	*fliC* RFLP (*Hha*I)	*fljB* RFLP (*Hha*I)
Albany **	3	A15	-	M1	-
Bovismorbificans**	1	A5	B3	M3	N5
1,4,12:d:-**	1	A6	-	M14	-
Rissen**	1	A8	-	M15	-
Typhimurium	2	A4	B3	M3	N3
Agona	1	A2	-	M15	-
Blockley**	2	A3	B9*	M18	N10*
Mbandaka	1	A4	B4	M4	N2
Virginia**	1	A6	B3	M14	N4
Livingstone**	1	A6	B5	M1	N9
Hadar	10	A4	B3	M4	N2
Schwarzengrund	4	A6	B2	M14	N8
Orion**	1	A16*	B4	M17	N2
Newport	4	A1	B3	M19	N4
Weltevreden**	2	A5	B8	M14	N9
Virchow**	2	A4	B3	M4	N2
Emek**	2	A15*	-	M1	-
Give	1	A3	B2	M1	N8
Corvallis**	4	A7	-	M1	-
Enteritidis	3	A13	-	M6	-
Total[a]	**47**	**11**	**6**	**9**	**7**

* = RFLP patterns not mentioned in Hong *et al*. (2003).

** = *Salmonella* serovars not treated in Hong *et al*. (2003).

[a] = Total number of isolates/ total number of RFLP patterns.

The patterns were named following the style in Hong *et al*. (2003).

and Hong *et al*. (2003) suggested that the assay was reproducible.

Since the whole process for sera preparation and serotyping itself is expensive, laborious and time-consuming, the molecular subtyping method is promising as a simpler and rapid alternative approach. However, it should be noted that the great value of traditional serotyping is important for the epidemiological study of a high variety of *Salmonella* serovars. Advantageously, the combination of the conventional serotyping with a molecular screening approach will offer more rapid traceability of the strain contamination.

ACKNOWLEDGEMENTS

This study was supported by the Thailand Research Fund. Srisamai Wiriyarumpa, from the Department of Veterinary Public Health, Kasetsart University, is thanked for providing laboratory facilities.

LITERATURE CITED

Chansiripornchai, N., P. Ramasoota, A. Bangtrakulnonth, J. Sasipreeyajan and S. B. Svenson. 2000. Application of randomly amplified polymorphic DNA (RAPD) analysis for typing avian *Salmonella enterica* subsp. *enterica*. **FEMS Immuno. Med. Microbiol.** 29: 221-225.

Dauga, C., A. Zabrovskaia and P. A. D. Grimont. 1998. Restriction fragment length polymerphism analysis of some flagellin genes of *Salmonella enterica*. **J. Clin. Microbiol.** 36: 2835-2843.

Gallegos-Robles M. A., A. Morales-Loredo, G. Alvarez-Ojeda, A. Vega-P, Y. Chew-M, S. Velarde and P. Fratamico. 2008. Identification of *Salmonella* serotypes isolated from cantaloupe and chile pepper production systems in Mexico by PCR-restriction fragment length polymorphism**. J. Food Prot.** 71: 2217-2222.

Hong, Y., T. Liu, C. Hofacre, M. Maier, D. G. White, S. Ayers, L. Wang and J. J. Maurer. 2003. A restriction fragment length polymorphism-based polymerase chain reaction as an alternative to serotyping for identifying *Salmonella* serotypes. **Avian Dis.** 47: 387-395.

Luk, J. M., U. Kongmuang, P. R. Reeves and A. A. Lindberg. 1993. Selective amplification of abequose and paratose synthase genes (*rfb*) by polymerase chain reaction for identification of *Salmonella* major serogroups (A, B, C2, and D). **J. Clin. Microbiol.** 31: 2118-2123.

Popoff, M.Y. 2001. **Antigenic Formulas of the *Salmonella* Serovars**, 8th ed. W. H. O. Collaborating Centre for Reference and Research on *Salmonella*. Institute Pasteur, Paris, France.

Radu, S., W. L. Ooi,. G. Rusul, M. I. A. Karim and M. Nishibuchi. 2001. Detection of *Escherichia coli* O157:H7 by multiplex PCR and their characterization by plasmid profiling, antimicrobial resistance, RAPD and PFGE analyses. **J. Microbiol. Meth.** 46: 131-139.

Sarwari A. R., L. S. Magder, P. Levine, A. M. McNamara, S. Knower, G. L Armstrong, R. Etzel, J. Hollingsworth and J. G. Jr Morris. 2001. Serotype distribution of *Salmonella* isolates from food animals after slaughter differs from that of isolates found in humans. **J. Infect. Dis.** 183: 1295-1299.