

## MOLECULAR DIFFERENTIATION BETWEEN *SALMONELLA ENTERICA* SUBSP *ENTERICA* SEROVAR PULLORUM AND *SALMONELLA ENTERICA* SUBSP *ENTERICA* SEROVAR GALLINARUM

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Submitted: September 01, 2007; Returned to authors for corrections: March 03, 2008; Approved: February 15, 2009.

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### ABSTRACT

*S. Pullorum* (SP) and *S. Gallinarum* (SG) are very similar. They are the agents of pullorum disease and fowl typhoid, respectively, and the two diseases are responsible for economic losses in poultry production. Although SP and SG are difficult to be differentiated in routine laboratory procedures, the ability to metabolize ornithine is a biochemical test that may be used to achieve this aim. While SP is able to decarboxylate this amino acid, SG is not. However, the isolation of strains showing atypical biochemical behavior has made this differentiation difficult. One of the genes associated with the metabolism of the amino acid ornithine is called *speC*, and is found in both serovars. The analysis of 21 SP and 15 SG strains by means of PCR did not enable the differentiation of the two serovars, because fragments produced were identical. However, after enzymatic treatment with restriction enzyme *Eco* RI, the band pattern of each serovar showed to be different, even in samples of atypical biochemical behavior. This fact enabled the standardization of the technique for a quick and safe differentiation of serovars Pullorum and Gallinarum.

**Keywords:** *Salmonella* Pullorum, *Salmonella* Gallinarum, differentiation, PCR

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### INTRODUCTION

Salmonellosis are among the main infections affecting commercial poultry. They are responsible for direct and indirect losses to poultry production, besides their public health importance (1,3). These bacteria infect poultry and may lead to pullorum disease, caused by *Salmonella* Pullorum (SP); fowl typhoid, caused by *Salmonella* Gallinarum (SG) and fowl paratyphoid, caused by any other salmonella but these ones (2).

Serovars Pullorum and Gallinarum are characterized as *Salmonella enterica* subsp *enterica*, group D (somatic antigens 1, 9 and 12) and show antigenic and biochemical similarities. Both do not show flagella and grow slowly in culture media, different from the other salmonellas (2).

Although pullorum disease affects birds at any age, mortality rates are higher in young animals. Animals that survive may become carriers, may not meet expected animal production parameters and may produce contaminated eggs. The history of the disease and the development of industrial poultry breeding are mingled; artificial incubation of eggs was highly influenced by the occurrence of the disease, because it led to high mortality and culling rates among chicks. As eggs of different origins were incubated together, the agent of pullorum disease was transferred to other birds of commercial interest. In Brazil, there were several pullorum disease outbreaks in the 1980s and 1990s, even involving the occurrence of a strain of atypical biochemical behavior (2,15).

Although fowl typhoid is caused by *Salmonella* very similar to the pullorum disease agent, its host-parasite relationship

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with the bird is markedly different. *S. Gallinarum* is highly pathogenic, capable of causing systemic infection and may affect birds of any age. However, its occurrence is more common among adult birds. Mortality caused by fowl typhoid may reach 40-80% of the flock. Fowl typhoid was initially described in England, at the end of the 19<sup>th</sup> century, and it is considered to be a disease of developing countries (14). In Brazil, it was diagnosed in laying poultry facilities, but it may also affect adult breeding birds used in the production of egg-laying or meat chicks. According to data from the *Laboratório de Patógenos Entéricos* [Enteric Pathogens Laboratory] at Instituto Adolfo Lutz (São Paulo – Brazil), among the 372 *Salmonella* samples identified in poultry breeding facilities from 1991 to 1995, 21 strains were *S. Pullorum* and 35 were *S. Gallinarum* (2,10,15).

These two salmonellas are very similar in relation to antigenic and biochemical characteristics, but some tests have been used to differentiate between them, such as glucose, maltose and dulcitol fermentation, as well as utilization of d-tartrate, mucate, cellobiose, salicine and gelatin. According to TRABULSI & EDWARDS (17), the ability or inability to assimilate ornithine would be the main biochemical test enabling the differentiation between SP and SG. However, the isolation of strains showing atypical biochemical behavior in this test makes it difficult to separate them (4,14).

As early as 1935, NOBREGA (9) mentioned the difficulty in differentiating between *S. Pullorum* and *S. Gallinarum* only considering the biochemical and serological characteristics these strains. Although LANGENEGGER *et al.* (6) just observed the occurrence of strains showing typical biochemical and serological reactions, they mentioned the occurrence of atypical strains, with characteristics different from those observed in standard bacteria.

The differentiation between these two salmonellas is very important both in an epidemiological standpoint and in relation to control programs, once sanitary measures to be adopted would be different (14).

Kauffmann-White scheme (12) enables the classification of the genus *Salmonella* in more than 2,500 serovars using the combination of flagellar and somatic antigens. However, the differentiation between SP and SG is still not possible, once they belong to the same serogroup (1,9,12 :-:-) and they do not have flagella. Because of these difficulties, alternative measures were developed, such as the use of molecular methods (13).

OLSEN *et al.* (11) analyzed SP and SG strains using molecular typing methods and reported their extreme similarity in terms of chromosome constitution. The genetic component that could be used in the differentiation between SP and SG would be genes related to ornithine assimilation (*speC* and *speF*), once the expression is different in the two serovars.

CUNNINGHAM-RUNDLES & MAAS (5) observed mutant *Escherichia coli* colonies that did not grow adequately because they lacked ornithine decarboxylase (ODC) synthesis,

determined by the gene *speC*. The same authors demonstrated that supplementation with putrescine or spermidine enabled normal growth.

Putrescine is an amine of living cells that has an important role as a precursor of spermidine. These two substances are related to cell growth and its regulation. Except in certain mutants, all organisms studied were able to synthesize putrescine, mostly by means of an enzyme called ornithine decarboxylase, which converts ornithine into putrescine (17).

The genes *speC* and *speF*, related to ornithine decarboxylation, are present both in SP and SG. Its size are similar in serotypes Pullorum, Gallinarum, Typhimurium and different in *S. Typhi*. In SP, the genes are expressed and the bacterium is positive for ornithine; in SG, the genes are not expressed and ornithine results are negative (4,7,16). Although this is the standard result expected, the occurrence of atypical reactions both in SP and SG makes it difficult to differentiate between these serovars.

Although genes *speC* and *speF*, are similar in SP and SG, it could show differences in relation to the action of restriction enzymes (8,14).

## MATERIALS AND METHODS

### Bacterial strains

Strains used were obtained by the Laboratório Nacional Agropecuário (LANAGRO-SP) in reference centers in Brazil. Strains kept and/or isolated in the poultry pathology laboratory at FCAV/UNESP – Jaboticabal were also used. These strains came from Brazilian commercial poultry flocks and two of them came from the ATCC (American Type Collection Culture). Strains were previously identified in Fundação Oswaldo Cruz (FIOCRUZ-RJ) and Instituto Adolfo Lutz (IAL-SP), based on biochemical behavior and antigenic tests using serum anti-somatic and flagellar antigens of *Salmonella*. Thirty six *Salmonella* samples kept in nutritive agar (DIFCO -213000) were used (21 *S. Pullorum* and 15 *S. Gallinarum*, including strains showing atypical biochemical behavior in relation to ornithine).

The biochemical characteristics of all strains were tested for: urease, indole, H<sub>2</sub>S/TSI, motility, sucrose, glucose, gás, dulcitol, maltose, mucate, salicin, cellobiose, lysine, ornithine, d-tartrate, Jordan tartrate, gelatinase, citrate e malonate.

SP and SG samples were cultured in LB broth (Luria Bertani - Invitrogen 12780-052), overnight at 37°C, under stirring. After that, samples were cultured in Petri dishes containing LB Agar and were incubated in the same conditions. A colony was lightly touched and directly immersed in a 1.5 mL eppendorf tube containing the PCR reagents.

### Primers and PCR conditions

The primers for PCR amplification of *speC* and *speF* genes were based on previously described sequences (8). A forward

primer, *speC*-1 (5'-GAAATC AAT GAA TAT TGC CG -3') and a reverse primer, *speC*-4 (5'-ATC GGC ATC GGT CTC GCT ATA TA -3'); a forward primer, *speF*-1 (5'-TTA GCC GTC ATT GCC CGG ATT -3') and a reverse primer, *speF*-4 (5'-ACG AGG TTT AAT GAC GTA GC -3') were used. Amplification reaction mixtures contained 30 µL X-mix (916 µL H<sub>2</sub>O milli-Q, 120 µL 10X buffer, 120 µL dNTP (2 mM), 36 µL MgCl<sub>2</sub>); 0,5 µL of each primer 1 e 4 (*speC* or *speF*) and 0,4 µL taq DNA polymerase (Invitrogen 10342-020). The cycling parameters were 92°C for 3 min, followed by 24 cycles including denaturation at 92°C for 20seg, annealing at 50°C for 1min, extension at 72°C for 3min, and a final extension cycle of 72°C for 5min. The amplification products were observed by electrophoresis on 1% agarose gel and the size of the products was analyzed in comparison to a 1Kb ladder M. W. size marker (GIBCO) after ethidium bromide staining.

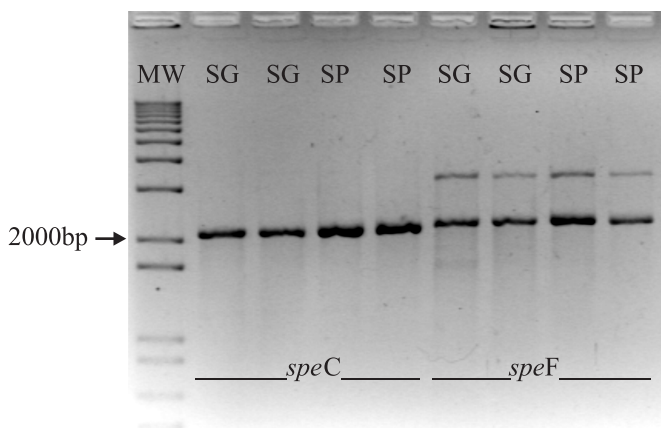
**Restriction enzyme - *Eco* RI, *Xba* I and *Sal* I**

Five microliters of PCR product was added to 1 µL of 10X reaction buffer, 1 µL of enzyme [*Eco* RI (Invitrogen 15202-013) or *Sal* I (Invitrogen 15217-011) or *Xba* I (Invitrogen 15226-012)] and 3 µL of DW and incubated at 37°C for 1:30h. The products were analyzed by the same conditions written above.

**RESULTS**

**Amplification of the *speC* and *speF* genes**

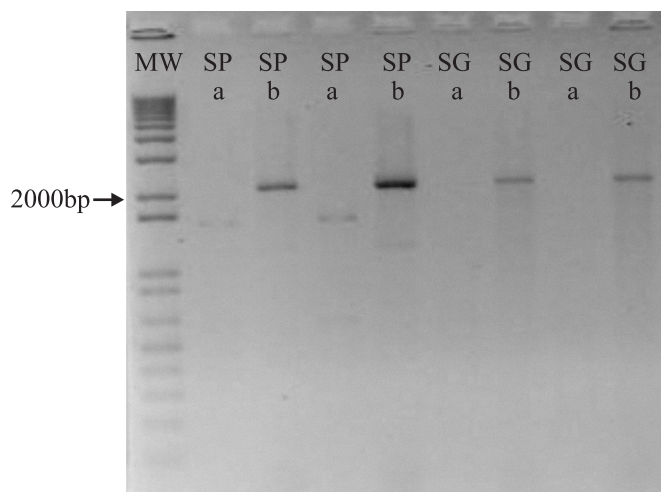
In this study, fragments of both genes was successfully amplified from all 21 strains of *S. Pullorum* and 15 of *S. Gallinarum* analyzed (Fig. 1).



**Figure 1.** Electrophoretic analysis of genes *speC* and *speF*. Lane 1: Molecular weight marker - 1Kb; lines 2, 3, 6, 7 (SG ATCC); lanes 4, 5, 8, 9 (SP ATCC); lines 2-5 (gene *speC*) and lines 6-9 (gene *speF*).

**Differentiation of *S. Pullorum* from *S. Gallinarum***

In this study, strains of *S. Pullorum* and *S. Gallinarum* were analyzed after treatment with restriction enzymes *Eco* RI, *Xba* I and *Sal* I. Differences in the banding pattern of serovars were noted after PCR amplification and treatment with both *Eco* RI and *Xba* I. In the Fig. 2, *S. Pullorum* showed one band and *S. Gallinarum* showed none band after PCR amplification and treatment with *Eco* RI. The enzyme *Sal* I not differentiated the two serovars because of the identical bands observed.



**Figure 2.** Electrophoresis on agarose gel of gene *speC* after treatment with enzyme *Eco* RI showing: Molecular Weight marker, 1Kb DNA ladder (lane 1); the reaction specific for *S. Pullorum* (lines 2-3 for strain SP ATCC and 4-5 for strain SP 449/87) and specific for *S. Gallinarum* (lines 6-7 for strain SG ATCC and 8-9 for strain SG FIOCRUZ 31). The letter “a” in the gel refer the strain after enzymatic treatment and the letter “b” refer the PCR amplification.

**DISCUSSION**

*S. Pullorum* and *S. Gallinarum* are very similar in relation to their antigenic and biochemical characteristics, and some biochemical tests have been used in order to differentiate between them, such as ability to metabolize ornithine (16). As this characteristic is also found in atypical strains, the separation between the two serovars have been difficult (4,14), a fact that have motivated the use of molecular methods.

Genes *speC* and *speF*, related to ornithine metabolism, are very similar in serovars *Pullorum*, *Gallinarum* and *Typhimurium*. These genes have already been sequenced in the latter strain (8). In the present study, the amplification of the two genes produced identical bands, both in SP and SG.

However, the comparison with the results by McClelland *et al.* (2001) showed that there was unspecific amplification of gene *speF*, leading to the production of a fragment greater than was expected. Because of this, the gene was excluded from the later stages of the study.

Even with similar size in the two serovars, it is possible that the use of restriction enzymes would produce different fragments of gene *speC* (14). During the standardization of the methodology, this gene was amplified and the PCR product was tested with

enzymes *Eco* RI, *Xba* I and *Sal* I with later analysis of the fragments. The use of enzyme *Sal* I was not able to differentiate between serovars Pullorum and Gallinarum. On the other hand, enzymes *Eco* RI and *Xba* I showed similar results. Due to the cost of these reagents, the enzyme *Eco* RI was preferred.

The amplification of gene *speC* and the treatment with enzyme *Eco* RI in SP strains enabled the visualization of one only band in electrophoresis. The same methodology in SG samples did not produce any band (Table 1).

**Table 1.** Results of biochemical analysis (ornithine), amplification of genes (*speC* and *speF*) and treatment of enzymes (*Sal* I, *Xba* I and *Eco* RI).

Strains	ornithine	PCR amplification (gene <i>speC</i> )	PCR amplification (gene <i>speF</i> )	Enzyme <i>Sal</i> I	Enzyme <i>Xba</i> I
SG FIOCRUZ 31	(-)	+	+	no band	no band
SG FIOCRUZ 32	(-)	+	+	no band	no band
SG FIOCRUZ 33	(-)	+	+	no band	no band
SG FIOCRUZ 34	(-)	+	+	no band	no band
SG FIOCRUZ 35	(-)	+	+	no band	no band
SG FIOCRUZ 36	(-)	+	+	no band	no band
SG LANAGRO 10	(-)	+	+	no band	no band
SG LANAGRO 15	(-)	+	+	no band	no band
SG LANAGRO 188-1C	(-)	+	+	no band	no band
SG LANAGRO 188-2	(-)	+	+	no band	no band
SG UNESP HAKIM LEBANM	(-)	+	+	no band	no band
SG UNESP 256/87	(-)	+	+	no band	no band
SG UNESP 291/90	(-)	+	+	no band	no band
SG UNESP 292/90	(-)	+	+	no band	no band
SG UNESP 293/90	(-)	+	+	no band	no band
SG UNESP 297/91	(-)	+	+	no band	no band
SG UNESP 372 GREEK	(-)	+	+	no band	no band
SG UNESP 5441-b	(-)	+	+	no band	no band
SG UNESP 72-805 NANABI	(-)	+	+	no band	no band
SG UNESP 7285-b	(-)	+	+	no band	no band
SG LANAGRO ATCC	(-)	+	+	no band	no band
SP 449/87	(-)	+	+	no band	no band
SP FIOCRUZ 1	(-)	+	+	no band	no band
SP FIOCRUZ 2	(-)	+	+	no band	no band
SP FIOCRUZ 3	(-)	+	+	no band	no band
SP FIOCRUZ 4	(-)	+	+	no band	no band
SP FIOCRUZ 5	(-)	+	+	no band	no band
SP FIOCRUZ 6	(-)	+	+	no band	no band
SP FIOCRUZ 7	(-)	+	+	no band	no band
SPLANAGRO 11	(-)	+	+	no band	no band
SPLANAGRO 335-26	(-)	+	+	no band	no band
SPLANAGRO 337-28	(-)	+	+	no band	no band
SP UNESP 21	(-)	+	+	no band	no band
SPLANAGRO ATCC	(-)	+	+	no band	no band

(\* +) and (\* -) = atypical strains in biochemical analysis (ornithine).

It was observed in the study of gene *speC* that enzymatic treatment with enzyme *Eco* RI may be applied to SP and SG differentiation, even when samples show atypical biochemical behavior in relation to ornithine metabolism.

#### ACKNOWLEDGEMENT

We thank LANAGRO-SP and FCAV-UNESP for providing strains of Pullorum and Gallinarum. This work was supported by the FCAV-UNESP.

#### RESUMO

##### **Diferenciação molecular entre *Salmonella enterica* subsp *enterica* serovar Pullorum e *Salmonella enterica* subsp *enterica* serovar Gallinarum**

A *S. Pullorum* (SP) é muito semelhante à *S. Gallinarum* (SG), agentes da Pulorose e Tifo aviário, respectivamente, sendo que as duas enfermidades são responsáveis por perdas econômicas no setor avícola. SP e SG são de difícil diferenciação em procedimento laboratorial rotineiro, mas uma prova bioquímica muito utilizada na distinção das duas refere-se à capacidade de assimilar o aminoácido ornitina: SP descarboxila este aminoácido enquanto SG não. No entanto, o isolamento de cepas com comportamento bioquímico atípico, tem dificultado tal diferenciação. Um dos genes relacionados à assimilação do aminoácido ornitina, denomina-se gene *speC*, o qual está presente nos dois sorovares. Analisando 21 amostras de SP e 15 de SG com a utilização da PCR não foi possível realizar a diferenciação dos dois sorovares pois os fragmentos gerados eram idênticos. Posteriormente, com o uso da técnica de tratamento enzimático com a enzima de restrição *Eco* RI, foi possível observar que o padrão de bandas gerado em cada sorovar era diferente, mesmo quando amostras que apresentavam comportamento bioquímico atípico eram analisadas. Tal fato permitiu a padronização da técnica para ser utilizada na diferenciação entre os sorovares Pullorum e Gallinarum de maneira rápida e segura.

**Palavras-chave:** *Salmonella* Pullorum, *Salmonella* Gallinarum, diferenciação, PCR

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