Evaluation of effectiveness, viability and potential of molecular techniques as a research tool of *Salmonella* spp. in eggs

**Avaliação da eficácia, potencial e viabilidade das técnicas moleculares como ferramenta de investigação da *Salmonella* spp. em ovos**

**Evaluación de la eficacia, potencial y viabilidad de técnicas moleculares como herramienta de investigación de *Salmonella* spp. en huevos**

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ABSTRACT
The majority of food intoxication is caused by *Salmonella* spp. originating from the intake of contaminated food, particularly of poultry and eggs. It is one of the most important zoonoses for Public Health, to be difficult to control, due to its high endemcity and morbidity and cause great socioeconomic impacts. This study aimed to investigate and identify the presence of *Salmonella* spp. in eggs and to verify the effectiveness of the Polymerase Chain Reaction (PCR) as a test for the diagnosis of *Salmonella* spp. For this, eggs commercialized in the city of Patos-PB were used. It was identified that the isolates have genes encoding virulence factors (*spvC* e *invA*), important indicators of pathogenicity and high risk to the consumer. Despite the high specificity, PCR showed high complexity in the identification of the colonies compared to conventional biochemical techniques, however, it was found that the use of more than one identification method contributes favorably, by increasing the probability of detection of *Salmonella* spp. Nevertheless, it is imperative more researchs of the identification of *Salmonella* spp., in order to standardize PCR techniques, with the purpose of making it more efficient and viable for microbiological control in the food industry.

**Keywords:** food infection, microbiological control, polymerase chain reaction (PCR), contaminated eggs.

RESUMO
A maioria dos casos de infecção alimentar é causada por *Salmonella* spp. oriunda do consumo de alimentos contaminados, em especial da carne de frango e ovos. É uma das zoonoses mais importantes para a Saúde Pública por ser de difícil controle, por sua elevada endemidade e morbidade e por causar grandes impactos socioeconômicos. O objetivo deste trabalho foi pesquisar e identificar a presença de *Salmonella* spp. em ovos, além de verificar a eficácia da técnica de Reação de Cadeia da Polimerase (PCR) como teste para o diagnóstico de *Salmonella* spp. Foram utilizados ovos comercializados no município de Patos-PB. Após a realização dos testes, foi identificado que os isolados avaliados apresentavam genes codificadores de fatores de virulência (*spvC* e *invA*), importantes indicadores de patogenicidade e de elevado risco ao consumidor. Apesar da elevada especificidade, a técnica de PCR mostrou de alta complexidade na confirmação da identificação das colônias analisadas, quando comparada às técnicas de bioquímica convencional, porém, foi constatada que a utilização de mais de um método de identificação contribui favoravelmente, por aumentar a probabilidade de detecção de *Salmonella* spp. É imperativo repetir trabalhos dedicados à identificação de *Salmonella* spp., visando padronizar técnicas de PCR, com fins de torná-la mais eficiente e viável para o controle microbiológico na indústria alimentícia.

**Palavras-chave:** infecção alimentos, controle microbiológico, reação de cadeia da polimerase (PCR), ovos contaminados.

RESUMEN
La mayoría de los casos de infecciones transmitidas por alimentos son causados por *Salmonella* spp. originados por el consumo de alimentos contaminados, especialmente carne de pollo y huevos. Es una de las zoonosis más importantes para la Salud Pública porque es de difícil control, por su alta endemicidad y morbilidad y porque causa importantes impactos socioeconómicos. El objetivo de este trabajo fue investigar e identificar la presencia de *Salmonella* spp. en huevos, además de verificar la efectividad de la técnica de Reacción en Cadena de la Polimerasa (PCR)
como prueba para el diagnóstico de Salmonella spp. Se utilizaron huevos comercializados en el municipio de Patos-PB. Luego de realizar las pruebas, se identificó que los aislados evaluados presentaban genes que codifican factores de virulencia (spvC e invA), importantes indicadores de patogenicidad y alto riesgo para el consumidor. A pesar de la alta especificidad, la técnica PCR demostró ser altamente compleja para confirmar la identificación de las colonias analizadas, en comparación con las técnicas bioquímicas convencionales, sin embargo, se encontró que el uso de más de un método de identificación contribuye favorablemente, al aumentar la probabilidad. detección de Salmonella spp. Es fundamental replicar el trabajo dedicado a la identificación de Salmonella spp., con el objetivo de estandarizar las técnicas de PCR, con el fin de hacerlas más eficientes y viables para el control microbiológico en la industria alimentaria.

Palabras clave: infección de alimentos, control microbiológico, reacción en cadena de la polimerasa (PCR), huevos contaminados.

1 INTRODUCTION

According to the Ministry of Health, foodborne illnesses, commonly known as FBIs, are caused by the ingestion of contaminated foods or beverages. There are over 250 types of FBIs, most of which are infections caused by bacteria and their toxins, viruses, and parasites (Brazil, 2014). FBIs have been characterized as significant causes of morbidity and mortality worldwide. In many countries, over the last two decades, they have emerged as a growing economic and public health problem. Numerous FBI outbreaks attract media attention and increase consumer interest. Notably, trends with the progression and scope of cases are observed, especially with various global changes, including population growth, poverty, food and animal feed exports, which influence international food security (World Health Organization, 2012).

According to the World Health Organization, it is estimated that over two million people die each year from diarrheal diseases, mostly acquired by ingesting contaminated food (Brazil, 2014). In the United States alone, about 76 million cases, 325,000 hospitalizations, and five thousand deaths are reported each year (Centers for Disease Control and Prevention, 2006).

In Brazil, epidemiological surveillance is conducted for FBI outbreaks and not individual cases, except for cholera, typhoid fever, and botulism. Among the pathogens involved in these outbreaks, the Ministry of Health identified Salmonella as the etiological agent with the highest number from 2010 to 2011. However, it was estimated that only 10% of cases of foodborne outbreaks are reported in Brazil, indicating failures in the identification and notification system of causative agents (Brazil, 2014).
According to Barancelli et al. (2012), salmonellosis represents a significant threat, leading to outbreaks of diseases in humans, both through contamination from chicken meat and eggs. *Salmonella* spp. has been isolated from foods of various compositions. However, it is most commonly isolated from beef, eggs, and poultry meat, especially chicken meat. Human infections are usually associated with the consumption of undercooked or improperly handled chicken meat or eggs, allowing *Salmonella* spp. to multiply in the food (Pan American Health Organization, 2003).

Belonging to the *Enterobacteriaceae* family, this genus is subdivided into two species: *Salmonella enterica* (divided into six subspecies) and *Salmonella bongori*. Currently, more than 2,500 serotypes/serovars are known, of which 80 to 90 are important for the health of animals and humans. Domestic poultry and their products (eggs) are the main sources of *Salmonella* spp. for humans. This pathogen can infect birds horizontally and vertically, after ingesting contaminated water or food. The egg is contaminated during passage through the cloaca because *Salmonella* can permeate the eggshell due to its porosity, cracks, and shell thickness (Hirsh, 2014).

The conventional bacteriological isolation method for *Salmonella* spp., for the microbiological quality control of animal origin products, is standardized by specific legislation (Brazil, 1995; 2003b). This method consists of pre-enrichment, selective enrichment, isolation and selection, biochemical identification, and agglutination serological testing stages. However, it is a laborious technique that requires a minimum time of up to seven days to obtain the result (Dickel, 2004; Andreatti Filho, 2011). Rapid diagnostic methods are extremely necessary for the food industry, as they reduce possible economic losses due to product retention until diagnostic results are released and favor quick decision-making (Von Rückert, 2006).

According to Benetti (2009), techniques for the rapid detection of *Salmonella* spp. have been developed and used in the food industry, based on nucleic acid hybridization methods, immunological methods, and molecular methods, such as Polymerase Chain Reaction - PCR. The application of PCR in the detection of contaminated foods, animals, and infected people is an innovative strategy, used and cited by many authors as efficient and fast, being successfully employed for the detection of various microorganisms, such as *Listeria monocytogenes*, *Campylobacter* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Shigella flexneri*, and *Escherichia coli*, which has attracted the attention of the poultry industry for its use in a wider range of
products, such as eggs, and on a commercial scale, aiming to maximize food control and quality actions (Aabo et al., 1993; Bennett et al., 1998; Santos et al., 2001; Malorny et al., 2003).

The study aimed to identify the presence of *Salmonella* spp. in isolated samples using the Polymerase Chain Reaction (PCR) technique and to determine its effectiveness and potential compared to methods commonly used for the diagnosis of *Salmonella* spp.

2 METHODOLOGY

Eggs commercialized in the market of Patos, Paraíba (PB), Brazil, were collected for the isolation and identification of *Salmonella* spp. Total egg samples were analyzed, including the shell (obtained by crushing the egg in a sterilized plastic bag) and the internal parts of the eggs. After disinfecting the eggs with iodine alcohol, they were aseptically opened, and their internal contents were separated into sterile bottles. Twenty-five grams and 25 mL of each fraction were taken, and 225 mL of 1% peptone water was added.

The isolation of *Salmonella* spp. was based on Normative Instruction No. 62 of the Ministry of Agriculture, Livestock, and Supply, as well as the biochemical analyses after isolation (Brazil, 2003). For DNA extraction, a colony of each isolate grown on Hektoen and Bismuth Sulfite Agar (HiMedia) at 35°C for 24 hours and subcultured in Brain Heart Infusion broth (HiMedia) at 37°C for 20 hours was used. After incubation, 1.0 mL of the culture was centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded, and the pellets were kept at the bottom of the Eppendorf tubes. Brazol reagent (LGC Biotechnology, Brazil) was used for DNA extraction following the manufacturer's recommendation.

The extracted DNAs were subjected to genotypic confirmation through PCR technique, using the *hilA* primer (Craciunas et al., 2012), common to the *Salmonella* genus, resulting in an amplification product of 413 bp. Additionally, the presence of virulence-associated genes was evaluated: *spvC* and *invA*, obtaining amplification products of 700 bp (Craciunas et al., 2012) and 248 bp (Rahn et al., 1992), respectively. The oligonucleotide sequence can be observed in Table 1. The positive control used was a strain of *Salmonella* spp. isolated in the Microbiology Laboratory of the Veterinary Hospital of UFCG, Campus Patos. And for the negative control, a reaction without bacterial DNA was used, adding water to the mixture, with a value equivalent to the lysate.
Table 1. Sequence of the primer oligonucleotides used in PCR reactions for amplification of genes for *Salmonella* spp. and detection of virulence-associated genes in egg samples.

<table>
<thead>
<tr>
<th>Oligonucleotides (5’–3’)</th>
<th>Target Gene</th>
<th>Amplified Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>hilA1: GCG AGA TTG TGA GTA AAA ACA CC</td>
<td>hilA</td>
<td>413 bp</td>
</tr>
<tr>
<td>hilA2: CTG CCC GGA GAT ATA ATA ATC G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spvC1: TAT GAT GGG GCG GAA ATA CC</td>
<td>spvC</td>
<td>700 bp</td>
</tr>
<tr>
<td>spvC2: GCGTTTACTGTTCCGTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>invA1: GTG AAA TTA TCG CCA CGT TCG GGC AA</td>
<td>invA</td>
<td>284 bp</td>
</tr>
<tr>
<td>invA2: TCA TCG CAC CGT CAA AGG AAC C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Authors.

The amplification reactions were prepared for a final volume of 20 µL, containing: 10X PCR buffer (10 mM Tris-HCl, pH 8.5; 50 mM KCl), 10 µM dNTPs, 1 µM of each primer, 1.5 mM MgCl2, 2 units of Taq DNA polymerase enzyme, and 5 µL of bacterial DNA, completing with 7.8 µL of ultrapure water.

The amplification parameters used with the specific primers for the hilA and spvC genes were: initial denaturation at 94°C for 4 minutes; 30 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Regarding the primer for genomic identification of the invA region, an initial denaturation step at 94°C for 5 minutes was followed by 30 consecutive cycles of 94°C for 1 minute for denaturation, 54°C for 1 minute for annealing, and 72°C for 1 minute for extension, with a final extension step at 72°C for 7 minutes.

The results of the reactions were visualized by agarose gel electrophoresis.

3 RESULTS AND DISCUSSION

During the study execution, 30 eggs were analyzed, where seven (23.33%) showed colonies suggestive of *Salmonella* spp. isolation, totaling 11 samples, both from egg contents and shells. Of these, 32 colonies were collected for biochemical analysis and identification through PCR. Based on the biochemical species identification, it was inferred that nine samples were *Salmonella* spp., while the other two were *Escherichia coli* and *Klebsiella* (Table 2). Using selective enrichment for *Salmonella* isolation allows the growth of these microorganisms, with partial inhibition of accompanying flora. Similarly, when performing selective plating, the employed media should have minimal inhibitory effect on *Salmonella* and maximum action on
accompanying flora. However, there is a possibility of growth of other Enterobacteria, such as Proteus, Escherichia coli, and Klebsiella.

Regarding identification through PCR, using the specific primer for the hilA gene, low yield was obtained, identifying only two samples with Salmonella spp. (one from egg contents and one from the shell) (Table 2). With the spvC primer, it was possible to identify that five samples possessed the coding region of the spvC gene. Finally, using the invA primer, four samples were identified as containing this gene.

Table 3 demonstrates that the biochemical test identified nearly twice as many samples as PCR, proving to be more effective in this identification. However, a highly relevant aspect when using molecular techniques was the detection of virulence-associated genes (spvC and invA), demonstrating the pathogenic potential of Salmonella spp. isolates circulating in the region of Patos-PB. Where the spvC gene is located on a virulence plasmid, being involved in systemic infection. And the invA gene is responsible for bacterial internalization through the stimulation of non-phagocytic cells. This gene functions to increase the invasion capacity of Salmonella, since serotypes that do not possess the invA gene are unable to express invABC, thus being deficient in invading mammalian cells, indicating that the invABC operon plays an important role in bacterial invasion of cells (Haneda et al., 2001; Swamy et al., 1996; Woodward, Kirwan, 1996).

Table 2. Results of PCR and biochemical tests on 11 samples of eggs commercialized in Patos-PB.

<table>
<thead>
<tr>
<th>Samples</th>
<th>hilA</th>
<th>spvC</th>
<th>invA</th>
<th>Biochemical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC1</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>OC2</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>CC2</td>
<td>Pos</td>
<td></td>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>OC6</td>
<td></td>
<td></td>
<td></td>
<td>Klebsiella</td>
</tr>
<tr>
<td>CC6</td>
<td></td>
<td></td>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>CC18</td>
<td></td>
<td></td>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>CC19</td>
<td>Pos</td>
<td></td>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>OC20</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>CC20</td>
<td></td>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>OC21</td>
<td></td>
<td></td>
<td></td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>CC21</td>
<td></td>
<td>Pos</td>
<td></td>
<td>Salmonella spp.</td>
</tr>
</tbody>
</table>

Source: Authors.

According to Haubert et al. (2014), Salmonella is a concern in the food industry, especially regarding food safety, and it is important to evaluate its presence in food and also the presence of virulence genes. Pathogenic microorganisms are distinguished from non-pathogenic...
ones by possessing and expressing genes that encode virulence factors, i.e., factors that promote colonization and occurrence of various events that subvert the host's physiology, leading to the appearance of abnormal signs and symptoms, which will ultimately define the disease state (VIEIRA, 2009).

The identification rate of 36.36% using the invA gene was higher than expected compared to Flores et al. (2003), who obtained a rate below 10.00%, and also slightly above the results of Fortuna (2013), who achieved 22.50% success using the same primer. In the study conducted by Craciunas et al. (2012), a percentage of 38.46% was reported using the spvC gene, a value close to the one presented in this work (45.45%) using the same gene.

Table 3. Percentage of PCR test identification compared to biochemical identification of Salmonella colonies isolated from eggs commercialized in Patos-PB, Brazil.

<table>
<thead>
<tr>
<th>Test</th>
<th>Positives</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical</td>
<td>9</td>
<td>81.81</td>
</tr>
<tr>
<td>spvC</td>
<td>5</td>
<td>45.45</td>
</tr>
<tr>
<td>invA</td>
<td>4</td>
<td>36.36</td>
</tr>
<tr>
<td>hilA</td>
<td>2</td>
<td>18.18</td>
</tr>
</tbody>
</table>

Source: Authors.

On the other hand, the result of only 18.18% in Salmonella spp. identification using the hilA gene was below expectations compared to Craciunas et al. (2012) and Haubert et al. (2014), who obtained rates above 90.00% in their tests. This discrepancy may have occurred due to inadequate standardization for the test with the hilA gene (Sousa et al., 2007).

Regarding the percentage of contaminated eggs, this study presented 23.33% (11 samples) of contaminated eggs. These values are higher than the 4.98% reported by Flores et al. (2003), which does not allow for direct comparison as the studies were conducted in commercial regions of different cities, but it underscores the urgency for stricter inspection measures to ensure that this product meets sanitary standards before being marketed.

4 CONCLUSION

The occurrence of Salmonella spp. in eggs sold in the municipality of Patos-PB has been confirmed, with the evaluated isolates presenting significant virulence factors for their pathogenicity, representing a high risk to consumers.
The large number of eggs infected with *Salmonella* spp. in the commerce of Patos-PB highlights the urgent need for stricter inspection to ensure that these eggs meet sanitary surveillance standards before being sold.

Regarding the use of PCR as a method for identifying *Salmonella* spp. in eggs, there is a need for more studies and better standardization before its widespread application by the food industry.
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