Identification of *Clostridium perfringens* and *Salmonella* spp. in Swine Through PCR

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**ABSTRACT**

**Background:** The presence *Clostridium* spp. and *Salmonella* spp. is the main concern in swine sector, since they interfere in the microbiological quality of meat and can be responsible for several diseases, presenting occurrences such as intestinal infections and enterotoxaemia. It is necessary to improve production and quality of meat and by-products swine ensuring good quality origin of products marketed. Considering these aspects, the objective of the present study was to assess the presence of *Clostridium perfringens* and *Salmonella* spp. in pigs destined for consumption, with bacterial counts and identification using PCR technique.

**Materials, Methods & Results:** The collections were made at slaughterhouse and confinement, with bacterial count by the pour-plate technique with Reinforced Clostridial Agar, and the identification by PCR with *cpa*, *cpb*, *IA*, *cpe*, *etx* and *cpb-2* genes for *C. perfringens*; and *invA* and *flIC* for *Salmonella* spp. The scores presented average values of $6.04 \times 10^4$ CFU/mL for confinement, and $1.8 \times 10^4$ CFU/mL in the slaughterhouse. The means counts between the confinement and slaughterhouse groups showed significant difference ($P < 0.01$). The samples taken at the slaughterhouse were from several farms at random, and allowed the appraisal of hygienic conditions, since animals of confinement remained sanitized and the accumulation of feces on floor was avoided. The PCR technique presented positive results for *C. perfringens* alpha toxin (*cpa*) in 25% of the confinement samples, and 46.4% in the slaughterhouse. *Salmonella* spp. was identified in 9.5% of confinement and 21.4% of the slaughterhouse.

**Discussion:** The presence of *C. perfringens* and *Salmonella* spp. is directly related to losses in the entire system of creation of pigs, since infected animals are susceptible to severe diseases, or without clinical signs can exhibit apathy and loss of appetite making weight gain difficult and causing deficiency of the immune system. The difference between the averages counts of the confinement and the slaughterhouse may be due to several factors, but environmental conditions and hygienic are the most influence these differences. When the slaughterhouse samples were collected, the contact with swine of the other farm and the stress of transport may allow the excretion of *C. perfringens* in greater quantity than usual, while the animals of confinement remained in stalls separate and stress-free the whole period. Many researches have been demonstrating that the identification of pathogenic bacteria by PCR has been more sensitive and faster than conventional microbiology. For this work, this technique gave swift and reliable results, since it consisted of amplification of specific genes to the species of *C. perfringens* and *Salmonella* spp. and *S. Typhimurium*. This fact is of great relevance to contribute preventively with the diagnosis and treatment of animals before they can cause damage to the sector. This comparison can be confirmed when observed the correlation coefficient (-0.25) between detection of bacterial and weight of animals confined. These results demonstrate that animals with high count of bacteria were those who had greater difficulties in gaining weight, and consequently were slaughtered below the ideal weight. It could be concluded that *C. perfringens* and *Salmonella* spp. are often related directly to negative influence on pig production, mainly in initial confinement hindering the weight gain of the animals, and in the industry sector contaminating the meat and its derivatives. Moreover, the molecular biology methods showed to be quite efficient when compared to the conventional ones, thus, PCR can be considered a useful tool to improve pig production.

**Keywords:** anaerobic bacteria, swine production, slaughterhouse.
INTRODUCTION

The pathogens *Clostridium* spp. and *Salmonella* spp. are directly related to diseases in piglets, such as intestinal infections and enterotoxaemia. This are pathogens found in the intestinal tract of animals and are identified as responsible for a wide range of diseases in human and animal.

The pathogenicity of *C. perfringens* in the intestinal tract of pigs is associated with the occurrence of toxin, mainly toxins α (type A) and β (type C) [15,17]. Which can cause diarrhea, hemorrhagic, necrotic enteritis and malignant edema and is considered the leading cause of diarrhea in newborns.

Infections by *Salmonella* spp. have worldwide dissemination and spreads quickly among animals why contact with the stool, causing great economic losses and public health [13]. As well as, failures in management at the industrial process carries those microorganisms from infected animals during slaughter, extending the scale of contamination to the consumer. It is considered of utmost importance the microbiological control of these agents to minimize economic losses and ensure food and trade security in products derived from pork.

The growing market expansion, care with production and marketing of meat are essential for better product quality and food safety, since *C. perfringens* food poisoning ranks among the most common foodborne diseases worldwide [11].

As infected pigs are still a major source of introduction of bacteria into the human food chain [8], the present study has as objective to evaluate the presence of *C. perfringens* and *Salmonella* spp. in pigs. Using bacterial counts and molecular biology techniques allow rapid and accurate identification results.

MATERIALS AND METHODS

**Sampling**

Samples were collected in the pig confinement of the College of Agricultural and Veterinarian Sciences - UNESP/FCAV, Jaboticabal *Campus* and in slaughterhouse in the countryside of the State of São Paulo in Brazil, being 84 animals from a slaughterhouse and 84 from the confinement, without signs of infection. Anorectal swabs were collected of each animal, and placed onto tubes with water peptone 0.1% sterile.

**Methodology**

The seek for *Clostridium* spp. began with serial dilutions subjected to thermal shock [4] to active germination of spores and elimination of vegetative cells and possible contaminants.

After thermal shock, an aliquot of 1 mL of each dilution was transferred to a Petri dish, and Reinforced Clostridial Agar was added by the pour plate method and incubated in anaerobic conditions with GasPack® system at 37°C for 48 h. Typical colonies (3 to 5) were inoculated in BHI broth tubes (Brain Heart Infusion) and incubated at 35°C for 24 h. DNA extractions were performed by the technique of Marmur [12].

The PCR reactions were directed for the detection of genes encoding toxins alpha, beta, Epsilon, iota (Table 1). The reactions followed 1X buffer [100 mM Tris-HCl pH 8.8; 500 mM KCl; 0.8% (v/v) Nonidet P40]; MgCl₂ 2 mM; dNTP’s 0.2 mM, 1.5 U of Taq DNA polymerase, 5 pmol of each primer, 60 ng of genomic DNA and sterile pure water 20 µL. The amplifications were in thermal cycler Veriti Thermal Cycler® programmed with annealing temperature of 55°C for 1 min. The electrophoretic used agarose gel 1% (p/v) with SYBR® Green 1X and molecular size standard 100 pb DNA Ladder®, and photo documentation GEL DOC™ EQ.

For *Salmonella* spp., samples were incubated at 42°C for 24 h, transfer 1 mL in 10 mL Selenite broth-Novobiocin® and Rappaport Vassiladis for enrichment at 37°C and 42°C, for 24 h. After enrichment, samples were isolated on MacConkey Agar and XLD with incubation at 37°C for 24 h. Suggestive colonies of *Salmonella* spp. were place onto Radhanjs Agar®, Agar Rambach® and TSI (Triple Sugar Agar) and incubated under the same conditions. The extraction of DNA of *Salmonella* also followed the technique of Marmur [12], and the identification with PCR reactions using oligonucleotides of *invA* gene (Table 2). Amplifications were executed in thermal cycler Veriti Thermal Cycler® with annealing temperature of 60°C for 1 min. The samples were subjected to electrophoresis in agarose gel 1% (p/v), with SYBR® Green 1X and molecular size standard 100 pb DNA Ladder®, and photo documented on GEL DOC™ EQ.

Positive samples to *invA*, were submitted also to gene *fliC* (Table 2) in order to identify a possible *Salmonella* Typhimurium. The PCR executed in
RESULTS

Seventeen of the 84 samples from confinement presented anaerobic bacteria with counts of $1.2 \times 10^5$ CFU/mL in the first assessment, and $7.88 \times 10^2$ CFU/mL in 24 animals in the second one. Samples of the slaughterhouse showed counts of $1.8 \times 10^4$ CFU/mL. These samples characterized greater frequency of anaerobic when compared with confinement showing higher variation DMS (5%). There were no significant differences between the two assessments (DMS 5% = 0.7024). The slaughterhouse samples compared to those of the confinement showed an increased frequency of anaerobic count in the samples collected in the slaughterhouse (Table 3).

The weight gain of animals of the confinement varied according the bacterial count, with negative coefficient of correlation between the count of Clostridium spp. and weight gain of animals, with statistical analysis

Data are presented as mean values ± sd of duplicate measurements. Statistical analysis was done by Tukey’s test, $P < 0.01$ was considered significant, and the coefficient of variation stabilized by transformation of values for log ($x + 5$).

Table 1. Nucleotides sequences used to amplify and confirm the Clostridium perfringens species in the collected samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Size (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa</td>
<td>Forward</td>
<td>AGTCTACGCTTGGGATGGAA</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTCCCTGGGTTGTCATTTTC</td>
<td></td>
</tr>
<tr>
<td>cpb</td>
<td>Forward</td>
<td>TCTTTTCTTGAGGAGGATAAA</td>
<td>611</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGACCCTCCTATTTGTATCCCA</td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>Forward</td>
<td>AAACGCATTAAAGCTACACC</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGCATAACCTGGAATGGCT</td>
<td></td>
</tr>
<tr>
<td>cpe</td>
<td>Forward</td>
<td>GGGGAACCCTCAGTGTTC</td>
<td>506</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCAGCTGAGTTGATTTTAATG</td>
<td></td>
</tr>
<tr>
<td>etx</td>
<td>Forward</td>
<td>TGGGAACCTTGATAAGCA</td>
<td>396</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTAACCTCATCTCCCTAATGCAC</td>
<td></td>
</tr>
<tr>
<td>cpb-2</td>
<td>Forward</td>
<td>CAAGCAATGCGGAGGTATTA</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAGAATCAGGATTTTGACCA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Nucleotide sequences used for amplification and confirmation of the species of Salmonella spp. and Salmonella Typhimurium in the collected samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>Microorganisms</th>
<th>Size (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>GTGAAATTATCGCCACGTTCGGGCA</td>
<td>Salmonella spp.</td>
<td>284 pb</td>
</tr>
<tr>
<td></td>
<td>TCATCGCACCCTCAAAGGAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fliC</td>
<td>CGGTGTTGCCCAGGTGGTAAT</td>
<td>Salmonella</td>
<td>620 pb</td>
</tr>
<tr>
<td></td>
<td>ACTGGTAAGAGATGGCT</td>
<td>Typhimurium</td>
<td></td>
</tr>
</tbody>
</table>
value of -0.25, showing that the mean samples group with larger bacterial counts showed lower weight gain.

Molecular research of Alpha toxins (cpa), beta (cpb), Epsilon (cpe) and iota (τ), allowed the identification of *Clostridium perfringens* type A in 21 (25%) and confinement of animals (46.4%) of 39 individuals in the slaughterhouse (Figure 1).

*Salmonella* spp. were detected on eight samples (9.5%) at the confinement and 18 (21.4%) at slaughterhouse, using PCR with gene *invA* (Figure 2). For *Salmonella* Typhimurium, the number of positive samples in a slaughterhouse was greater than in confinement, reaching 8.33% of positivity in the samples (Figure 3), while in confinement the percentage was 3.57%.

<table>
<thead>
<tr>
<th></th>
<th>Average Values</th>
<th>Significance</th>
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<tbody>
<tr>
<td>First Evaluation</td>
<td>$1.2 \times 10^5$</td>
<td>b</td>
</tr>
<tr>
<td>Second Evaluation</td>
<td>$7.88 \times 10^2$</td>
<td>b</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>$1.85 \times 10^4$</td>
<td>a</td>
</tr>
<tr>
<td>CV</td>
<td>49.7</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>28.89*</td>
<td></td>
</tr>
<tr>
<td>$P$</td>
<td>$&lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>DMS (5%)</td>
<td>0.7024</td>
<td></td>
</tr>
</tbody>
</table>

*Transformed data for log (x+5), the equal lyrics represent data what no significant differences and lyrics different which obtained significance with each other. Statistical results are showed according to Tukey’s test ($\alpha = 5\%$).

**Figure 1.** Amplification of samples of *Clostridium perfringens* type A with primer encoding the *cpa* gene by PCR. Channels: 1. Standard molecular 100 bp DNA Ladder; 2. Positive Control; 3. Negative Control; 4 to 6. Samples collected from confinement; 7 to 9. Samples collected in the slaughterhouse.

**Figure 2.** Amplification of *Salmonella* spp with primer encoding the *invA* gene by PCR. Channels: 1. Standard molecular bands of 100 bp DNA Ladder; 2. Positive Control; 3. Negative Control; 4 to 6. Samples collected from confinement; 7 to 10. Samples collected in the slaughterhouse.

**Figure 3.** Amplification of Salmonella Typhimurium with primer encoding fliC gene by PCR. Channels: 1. Standard molecular bands of 100 bp DNA Ladder; 2. Positive Control; 3. Negative Control; 4 to 7. Samples collected from the slaughterhouse; 8 to 10. Samples collected in the confinement.

**DISCUSSION**

The bacterial counts between the confinement and slaughterhouse groups showed significant difference ($P < 0.01$). The samples taken at the slaughterhouse were from several farms at random, and allowed the appraisal of hygienic conditions, since animals of confinement remained sanitized and the accumulation of feces on floor was avoided. The difference in bacterial counts can be linked to this factor, where the group with controlled conditions showed lower scores of colonies.

When comparing the two samples collected on confinement, it is observed that there were more positives in the second ones. This fact can occur when animals kept in confinement suffer stress, this may affect the immune system and makes them susceptible to infections by environment pathogens. During the confinement period, there were no characteristic signs of infection by *C. perfringens*, however, the typification by PCR showed positivity for this microorganism in the pigs. *C. perfringens* is commonly found in the intestines of warm-blooded animals without clinical signs, which occur only when there is production of toxins [7].

Molecular biology techniques are gain headway by allowing the identification of potentially pathogenic microorganisms, prior to the onset of clinical signals. This fact is of great relevance to contribute preventively with the diagnosis and treatment of animals before they can cause damage to the sector. *C. perfringens* are responsible for reducing the feed conversion ratio and the gain weight of animals, and that demands greater investments with food and supplements in order to minimize severe cases of economic loss.

This comparison can be confirmed when observed the correlation coefficient between bacteria and weight of confined animals. These results demonstrate that animals with high count of bacteria were those who had greater difficulties in gaining weight, and consequently were slaughtered below the ideal weight. This fact corroborates with the ones described by Baker [1], where this occurrence was also verified.

In all cases of piglets diagnosed with *C. perfringens*, they were found to have enteritis and diarrhea, which leads to weight loss and often deaths of less resistant individuals. Among the toxins of this agent, the most commonly associated with the disease is type *alpha* toxin, which is characterized by its aggressiveness in its action on the intestinal villi [6]. However, more recent researches have demonstrated that the expression of *alpha* toxin alone did not show worrying cases of injury, only when it is associated with other toxins that expressed its cytotoxic power [17]. Recently, studies shows that *beta2* toxin produced by *C. perfringens* type A has been linked to frames of illnesses in pigs, but the specific role of this toxin in the manifestation of enteric diseases is not fully elucidated [15]. In a recent and extensive study, 85% isolates from pigs with diarrhea or enteritis were *cpb-2*-harbouring, and is correlation was even higher (91%) in neonatal pigs with enteritis or diarrhea [16].

This relationship between the cytotoxic power of toxins allows the understanding of the results of this work, where even in the absence of clinical signs it was positive for the presence of gene *cpa* (*alpha* toxin encoder) of *C. perfringens* found by molecular techniques.
This data permit to conclude that the illness in confined pigs is directly related to the presence or absence of the gene expression for the toxin beta2. The absence of illness in the pigs was confirmed because the expression of this gene was not identified molecularly in the samples.

As for the presence of Salmonella spp. and S. Typhimurium the literature recognizes the presence of this bacteria intermittently and systematically frequent in animals from grange. This fact worries the industrial sector requiring greater care during the slaughter and evisceration in order to prevent the spread of this agent in the industrial process.

The results for Salmonella spp. showed higher incidence at the slaughterhouse (21.4%) when compared to confinement (9.5%). This difference can be attributed to hygienic quality of the finishing farms and the fact that animals over stress suffered during transport to slaughter, since this can trigger the intermittent elimination of the agent to environment, infecting healthy animals. The detection of Salmonella spp. in slaughterhouse is considered a risk factor for the steps of slaughter, evisceration and cutting of carcasses, since the industrial process is dynamic and an effective control of this agent during the production line is difficult. Bessa [3] found significant correlations between the presence of Salmonella spp. in carcasses on slaughter line and the bacteria in intestinal contents of animals.

Up to 10% of cases of salmonellosis in humans is motivated by consumption of pork meat, and that 17% of the pigs with age of slaughter are infected with Salmonella spp., but estimate that 4% of the animals eliminate the microorganism in their feces for a long period [10].

Many researches have been demonstrating that the use of PCR to identify Salmonella spp. have more sensitivity than the conventional microbiology. For this work, this technique gave swift and reliable results, since it consisted of amplification of specific genes to the species of C. perfringens and Salmonella spp. and S. Typhimurium. PCR technique was much more swifter and effective than conventional methods [9], since in just three days the author obtained relevant and reliable results while traditional methods extended for more than seven days.

Also comparing standard microbiological techniques with PCR technique for the detection of the genus Salmonella spp. [5], concluded what on PCR resulted in 21% more positive samples detected than by standard microbiology technique. These results have established that PCR is proven to be a fast and secure method for identification, in addition to preventing the occurrence of dubious results before the interpretation of biochemical tests.

It was concluded that technique is frequently used in research labs, scientific papers and has gained headway in other sectors, such as food industries, production and diagnostic laboratories, in order to improve the control and speed identification. Studies that seek to identify the presence of C. perfringens and Salmonella spp. in finishing pigs leads to better understanding of the action of disease and of prevention methods, thus avoiding serious economic losses in the sector and improving sanitary control of farms and slaughterhouse.

CONCLUSION

The presence of C. perfringens and Salmonella spp. is related directly to negative influence on pig production, mainly in initial confinement hindering the weight gain of the animals, and in the industry sector contaminating the meat and its derivatives.

In these cases the molecular biology methods showed to be quite efficient when compared to the conventional ones, using the gens of toxins in the detection of such pathogens. Thus, PCR can be considered an useful tool to improve pig production.

MANUFACTURERS
1BD BBLTM. Franklin Lakes, NJ, USA.
2Applied Biosystems Inc. CA, USA.
3Invitrogen. Carlsbad, CA, USA.
4Fermentas AB. Vilnius, Lithuania.
5BIO-RAD. Benicia, CA, USA.
6HiMedia Laboratories, L.B.S. Marg. Mumbai, India.
7Merck, Sharp & Dohme S.A. Madrid, Spain.

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

REFERENCES


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