Revista Brasileira de Ciência Avícola
ISSN: 1516-635X
revista@facta.org.br
Fundação APINCO de Ciência e Tecnologia Avícolas
Brasil

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Revista Brasileira de Ciência Avícola, vol. 16, núm. 2, abril-junio, 2014, pp. 57-61
Fundação APINCO de Ciência e Tecnologia Avícolas
Campinas, SP, Brasil

Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X Apr - Jun 2014 / v.16 / n.2 / 57-62

http://dx.doi.org/10.1590/1516-635x160257-62

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Keywords

Salmonella serovars, laying hens, chicks, feces, eggs.

Submitted: January/2014
Approved: June/2014

ABSTRACT

Salmonella spp. is the main originator of human foodborne diseases worldwide and is mainly transmitted by food containing eggs. In Brazil, as a result of the lack of studies and data collection very little is known about the prevalence of *Salmonella* spp. in laying hen flocks and commercial table eggs. Consequently the present study was elaborated and aimed at generating data about *Salmonella* spp. in part of the Brazilian egg production chain. Eight flocks of day-old chicks, eight flocks of adult laying hens (four vaccinated with bacterin against *Salmonella* Enteritidis and four unvaccinated) and commercial table eggs from four supermarkets were examined. *Salmonella* spp. was isolated in 50 % of the newly hatched chicks, 25 % of the adult flocks and 1.5 % of egg samples examined. *S. enterica* subsp. *enterica* 4,12:r:-, *S*. Mbandaka, *S. enterica* subsp. *enterica* 6,7: z10:-, *S*. Enteritidis and *S*. Havana were the serovars isolated in birds. In commercial table-eggs *S*. Mbandaka, *S. enterica* subsp. *enterica* 6,7: z10:- and *S*. Braenderup were isolated. These results show that *Salmonella* spp. is present in laying hen flocks and consequently in eggs destined for human consumption. Probably, some of the *Salmonella* serovars are being introduced in egg farms by vertical via.

INTRODUCTION

Samonella spp. is a major zoonotic pathogen being the originator of several foodborne diseases outbreaks worldwide each year. These microorganisms may cause human suffering as well as economic losses to food production and to the food industry (Foley *et al*., 2008; IFAH, 2012). Poultry and eggs remain the major sources of *Salmonella* spp. in developed countries. Eggs and dairy products accounted for 42 % of the outbreaks caused by *Salmonella* spp. in Europe (EFSA, 2009). According to the Brazilian health surveillance service, from 1999 to 2009, 6,349 foodborne diseases outbreaks were notified, and eggs and foods containing egg were responsible for 15 % of them (COVEH, 2009).

There are over 2,600 *Salmonella* serovars identified but about 90 are responsible for human and animal salmonellosis (EFSA, 2009, CDC, 2011). In 2009, Enteritidis, Typhimurium, Newport, Javiana and Heidelberg were the five most frequently reported *Salmonella* serovars from human sources in the United States (CDC, 2011). Food of animal origin is the main source of *Salmonella* spp. for human beings (Pires *et al*., 2010). Over the last decades, there has been an increase in reported cases of human salmonellosis, making the public health authorities focus attention on controlling *Salmonella* spp. in livestock, poultry, and their products (Newell *et al*., 2010).

To take measures able to reduce human foodborne salmonellosis is essential. It is also important to quantify the burden of human illness and also to identify the sources, route of infection and the main serovars involved (Pires *et al*., 2010). Considering eggs and food containing eggs as the major sources of human foodborne salmonellosis, measures and control programs to reduce *Salmonella* spp. and minimize the human exposure must be addressed to chicken farms (Barrow, 2007; Gast, 2007). However, to elaborate a control program it is necessary to know the prevalence and epidemiology of the main *Salmonella* serovars in chicken flocks.

Little is known about prevalence of *Salmonella* serovars in Brazil. A piece of work was done by Zancan *et al*. (2000) who analysed meconium samples from transport cardboard boxes of chicks. According to this study, *Salmonella* Heidelberg and Mbandaka were detected in broiler breeder chicks. Meanwhile, Enteritidis, Mbandaka and Cerro were isolated in laying hen chicks. Moreover, Kanashiro *et al*. (2005) isolated *S*. Enteritidis, Heidelberg, Kentucky, Infantis, Mbandaka, Typhimurium, Senftenberg and other serovars in samples of faeces, meconium and eggs of breeders and broilers of several regions of Brazil from 1997 to 2004. *S*. Enteritidis was the most recovered serovar in the mentioned study.

Although few studies on the prevalence of *Salmonella* serovars have been carried out in Brazil (Zancan *et al*., 2000; Kanashiro *et al*., 2005; Fernandez *et al*., 2006; Kottwitz *et al*., 2008), the information about these microorganisms in humans, animals and food of animal origin are not updated and remain underestimated. The present study was carried out in order to generate data which could support the elaboration of new *Salmonella* spp. control programs for the poultry industry or to help in evaluating the existing ones. This study aimed at surveying for *Salmonella* serovars in flocks of newly hatched chicks, adult commercial laying hens and a representative amount of commercial table-eggs from supermarkets of a region of Sao Paulo state, Brazil.

MATERIAL AND METHODS

Samples and sampling

The presence of *Salmonella* spp. was assessed in flocks of laying hens from eight farms (A, B, C, D, E, F, G and H) and in commercial table-eggs commercialized by four supermarkets (I, J, K and L); all establishments from the same region of São Paulo State, Brazil.

To investigate whether the chicks were getting farm infected with *Salmonella* spp., samples of meconium from eight newly hatched chick flocks (one flock from each farm) were collected from transport boxes following the methodology described by Zancan *et al*. (2000). Each flock had about 3,000 birds and were transported in cardboard boxes with 100 birds each. In total, 240 boxes were examined using a large gauze swab moistened in 1 % buffered peptone water (BPW) (Oxoid® CM0509). Swabs from five boxes were placed into sterile glass with 225 mL of BPW and corresponded to one sample. Samples were taken to the laboratory under refrigeration.

Salmonella spp. was also searched in faecal samples of other eight flocks (four vaccinated with oil-emulsion bacterin against *S*. Enteritidis and four unvaccinated) of adult laying hens (aged twenty-three to thirty-eight weeks). Each chicken house was divided into four parts. Fresh samples of cecal faeces from each part were collected under the cages using sterile swabs moistened in BPW, which were placed into sterile glass and corresponded to one "pool" of samples. In total, sixty-four pools of samples, taken at different times from the eight flocks, were stored in sterile glasses with 225 mL of BPW and transported to the laboratory under refrigeration.

A total of 1,700 eggs from four supermarkets (I, J, K and L) of the same region were examined. Each egg sample consisted of five eggs (shell and contends); a total of 340 samples were examined. Eggs were bought in packages of a dozen at different moments (1, 2, 3 and 4).

Sample processing

Bacteriological exams

Glasses containing the samples in BPW were left at room temperature for one hour, followed by overnight incubation at 37 °C. Then, 2.0 ml of the BPW was transferred to tube containing 20 ml of selenite (Oxoid, CM 395) plus Novabiocin at concentration of 40 mg/L (Merial, 8041706) (SN) and Tetrationate (Biolife, 402125) plus Novobiocin (40 mg/L) broths and 0.2 ml to 20 mL of Rappaport – Vassiliadis (RV) (Oxoid, CM 669) broth. Broths were incubated overnight at 37 °C (Davies & Wray, 1994). After removed from the package, "pools" of 5 eggs were placed into sterile glass jars, broken and homogenised with a sterile wooden stick and then incubated overnight at 37 °C. After that, amounts of 2.0, 2.0 and 0.2 mL of this content were inoculated into tubes containing 20 mL of SN, TN and RV broths, respectively, and were then incubated overnight at 37 °C. Subsequently, broths from all samples were plated onto the following mediums: Brilliant Green agar (Oxoid, CM 0263), Mac

Conkey agar (Oxoid, CM 0115) and XLT4 agar (Difco, 223420). Plates were incubated overnight at 37 °C. From each plate, five typical colonies were inoculated in triple Sugar Iron agar (Oxoid, CM 277) and in Lysine Iron agar (Oxoid, CM 381), which were incubated overnight at 37 °C and submitted to serology using polyvalent sera against O and H *Salmonella* antigens (Probac). Isolates were sent to Adolfo Lutz Institute, São Paulo, Brazil, for complete identification and serotyping.

RESULTS AND DISCUSSION

Salmonella spp. was isolated from transport boxes used to deliver newly hatched chick flocks in four different farms. *S.* Mbandaka was isolated in flocks of farms B, C, F and G; *Salmonella enterica* subspecies. *enterica* 4,12: r: - was recovered in flock from farm C and *Salmonella enterica* subsp. 6,7 *enterica*: Z10: - on farm G (Table1).

Table 1 – *Salmonella* serovars isolated in meconium samples from transport boxes of newly hatched chicks.

Flocks of newly hatched chicks from FARMS	serovars
A	-
B	*Salmonella* Mbandaka
C	*Salmonella* Mbandaka *S. enterica* subsp. *enterica* 4, 12: r: -
D	-
E	-
F	*Salmonella* Mbandaka
G	*Salmonella* Mbandaka *S. enterica* subsp. *enterica* 6, 7: z10: -
H	-

- = Absence of *Salmonella* spp.

It is known that when poultry become infected with *Salmonella* spp. at the beginning of life is more difficult to control because newly hatched chicks are very susceptible, and they may shed this bacterium in high amount and for long periods (Barrow *et al*., 1988). Although many efforts have been done in breeder farms and hatcheries, *Salmonella* spp. still can be found in newly hatched chicks inside the hatchery or at the moment of arrival on the farm (Cox *et al*., 1991; Cox *et al*., 2000; Snow *et al*., 2008). Reports have shown that the detection of *Salmonella* spp. may vary from 11% to 77%, being *S.* Enteritidis the most common one (Cox *et al*., 1990; Zancan *et al*.,2000; Cox *et al*., 1991; Gama *et al*., 2003; Rocha *et al*., 2003). The work done by Zancan *et al*. (2000) that reported positive results in day-old bread birds, suggesting that also grand-parent flocks would be infected by *Salmonella* serovars calls our attention. In the present study, four of the eight farms received chicks infected with four *Salmonella* spp. showing that the control program in breeder farms and hatcheries should be improved.

S. Havana was isolated in cecal faeces from flocks of farms C and G; meanwhile *S*. Enteritidis was detected only in flock from farm G (Table 2). Faeces are the potential vehicle in transmitting *Salmonella* spp. to birds and are also an important source of contamination for eggs (Gast & Beard, 1992, Gast, 2003). Infected birds can shed intermittently about 10^8 cells of *Salmonella* spp. per gram of feces (Bryan & Doyle, 1995). In this work, 25 % of the adult flocks were shedding *Salmonella* spp. Similar results were reported by Salles *et al*. (2008), who detected *Salmonella* spp. in 25% of the inspected flocks and by Kottwitz *et al*. (2008) that detected these microorganisms in 23% of examined flocks. These findings are higher than those reported by Castellan *et al*. (2004) who found *Salmonella* spp. in 10.5% of laying hen flocks in The United States and also higher than those presented by Snow *et al*. (2007), who isolated *S*. Enteritidis, Typhimurium, Mbandaka, Havana and other serovars in 11.7% of commercial flocks in the United Kingdom. It is known that factors such as weather, geographic location of the farm, farming conditions and management of the flock can influence the prevalence of *Salmonella* spp. in poultry (Khakharia *et al*., 1997; Angulo & Swerdlow, 1999).

Table 2 – *Salmonella* serovars isolated in cecal faeces of layers in flocks vaccinated and unvaccinated against *Salmonella* Enteritidis.

Flocks of adult laying hens from FARMS	Serovars
A	-
B	-
C	*Salmonella* Havana
D	-
E	-
F	-
G	*Salmonella* Enteritidis *Salmonella* Havana
H	-

- = Absence of *Salmonella* spp.

There are few reports mentioning the isolation of *S.* Havana in faeces of commercial layers (Hussein *et al*., 2010), even though it was isolated from faeces of two flocks examined in the present study. *S*. Havana was isolated from poultry food and supplements (Berchieri Junior et a., 1989; Okamura *et al*., 2001, Davies & Breslin, 2004), and this could be the source of contamination for the chickens in the present study. Hygiene and biosecurity in addition to the administration of an appropriate form of oil emulsion bacterium against *S*. Enteritidis in flocks of farms A, B, C and D could be some of the factors that contributed to the absence of this serovar. In a study conducted in Japan, *S*. Enteritidis was isolated in several samples of faeces, eggs and the environment from farms containing vaccinated and unvaccinated flocks and the percentage of isolation was lower in flocks where the oil-emulsion bacterin against *S*. Enteritidis was administered (Toyota-Hanatani *et al*., 2009). Freitas Neto *et al*. (2008) also reported reductions in shedding of *S*. Enteritidis and egg contamination in laying hens experimentally vaccinated with bacterin. In the present study, the isolation of *S.* Havana in faeces of birds from farm C, vaccinated against *S*. Enteritidis, demonstrates a lack of cross protection between these serovars.

In this study, three of the four supermarkets had at least one egg sample positive for *Salmonella* spp. Among 340 egg samples examined, five (1.47 %) were contaminated. It was isolated *S*. Mbandaka and *S*. enterica subspecies enterica 6,7: z10:- in the supermarkets I and J, and *S*. Braenderup in the supermarket L. No *Salmonella* spp. was recovered in egg samples from supermarket K (Table 3).

Table 3 – Salmonella serovar isolated from table egg samples from four supermarkets.

Sample collection	SUPERMARKETS				Serovar
	I	J	K	L	
1	+	-	-	-	*S*. Mbandaka
2	+	-	-	+	*S*. Mandaka; *S*. *enterica* subspecies enterica 6,7: z10:- ; *S*. Braenderup
3	+	+	-	-	*Salmonella* spp.; *S*. Mandaka; *S*. *enterica* subspecies enterica 6,7: z10:-
4	-	-	-	-	-

- = Absence of *Salmonella* spp.

Lower percentages of *Salmonella* spp. in eggs have been described. For instance, Ebel & Schosser (2000) reported 0.005 % of contamination in eggs examined in The United States. In European countries, the percentage of *Salmonella* spp. in eggs was about 0.8 % (EFSA, 2010). On the other hand, percentages of *Salmonella* spp. similar to what was found in the present study have also been reported by Humphrey (1994) and Okamura *et al*. (2001). According to Humphrey (1994) and Gast (2003) there are many factors that can contribute to variations in the percentages of *Salmonella* spp. in eggs. Factors such as the sample size, season of sampling, bacteriologic method adopted for examination and the bacterial load present in eggs would be some of them.

During the 2000s, *S*. Enteritidis was one of the most frequent serovars associated with worldwide outbreaks of diseases transmitted by food containing poultry or eggs (EFSA, 2007; CDC, 2011; Fernandes *et al*., 2006; O'Brien, 2012). Although, in lower numbers, reports of human foodborne salmonellosis caused by *S*. Havana, *S*. Mbandaka, *S*. Braenderup (other serovars isolated in this study) have been described (Taunay *et al*. 1996; Tavechio *et al*. 1996; Fernandes *et al*., 2006; CDC, 2011). Therefore, their presence in table eggs and laying hen flocks represent risk for public health.

The results of this study demonstrate that, in addition to *S*. Enteritidis, other serovars are present in commercial laying hens and table eggs. As could be noticed in our results, some of these serovars are possibly being introduced in the egg production chain by infected newly hatched chicks when they arrive at the farm. In order reduce the prevalence of *Salmonella* serovars in the laying hen flocks and eggs, the control programs should be improved not only in egg farms but also in hatcheries and breeder farms.

ACKNOWLEDGEMENTS

The authors would like to thank FAPESP and CNPq for financial support and the Adolfo Lutz Institute of São Paulo, Brazil, for complete identification and serotyping of the isolates.

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