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Detection of Salmonella spp. by Conventional Bacteriology and by Quantitative Polymerase-Chain Reaction in Commercial Egg Structures

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ABSTRACT

Conventional bacteriology techniques and quantitative polymerase-chain reaction (qPCR) were applied to the eggshell, albumen, and yolk of washed and unwashed commercial white and brown eggs, to detect *Salmonella* spp. Pooled samples of eggshells, albumen, and yolk of white and brown eggs were collected at the poultry house and at the egg-storage room. *Salmonella* spp. was detected by conventional bacteriology in 5.4% (21/387) of analyzed samples and in 16% (68/387) by qPCR. In the 114 unwashed white eggs samples of eggshell, albumen and yolk, the bacterium was identified in 2.6% of the eggs (3/114) by conventional bacteriology and in 13.2% (15/114) by qPCR. In the 90 samples of washed eggs, 6.7% (6/90) were contaminated as detected by conventional bacteriology and 10.0% (9/90) by qPCR. In the 81 samples of unwashed brown eggs, *Salmonella* spp. was detected in 6.1% of the eggs (5/81) by conventional bacteriology and 27.2% (22/81) by qPCR. In the 102 samples of brown washed eggs, 6.9% (7/102) where positive by conventional bacteriology and 35.3% (16/102) by qPCR. All samples detected as positive by conventional bacteriology were also positive by qPCR. *Salmonella* Agona represented 18.2% (4/22) of identified serovars, *Salmonella enterica* subs. *enterica* O: 4.5 18.2% (4/22), *Salmonella* Schwarzengrund 18.2% (4/22), *Salmonella* Cerro 13.6% (3/22), *Salmonella* Anatum 13.6% (3/22), *Salmonella* Enteritidis 9.1% (2/22), *Salmonella* Johannesburg 4.5% (1/22), and *Salmonella* Corvallis 4.5% (1/22). The qPCR method provided better detection of *Salmonella* spp. in commercial eggs than conventional bacteriology. The conventional egg washing and disinfection procedures are not efficient to eliminate *Salmonella*.

INTRODUCTION

Fresh eggs have high nutritional value, because it contains all essential amino acids and it is rich in vitamins and minerals (Nepa, 2011). In addition, it is an inexpensive protein source. The egg production industry has faced several environmental, animal welfare, and animal health challenges. *Salmonella* contamination stands out among animal health issues, as it may cause significant production losses and severe public health problems.

Epidemiological research has pointed the egg as a source of human contamination with *Salmonella* spp. in foodborne infection outbreaks. This bacterium was found to be the main agent of foodborne diseases (FBD) and during 2009, fresh eggs were considered the main cause of FDB in the United States (Cdc, 2010). In the European Union in 2008, salmonellosis was associated with food poisoning caused by the consumption of eggs and egg by-products (Efsa, 2010). In Brazil, the same scenario is observed. According to Brazilian Health Surveillance

Secretariat, *Salmonella* spp. was the most prevalent agent in food poisoning outbreaks from 1999 to 2008 (Svs, 2008).

Eggs may be contaminated with *Salmonella* spp. in the reproductive tract during their formation, and immediately after lay by direct contact with contaminated fomites, such as poultry litter, nests, cages, trays, transport crates, as well as by handlers (Cox *et al.*, 2000). The contamination of egg structures depends on the site of infection in the reproductive tract. The yolk, yolk membrane, albumen, eggshell membrane, and eggshells may be contaminated by *Salmonella* spp. when the ovarian follicles, infundibulum, magnum, isthmus, and shell gland, respectively, are infected (Gantois *et al.*, 2009).

The yolk is a suitable substrate for bacterial multiplication and differently from other egg structures, it does not have mechanisms to prevent bacterial growth (Tranter & Board, 1982). In addition of infecting the ovaries, the yolk can be infected by the penetration of *Salmonella* spp. through the contaminated eggshell and yolk membrane. Yolk invasion rate is affected by egg storage time and temperature (Kanashiro *et al.*, 2002).

The albumen contains antimicrobial substances (Gantois *et al.*, 2009). It has a high concentration of ovotransferrin, which chelates iron, inhibiting bacterial growth, as well as lysozymes, which are cationic peptides capable of interacting with the lipopolysaccharide layer (LPS) of the cell wall of Gram-negative bacteria, forming pores that allow albumin overflow into the bacterial cytoplasm (Clavijo *et al.*, 2006).

The intact eggshell has physical barriers that prevent bacterial penetration, including the cuticle, the eggshell membrane, and a thick layer of material that separates the shell membrane from the albumen (Lunam & Ruiz, 2000).

Chemical compounds are used to disinfect the eggs in order to reduce the number of existing microorganisms in the eggshell (Marques *et al.*, 1994). Aragon-Alegro *et al.* (2005) stated that the washing process and use of disinfectants reduce the contamination risk. According to Stringhini *et al.* (2009), washed eggs have better eggshell bacteriological quality than unwashed eggs, even they did not detect *Salmonella* spp. in the eggshells analyzed in their study.

In Brazil, the *Salmonella* spp. survey methodology recommended by Brazilian Ministry of Agriculture is the traditional method of conventional bacteriological test (Brasil, 1997). However, this is a time-consuming and labor-intensive technique. Therefore, new pathogen-detection methodologies, especially used for foodborne infections, have been developed, and include PCR, which is faster and requires less labor (Sachse, 2003).

The European Committee for Standardization recommends that the PCR method should be standardized and meet some criteria, such as accuracy of analysis and diagnosis, high sensitivity, low contamination, accessible and easy-to-interpret protocols. The quantitative PCR (qPCR) assay was developed to meet those criteria, combining amplification and detection in a closed tube, thereby reducing contamination risks (Malorny *et al.*, 2003).

In the light of the above, this study aimed at analyzing the presence of *Salmonella* spp. in the eggshell, yolk, and albumen of washed and unwashed brown and white commercial eggs by conventional bacteriological methods and by qPCR.

MATERIAL AND METHODS

Location

The experiment was conducted at the Bacteriology Laboratory and at the Molecular Diagnostics Laboratory of the Department of Veterinary Medicine of the School of Veterinary Medicine and Animal Science of the Federal University of Goiás, Brazil.

Sampling

Samples were collected on small, medium, and large layer farms with hens of different breeds and ages during 2012 and 2013. During the 24-month period, 68 dozens of white eggs and 61 dozens of brown eggs were randomly collected in poultry houses and the egg-storage room of the farms every two weeks.

Each collected dozen corresponded to three sample units: eggshell, albumen and yolk, totaling 387 samples. Out of the 68 dozens of analyzed white eggs, 38 were collected directly from production sheds, and were identified as unwashed eggs; and the remaining 30 dozens were collected from the egg-storage room and were identified as washed eggs. These were mechanically washed with chlorinated water, with approximately 10ppm free chlorine, at 35-40°C. Out of the 61 dozens of brown eggs collected, 27 were unwashed and 34 were washed.

After an aseptic collection, eggs were placed in cardboard egg cartons (12 eggs per carton), which were identified and placed in coolers containing reusable ice packs, and directly submitted to the laboratory, where they were processed according to Brasil (2003) with some modifications.

Conventional bacteriological *Salmonella* spp. research

At the laboratory, after sterilization of smaller diameter end of the eggs with 70% alcohol, each dozen eggs were broken. Parts of the eggshell were discarded, and the remaining eggshell, the albumen and the yolk were separated and individually placed in a sterilized container.

Each dozen eggs originated three samples: a 12-shell pool, a 12-albumen pool, and a 12-yolk pool. Samples were individually homogenized and 25g of shell, 25mL of yolk, and 25mL of albumen were placed in an Erlenmeyer flask containing 225mL of 1% peptone solution.

Samples in 1% peptone water were incubated at 37°C for 18-20h. After this period, samples were homogenized, and 1mL was transferred to 9mL of Selenite Cystine Broth (SC) and 1mL to 10mL of Rappaport Vassiliadis broth (RV), followed by incubation at 37°C for 24h. Aliquots of 2mL of SC were placed in Eppendorf tubes and stored at - 20ºC for qPCR. Using a nichrome inoculation loop, aliquots were the streaked on the surface of XLT4, Hektoen, and brilliant green agar plates, and again incubated at 37°C for 24h. Colony-forming Units (CFU) with characteristic *Salmonella* morphology were selected, and three to five CFU per plate were transferred to tubes containing triple sugar iron agar (TSI) and incubated at 37ºC for 24h. TSI cultures suggestive of *Salmonella* were subjected to urease, indole production, methyl red, motility, lysine decarboxylase, Simmons citrate, and malonate tests. When the biochemical test results indicated the presence of *Salmonella*, the samples were subjected to serological tests with polyvalent o-antisera, and those positive were referred to the Oswaldo Cruz Foundation (FIOCRUZ)-RJ on nutrient agar for serological typing.

Salmonella spp.

Before the extraction procedure, the frozen samples in selenite cystine broth were again subjected to bacterial enrichment using 9mL of selenite-cystine broth. Total DNA was isolated by boiling lysis (Santos *et al.*, 2001). A 400µL sample was placed in a 1.5mL polypropylene tube, free from DNA and RNA (Axygen). The tube containing the sample was centrifuged at 2,000 g for four minutes. The supernatant was discarded and suspended in 1mL TE (100mL Tris/HCL 1m + 20µL EDTA 0.5m + 9,880µL H_2O). The sample was mixed by vortexing for ten seconds and centrifuged at 2,000g for eight minutes. After discarding the supernatant, the pellet was suspended in 100µL TE. The mixture was washed, vortexed for ten seconds, and placed on a hotplate at 95°C for 20 minutes, aliquoted, and stored at -20°C in a freezer for later use.

The qPCR assays for the detection of *Salmonella* spp. were performed according to Calvó *et al.* (2008) with some modifications. The eluates obtained from the extracted samples were used for qPCR using the TaqMan® (Life®) system. A sample volume of 20µL was used, with 4.6µL of milli-Q water, 10µL of Master Mix (1x), 2µL of IPC mix (10x), 0.4µL of IPC DNA (50x) and 1µL of oligonucleotide primers (concentration 30 mM), and probe (concentration of 10 mM) adding 2µL of DNA samples. As a reaction internal control, IPC DNA was placed in one of the wells of a 96-well plate, along with an IPC reagent blocker (negative control blocked IPC, Life®) and another with IPC DNA without blocking. Samples were tested for presence or absence of *Salmonella* spp. by the StepOnePlus™ qPCR System (Applied Biosystems) under the following conditions: pre-PCR at 60°C for 30 seconds followed by 95°C for 10 minutes, 40 cycles at 95ºC for 15 seconds (denaturation step) and 60°C for 1 minute and 60ºC for 30 seconds for extension step.

The TaqMan® system was used to detect Salmonella spp. by qPCR, applying oligonucleotide primers SAL1410f5'-GGTCTGCTGTACTCCACCTTCAG-3' and SAL1494r 5'-TTGGAGATCAGTACGCCGTTCT-3' and probe SAL1441pr FAM– TTACGACGATATTCGTCCG-GGTGAAGTG – TAMRA, developed by Calvó et al. (2008).

Results were analyzed using the StepOne Software v2.1 (*Applied Biosystems*), at 95% confidence level.

Statistical Analysis

Results were interpreted using Binomial Distribution analysis with R Statistical Software (Core Team, 2015). The *Kappa* (K) coefficient was applied to analyze the consistency of the test results, using R Statistical Software (Core Team, 2015), and the conventional interpretation of the values of *k* were the following: 0.00-0.20 = weak consistency, 0.21-0.40 = regular, 0.41-0.60 = moderate, 0.61-0.80 = good, 0.81-1.00 = very good. Negative values were interpreted as 0.00.

RESULTS AND DISCUSSION

Table 1 shows the occurrence of *Salmonella* spp. in different fresh egg structures according to conventional bacteriology and qPCR results.

Out of the 387 eggshell, albumen, and yolk samples subjected to conventional bacteriology and qPCR, 5.4% (21/387) and 16.0% (62/387) samples, respectively, were positive for *Salmonella* spp. The 21 samples positive by conventional bacteriology were also positive by qPCR.

Table 1 – Frequency (%) of *Salmonella* spp. isolated from the eggshell, albumen and yolk of unwashed white eggs (UWE), washed white eggs (WWE), unwashed brown eggs (UBE), and washed brown eggs (WBE) as determined by conventional bacteriological (CB) and qPCR.

Samples	UWE			WWE			UBE			WBE			Total of samples	
	n	CB	qPCR	n	CB	qPCR	n	CB	qPCR	n	CB	qPCR	CB n + (%)	qPCR n + (%)
Shell	38	1	9	30	1	3	27	1	10	34	3	7	6/129(4.7)	29/129(22.5)
Albumen	38	2	4	30	3	3	27	2	6	34	2	5	9/129(7)	18/129(14.0)
Yolk	38	0	2	30	2	3	27	2	6	34	2	4	6/129(4.7)	15/129(11.6)
Total (%)	114	2.6	13.1	90	6.7	10.0	81	6.1	27.2	102	6.9	35.3	21/387(5.4)	62/387(16.0)

The qPCR technique detected a higher number of *Salmonella* spp. positive samples than conventional bacteriology in all types of samples (Table 1). The rates determined by both methods in the present study are higher than those described by Chemaly *et al.* (2009) in France, who detected *Salmonella* spp. by conventional bacteriology in 1.05% of the shells of eggs collected on 28 farms known to be positive for this bacterium. On the other hand, the obtained rates are close to the 3.8% determined in farm eggs and the 5.5% in eggs from grocery shops by Singh *et al.* (2010) using conventional bacteriology.

According to the qPCR results, shown in Table 1, the eggshells presented the highest contamination, with 22.5% (29/129), followed by the albumen, with 14% (18/129), and by the yolk, with 11.6% (15/129). Eggshells may be contaminated with bacteria by horizontal transmission, during egg passage through the cloaca, or immediately after oviposition, because during the first minutes after lay the cuticle is still immature and allows *Salmonella* penetration. However, for this agent to remain on the eggshell and migrate into the egg, it must overcome inherent barriers such as the cuticle, the shell membranes, and the thick layer of material that separates the shell membrane from the albumen. The bacteria can also be trapped in the membranes and be prevented from entering the egg because shell membranes function as filters and retain microorganisms (Lunam & Ruiz, 2000). These intrinsic barriers have probably contributed to lower *Salmonella* spp. contamination rates of the egg contents compared with those determined in the eggshells.

Table 2 shows the distribution of serovars isolated from the shell, albumen, and yolk of washed and unwashed white and brown eggs.

Using conventional bacteriological methods, six *Salmonella* spp. isolates were found and identified. The isolates belonged to the following serovars: *Salmonella enterica* subs. *enterica* O:4.5, *Salmonella* Schwarzengrund, *Salmonella* Agona, *Salmonella* Corvallis, *Salmonella* Enteritidis. The serovar Enteritidis detected in the eggshell of washed brown eggs collected from egg-storage room and handled after washing and disinfection suggests cross-contamination after lay.

As shown in Table 2, two positive samples were detected both in the eggshell and the albumen. One isolate was identified as *Salmonella* Agona and the other as *Salmonella enterica* subs. *enterica* O:4.5. The agents may have possibly migrated from the eggshell

Table 2 – *Salmonella* serovars isolated from shell (Sh), albumen (Al) and yolk (Yo) of unwashed white egg (UWE), washed white egg (WWE), unwashed brown egg (UBE) and washed brown egg (WBE) by conventional bacteriological methods.

SEROVARS	UWE			WWE			UBE			WBE			Total
	Sh	Al	Yo	Sh	Al	Yo	Sh	Al	Yo	Sh	Al	Yo	
Agona	1	2	-	-	-	-	-	-	-	-	-	-	3
subs.enterica O:4.5	-	-	-	-	1	-	-	1	-	1	1	-	4
Schwarzengrund	-	-	-	-	1	-	1	-	-	1	-	1	4
Cerro	-	-	-	-	-	-	-	1	1	-	-	1	3
Anatum	-	-	-	-	1	1	-	-	1	-	-	-	3
Enteritidis	-	-	-	-	-	1	-	-	-	1	-	-	2
Johannesburg	-	-	-	-	-	-	-	-	-	-	1	-	1
Corvallis	-	-	-	1	-	-	-	-	-	-	-	-	1
Total	1	2	0	1	3	2	1	2	2	3	2	2	21

to the albumen, in which case, the intrinsic defenses of the eggshell and albumen were not sufficient to inhibit or inactivate the replication of the bacteria, allowing their migration from the shell to the albumen. Egg contamination may also have occurred by transovarial transmission, which occurs during egg development prior to calcium deposition for eggshell formation (Messens *et al.*, 2005).

The greatest serovar diversity was identified in albumen (Table 2), where S*almonella* Agona, *Salmonella enterica* subs. *enterica* O:4.5, *Salmonella* Schwarzengrund, *Salmonella* Anatum, *Salmonella* Cerro, and *Salmonella* Johannesburg were detected. Out of the 21 identified isolates, one Agona, two *Salmonella enterica* subs. *enterica* O: 4.5, one Schwarzengrund and one Johannesburg isolates were detected only in the albumen, suggesting that the contamination occurred in the oviduct before lay. This result is supported by Gantois *et al.* (2008) and Gast *et al.* (2010), who reported the incorporation of *Salmonella* spp. at the time of albumen formation and preferably in the infected reproductive tract. Furthermore, according to Gantois *et al.* (2009), the colonization of the reproductive tract depends on bacterial genotypic and phenotypic characteristics, such as virulence, invasiveness, permanence and even on the development of mechanisms able to elude the avian immune system. The serovars identified in the albumen in the present study, *Salmonella* Agona and *Salmonella* Johannesburg, have lower capacity to survive in this structure than *Salmonella* Enteritidis, according to De Vylder *et al.* (2013).

The yolk presented the lowest detection rate of *Salmonella* spp. by qPCR, with 11.6% (15/129) (Table 1). In this structure, *Salmonella* Anatum, *Salmonella* Cerro, *Salmonella* Schwarzengrund, and *Salmonella* Enteritidis were identified (Table 2). The yolk is a suitable substrate for bacterial multiplication and, unlike other egg structures, it does not contain mechanisms to prevent bacterial growth (Tranter & Board, 1982), which suggests that contamination can be derived from ovarian follicle of apparently non-infected hens. Another aspect that must be considered is that no structure was contaminated in the samples of washed or unwashed white eggs. It is noteworthy that after lay, these eggs were kept at approximately 28 °C for 24-48 hours. Possibly, there was not enough time to occur changes in the yolk membrane that would allow iron ion and nutrient release from the yolk to the albumen, thereby attracting microorganisms (Gantois *et al.*, 2009). The obtained results differ from those found by Oliveira & Silva (2000), who determined that the invasion of the yolk by *Salmonella* Enteritidis from the eggshell occurs in 24 hours and with greater intensity in eggs stored at room temperature.

These results support those found by Kottwitz *et al.* (2008), who state that commercial eggs are one of *Salmonella* vehicles in the human food chain, and that this microorganism is one of the most frequent and relevant etiological agents of intestinal infections.

Out of the 387 samples of the eggs analyzed by conventional bacteriology, 21 were positive for *Salmonella* spp. and the isolates were typed by FIOCRUZ. Most frequently isolated serovars were: *Salmonella* Schwarzengrund and *Salmonella enterica* subesp. *enterica* (O:4.5), with 19.0% (4/21); followed by *Salmonella* Anatum, with 14.3% (3/21); *Salmonella* Cerro, with 14.3% (3/21); *Salmonella* Agona, with 14.3% (3/21); *Salmonella* Enteritidis, with 9.5% (2/21); *Salmonella* Johannesburg, with 4.8% (1/21); and *Salmonella* Corvallis, with 4.8% (1/21).

Serovar frequency varies according to region and country. The current results are partly different from those obtained by Shahzad *et al.* (2012), who determined contamination rates of eggs collected in Pakistan of 41.9% for *Salmonella* Enteritidis, 26.7% for *Salmonella* Typhimurium, 12.8% *Salmonella* Cerro and 8.1% *Salmonella* Pullorum. Chousalkar & Roberts (2012), on the other hand, did not find any *Salmonella* spp. in the egg content or eggshell crush in Australia; however, six *Salmonella* Infantis and one *Salmonella* subspecies 1 D 4:12, a type of *Salmonella* associated with rat feces were detected in the eggshells.

In the present study, the serovar Schwarzengrund presented the highest occurrence, with 19.0% (4/21), and was the only isolate detected in all three evaluated egg structures. According to Aarestrup *et al.* (2007), this serovar can cause severe cases of salmonellosis in humans. Furthermore, Chen *et al.* (2010) identified it as one of the main contaminants of food of avian origin in China.

Salmonella Agona was more frequent than Salmonella Enteritidis, with 18.2% and 9.1%, respectively. Outbreaks of *Salmonella* Agona have been associated with different foods, including meat, cereal and fruits. All identified serovars in the present study may potentially affect human health.

The primers and probes used for qPCR were developed by Calvó *et al.* (2008) and have the *bip*A gene as reference. According to those researchers, other PCR assays present issues regarding inclusion and specificity. For instance, the invA gene does not

detect *Salmonella* Saint Paul, and the virulence genes suffer silent mutations, which means that using them as targets for the detection of *Salmonella* spp. can lead to false negative results. All isolated serovars were detected by qPCR, confirming the efficacy of the method of Calvó *et al.* (2008), even though DNA samples were extracted from enriched selenite cystine broth and not from pre-enrichment peptone water as recommended by them.

The results obtained by the two techniques were submitted to the Kappa comparison test (Table 3).

Table 3 – Determination of the Kappa index, confidence intervals (CI) 95% and p-values of positive and negative test categories for overall kappa test, considering conventional bacteriology and qPCR.

	Kappa	CI 95%	P
Positive	0.452	Higher = 0.531 Lower = 0.373	< 0.001
Negative	0.452	Higher= 0.531 Lower= 0.373	< 0.001
General	0.452	Higher= 0.531 Lower= 0.373	< 0.001

Bacterial culture and qPCR results were considered. According to the Kappa test, the correlation was moderate (0.40 to 0.59), indicating that qPCR is a reliable tool for the quick detection of *Salmonella* spp. This finding is supported by researchers who reported that molecular techniques have been successfully used for *Salmonella* spp. research and for the identification of specific serovars (Malorny *et al.*, 2004; Dilmaghani *et al.*, 2011). However, Soria *et al.* (2012), when comparing bacteriological methods with PCR for the detection of different serovars of *Salmonella* spp. of artificially contaminated commercial egg samples, found poor consistency (0 to 0.19) in the evaluation of Kappa test.

Even though conventional bacteriology is considered the gold standard for identifying *Salmonella* spp. in samples of different origins, in the current study, only 21 samples were positive by conventional bacteriology out of 62 samples positive by qPCR. Therefore, 41 samples positive by qPCR were not isolated by bacteriology. According to Temelli *et al.* (2010), the marked presence of false negatives in conventional bacteriology is related to a large number of non-viable *Salmonella* in the samples or to a small amount of biological material. Other factors may be the overgrowth of lactose-fermenting bacteria, masking *Salmonella* spp. CFUs on the selective growth plates, and insufficient recovery of stressed cells.

Table 4 shows the results of the binomial distribution of the results, calculated pairwise, in brown and white eggs samples, washed and unwashed, subjected to conventional bacteriological techniques and qPCR.

Table 4 – Binomial distribution of the results obtained by conventional bacteriology (CB) and by qPCR in unwashed and washed brown and white egg samples.

Sample	n	CB n + (%)	qPCR n + (%)
White egg	204	9/204(4.4)	24/204(11.8)A
Brown egg	183	12/183(6.5)	38/183(20.8)B
p	-	0.4805	0.02313
Unwashed white egg	114	3/114(2.6)	15/114(13.1)
Washed white egg	90	6/90(6.7)	9/90(10.0)
p	-	0.2936	0.6339
Unwashed brown egg	81	5/81(6.2)	22/81(27.2)
Washed brown egg	102	7/102(6.9)	36/102(35.3)
p	-	1	0.3103
Unwashed white egg	114	3/114(2.6)	15/114(13.1)A
Unwashed brown egg	81	5/81(6.2)	22/81(27.2)B
p	-	0.3885	0.0230
Washed white egg	90	6/90(6.7)	9/90(10.0)A
Washed brown egg	102	7/102(6.9)	36/102(35.3)B
p	-	1	0.0007

A,B Means followed by different letters in the same column present different binomial distribution ($p<0.05$).

As seen in Table 4, brown eggs, with 20.8% (38/183), were more contaminated ($p <0.05$) than white eggs, with 11.8% (24/204), as determined by qPCR. This difference is observed both when unwashed brown eggs (27.2%; 22/81), are compared with unwashed white eggs (13.1%; 15/114), and when washed brown eggs (35.3%; 36/102) are compared with washed white eggs (10.0%; 9/90).

The greater contamination ($p <0.05$) of brown eggs in relation to white eggs may be related to genetic heritage. Brown layer strains seem more susceptible to *Salmonella* infection than white strains, as mentioned by Dunn *et al.* (2005). Another explanation may be the delay to start of the classification process of brown eggs, favoring *Salmonella* multiplication in the eggshell and its penetration into the egg. In most farms where eggs were collected, brown egg production was lower than that of white eggs, and brown eggs were classified after white eggs, at the end of the day. Another issue may be egg handling. Most white eggs were handled in automated storage rooms, where eggs are handled only at packing, after being classified. On the other hand, all washed brown eggs used in this study were manually collected in conventional sheds, packed in reusable plastic trays, and transported in trucks to the egg-storage room to be classified.

In addition, relative to the binomial distribution results shown in Table 4, there were no statistical differences between unwashed and washed white eggs or between washed and unwashed brown eggs. Therefore, it seems that the washing and sanitizing process did not interfere with the *Salmonella* spp. contamination levels of brown and white commercial eggs.

Table 5 shows the binomial distribution results, calculated pairwise, of the eggshells and egg contents of washed and unwashed brown and white eggs, according to conventional bacteriology and qPCR.

Table 5 – Binomial distribution of the results obtained by conventional bacteriology (CB) and by qPCR in the eggshells and contents of unwashed and washed brown and white eggs.

Sample	n	CB n + (%)	qPCR n + (%)
Eggshell, unwashed white eggs	38	1/38(2.6)	9/38(23.7)A
Content, unwashed white eggs	76	2/76(2.6)	6/76(7.9)B
p	-	1	0.0396
Eggshell, washed white eggs	30	1/30(3.3)	3/30(10.0)
Content, washed white eggs	60	5/60(8.3)	6/60(10.0)
p	-	0.654	1
Eggshell, unwashed brown eggs	27	1/27(3.7)	20/27(37.0)
Content, unwashed brown eggs	54	4/54(7.4)	12/54(22.2)
p	-	0.8703	0.2509
Eggshell, washed brown eggs	34	3/34(8.8)	7/34(20.6)
Content, washed brown eggs	68	4/68(5.9)	9/68(13.2)
p	-	0.8899	0.5004

A,B Means followed by different letters in the same column present different binomial distribution ($p<0.05$).

As shown in Table 5, the eggshells of unwashed white eggs presented higher ($p<0.05$) contamination than the egg contents. This finding has public health implications because, according to Braden (2006), the eggshell is considered a vehicle of *Salmonella* spp. contamination of the human food chain.

The eggshells of unwashed white eggs, with 23.7% (9/38), presented higher contamination ($p<0.05$) compared with the egg content, with 7.9% (6/76). The unwashed white eggs were collected directly from the sheds.

Some management aspects may be responsible for eggshell contamination. Hen age influences eggshell quality, as observed by Lapao *et al.* (1999) and Radkowski (2002). Also, according to Shahzad *et al.* (2012), a higher incidence of bacteria are found in market eggs when compared with farm eggs, as a result of their contamination between the poultry farms and the retail markets.

CONCLUSIONS

Washed and unwashed brown eggs showed greater contamination rates than white eggs. The eggshell of unwashed white eggs presented higher contamination rate than the other analyzed egg components. The process of washing and sanitizing commercial eggs does not seem to be an efficient method to eliminate *Salmonella* spp. serovars. In general, qPCR is more efficient than conventional bacteriological methods for the detection of *Salmonella* spp. in commercial egg structures.

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