Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Oct - Dec 2009 / v.11 / n.4 / 271 - 276

Molecular differentiation of *Salmonella* Gallinarum and *Salmonella* Pullorum by RFLP of *fliC* gene from Brazilian isolates

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Keywords

FliC gene, differentiation, *Salmonella* Gallinarum, *Salmonella* Pullorum.

ABSTRACT

Although *Salmonella* Pullorum and *Salmonella* Gallinarum cause different diseases in poultry, they are very similar. Both are non-motile and present the same somatic antigenic structure. They are differentiated by biochemical tests. Certain atypical strains are very difficult to distinguish. They do not produce the expected results when dulcitol and ornithine descarxboxylase tests are performed. Therefore, additional tests could be helpful. Many studies have chose the part I of the gene that encodes flagellin (fliC) to differentiate serotypes. Most *Salmonella* strains have two structural genes (fliC and fliB) that encode flagellins. Non-motile strains generally present these structural genes, but are not able to build a functional flagellum. It was demonstrated that enzymatic restriction of the amplified fliC gene using Hinp1I enzyme can differentiate SG from SP. In the present study, this method was adopted to analyze 14 SP and 22 SG strains, including some strains with atypical results in biochemical tests assessing the utilization of dulcitol and ornithine. The results showed that all SG strains were broken by the enzyme, whereas the 14 SP strains were not.

INTRODUCTION

According to Kauffman-White scheme (Grimont & Weill, 2007), more than 2,500 serovars have been described based on the antigen structure of lipopolysaccharide (O antigen) and flagellar proteins (H) (Boyd *et al*., 1993). However, only a limited set of *Salmonella* serotypes have been associated with poultry diseases and human salmonellosis. *Salmonella* enterica serotype Gallinarum (SG) and *Salmonella enterica* serotype Pullorum (SP) are non-motile host-specific avian pathogens. *Salmonella* Pullorum causes Pullorum disease, which is mainly characterized by septicemia in young birds, with white diarrhea and mortality (Berchieri Jr. & Freitas Neto, 2009). In general, adult birds do not show symptoms of the disease and may transmit SP to the progeny. *Salmonella* Gallinarum causes fowl typhoid, an acute septicemic disease with high mortality and morbidity that affects mostly adult birds, although it is very virulent to birds of any age. Some countries are considered free from SP and SG; however, these are sometimes reported, and are still a matter of concern in the poultry industry (Shivaprasad, 2000).

The differentiation between SP and SG is very important from epidemiological and preventive perspectives. They are very similar, and cannot be distinguished by conventional serological methods. The differentiation basically takes into account their biochemical characteristics. The standard methods take approximately 5 to 7 days, and are very time-consuming and expensive. The main biochemical characteristics assessed are the capacity to use dulcitol by SG, but not by SP, and ornithine decarboxylation by SP, but not by SG. Intermediate

Arrived: August/2009
Approved: October/2009

strains with atypical behavior in the dulcitol and ornithine descarboxilase biochemical tests were described by Li *et al*. (1993). SP and SG atypical strains were isolated in Brazil (Ribeiro *et al*., 2009).

Lately, biochemical methods have been complemented by DNA-based molecular techniques, because of their sensitivity, specificity, and swiftness. Such methods include restriction fragment length polymorphism (RFLP), which is sometimes associated to PCR (PCR-RFLP), IS200 profiling, ribotyping, pulse-field gel electrophoresis (PFGE), and single-strand conformational polymorphism (SSPC) (Christensen *et al*., 1992; Olsen *et al*., 1996; Kwon *et al*., 2000). Many studies used part I of the gene that encodes flagellin (fliC) to differentiate serotypes. Most *Salmonella* strains have two structural genes (fliC and fliB) that encode flagellins. Non-motile strains generally exhibit these structural genes, but are unable to build up a functional flagellum (Popoff *et al*., 1992).

The objective of the present study was to differentiate SP and SG isolated in Brazil, including strains with atypical biochemical behavior, by a single method: restriction of the amplified fliC gene using Hinp 1I enzyme.

MATERIAL AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. These strains were obtained from the Brazilian governmental laboratories LANAGRO, FIOCRUZ, and Adolfo Lutz Institute.

DNA extraction. Bacterial DNA was prepared as described by Soumet *et al*. (1994) with modifications. SP and SG strains were inoculated in Luria Bertani (LB) broth (Invitrogen 12780-052) and incubated 37ºC for 24h in shaking incubator (100rpm). One ml of each culture was centrifuged for 3min at 13000xg (4ºC).the resulting pellets were washed twice in 500µL 1X TAE buffer (Tris, Acetic Acid, and EDTA; pH 8.0), and centrifuged at 13000xg for 3min (4ºC). The pellet was resuspended in 200µL sterile water, boiled for 8min, and stored at -20ºC.

PCR primers. The following two primers were used for the amplification of flagellin gene phase 1: CTGGTGATGACGGTAATGGT (fliCF: 866-885) and CAGAAAGTTTCGCACTCTCG (fliCR: 1063-1044) (Kwon *et al*., 2000).

Amplification of the fliC gene by PCR. A reaction mixture containing 16.8µL ultra pure water (Gibco), 2µL PCR Buffer 10x, 0.7µL d-NTP (2mM), 0.8µL MgCl2 (50mM), 0.5µL of each primer, 0.5µL Taq DNA polymerase, and 3.2µL DNA, was prepared. The thermocycler was programmed with 1 cycle of 94ºC for 5min, 35 three-step cycles (denaturation at 94ºC for 30s, annealing at 58ºC for 10s, extension at 72ºC for 20s), and a final cycle at 72ºC for 7min. The amplicons were analyzed by electrophoresis in 1.5% agarose gel for 1h and 80V. Product size was compared to the 50 pb ladder (Fermentas SM1211) after ethidium bromide staining.

Table1. Strains of *Salmonella* Gallinarum and *Salmonella* Pullorum used in this study.

Strain nº	Isolate designation	*Salmonella*	ODC	Dulcitol
1	Fiocruz 31	Gallinarum	(-)	(+)
2	Fiocruz 33	Galinarum	(-)	(+)
3	Fiocruz 34	Gallinarum	(-)	(+)
4	Fiocruz 35	Gallinarum	(-)	(+)
5	Fiocruz 36	Gallinarum	(-)	(+)
6	Lanagro 10	Gallinarum	(-)	(+)
7	Lanagro 15	Galinarum	(-)	(+)
8	Lanagro 188-1C	Gallinarum	(-)	(+)
9	Lanagro 188-2	Gallinarum	(-)	(+)
10	Lanagro ATCC	Gallinarum	(-)	(+)
11	Unesp 256/87	Galinarum	(-)	(+)
12	Unesp 291/90	Gallinarum	(-)	(+)
13	Unesp 292/90	Gallinarum	(-)	(+)
14	Unesp 293/90	Gallinarum	(-)	(+)
15	Unesp 297/91	Galinarum	(-)	(+)
16	Unesp Greek	Gallinarum	(-)	(+)
17	Unesp 5441-6	Gallinarum	(-)	(+)
18	Unesp Nanabi	Gallinarum	(-)	(+)
19	Unesp 7285-b	Gallinarum	(-)	(+)
20	Unesp Hakim-Leban	Gallinarum	(-)	(+)
21*	Fiocruz 32	Gallinarum	(+)	(+)
22	449/87	Pullorum	(+)	(-)
23	Fiocruz 1	Pullorum	(+)	(-)
24	Fiocruz 2	Pullorum	(+)	(-)
25	Fiocruz 3	Pullorum	(+)	(-)
26	Fiocruz 4	Pullorum	(+)	(-)
27	Fiocruz 5	Pullorum	(+)	(-)
28	Fiocruz 6	Pullorum	(+)	(-)
29	Fiocruz 7	Pullorum	(+)	(-)
30	Lanagro 11	Pullorum	(+)	(-)
31*	Lanagro 335-28	Pullorum	(-)	(-)
32*	Lanagro 337-28	Pullorum	(-)	(-)
33	Lanagro ATCC	Pullorum	(+)	(-)
34	Unesp 21	Pullorum	(+)	(-)
35	SG9R	Gallinarum	(-)	(+)

ODC ornithine decarboxylase; *atypical strains.

PCR-RFLP analysis. Digestion solution was prepared with 5µL of the PCR product, 1µL of Hinp 1I buffer (10x), 0.1µl of *Hinp* 1I enzyme, and 3.9µl of ultra-pure water (Gibco). After incubation at 37ºC for 1h, RFLPs were determined by electrophoresis of the digested DNA in 4% agarose gel for 4h at 40V. Product size of the products was analyzed in comparison to the 50bp ladder (Fermentas SM1211).

RESULTS

Amplification of the fliC gene. The expected 197 bp fragment of the fliC gene was successfully amplified from all the 22 *Salmonella* Gallinarum strains and 14 *Salmonella* Pullorum strains tested. Nonspecific pairing was not observed. The amplicons are shown in Figure 1.

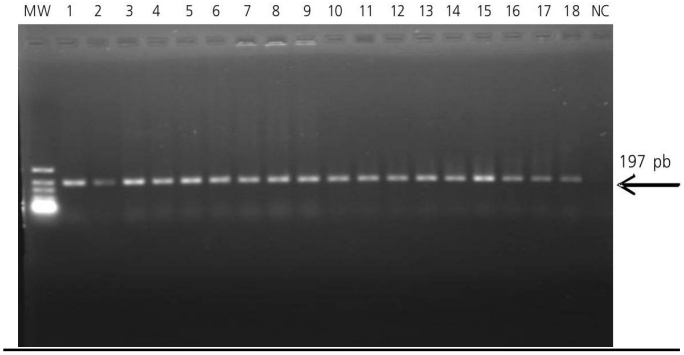

Figure 1. Electrophoresis of *fliC* gene amplicons from SP and SG samples. MW Molecular weight marker 50bp DNA ladder. SP samples (lanes 1 to 9); SG samples (lanes 10 to 18). NC negative control (ultra pure water).

PCR-RFLP analysis. Twenty-two SG strains and 14 SP strains were analyzed. Digestion of SG amplicons with Hinp 1I yielded two bands, of 115 and 82 bp, while no change in SP amplicons was observed, since no digestion occurred (Figure 2).

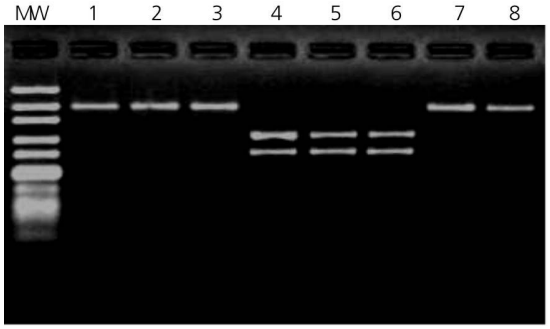

Figure 2. Eletrophoretic analysis of the *fliC* gene after enzymatic treatment with *Hinp* I1restriction enzyme. MW Molecular weight marker 50bp DNA ladder; lanes 1, 2, and 3: digestion product of SP samples; lanes 4, 5, and 6: restriction fragments of the *fliC* gene from SG samples; lane 7: P amplicons not submitted to enzymatic restriction (SP positive control); lane 8: SG amplicons not submitted to digestion (SG positive control).

DISCUSSION AND CONCLUSIONS

Salmonella Gallinarum and *Salmonella* Pullorum are non-motile pathogens that infect poultry and other galliform birds (Barrow *et al*., 1994; Shivaprasad, 2000). *Salmonella* Gallinarum is responsible for fowl typhoid, and *Salmonella* Pullorum causes pullorosis, which is characterized by white diarrhea in chicks (Pomeroy, 1984). SG, found mostly in adult birds, can also affect young birds. *Salmonella* Pullorum may infect older chickens, causing symptoms similar to those observed in fowl typhoid (Wray *et al*., 1996). Therefore, the proper identification of both *Salmonella* serovars is very important from the epidemiological and control standpoints. The differentiation between SG and SP is based on biochemical characteristics (Cox & Willians, 1976); Ewing, 1986). These serovars cannot be distinguished by conventional serological methods because they have very similar antigenic O-factors. Biochemical methods are currently complemented by DNA-based molecular techniques. Most *Salmonella* strains exhibit two structural genes (fliC and fliB) that encode flagellins. Only of these structural genes is expressed in the bacterium at a time. Non-motile strains generally have these structural genes, but are unable to build up a functional flagellum (Popoff *et al*., 1992). Early studies showed that *Salmonella* Pullorum and *Salmonella* Gallinarum have a cryptic structural gene, flagellin (Zinder & Ledeberg, 1952). Several sequences of the gene encoding phase 1 flagellin (fliC) are available (Selander *et al*. 1992; Li *et al*., 1993; Kwon *et al*., 2000). The distal parts of the fliC alleles are conserved regions, making this gene in any serotype suitable for easy amplification, whereas the central region of the fliC gene is hyper variable, making it a target for differentiation among *Salmonella* serotypes (Wei & Joys, 1985; Kilger & Grimont, 1993; Dauga *et al*., 1998). Genetic events, such as point mutations, lateral transfer, and recombination, may explain the genetic diversity of *Salmonella* flagellin genes (Smith *et al*. 1990); Li *et al*., 1994) The SG and SP fliC gene represent allelic variants, and differ only in two codons (316 and 339) (Li *et al*., 1993). Kwon *et al*. (2000) showed that the Hinp1I enzyme recognizes one cleavage site in SG (codon 316), but not in SP.

The PCR-RFLP system has been frequently used in differentiation techniques because it is cheap and easy to perform. In our study, the fliC gene in SP (14 strains) and SG (22 strains), including intermediate strains dulcitol and ornithine descarboxylase positive and negative isolates in Brazil, were amplified. PCR-amplicons (197bp) were digested with the Hinp1I enzyme. Two fragments were obtained (82bp and 115bp) for all SG strains, including one dulcitol and ornithine descarboxylase positive strain, whereas no digestion was observed in the 14 SP strains, which gene

remained unchanged (197bp), including the two atypical strains.

The RFLP-PCR flagellar typing scheme was successfully applied to serotype identification in 112 *Salmonella* isolates obtained from poultry and poultry environment (Hong *et al*., 2003). Kilger & Grimont (1993) showed practical application of restriction patterns of fliC gene using a mixture of endonucleases (TaqI and ScaI) to differentiate Pullorum and Gallinarum strains from non-motile *Salmonella* Typhi and some flagellar strains of *Salmonella*; however, this method was not useful to differentiate Gallinarum from Pullorum strains. Kwon *et al*. (2000) demonstrated that fliC gene RFLP-PCR using Hinp 1I enzyme can be successfully applied for differentiate these two *Salmonella* serovars in Korean isolates. However, the authors tested only eight strains of each serovar, all presenting typical biochemical behavior. In the present study, we were able to demonstrate that the use of fliC gene restriction patterns is an useful method to allow the differentiation between strains of *S.* Pullorum and *S.* Gallinarum isolated in Brazil, including those with atypical biochemical behavior. Therefore, our results reinforce that this method may be adopted to differentiate SP from SG.

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