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# IDENTIFICATION OF BACTERIAL AGENTS OF ENTERIC DISEASES BY MULTIPLEX PCR IN GROWING-FINISHING PIGS

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## ABSTRACT

In Brazil, the most common bacterial enteric diseases affecting growing and finishing pigs are porcine proliferative enteritis, porcine intestinal spirochetosis, swine dysentery, and salmonellosis. The diagnosis of these diseases by routine culture techniques is expensive, difficult, time-consuming, and even impossible, in cases of porcine proliferative enteritis. The detection of pathogens by polymerase chain reaction is a highly sensitive and specific method that can be an useful tool in veterinary diagnosis. Two multiplex PCR (M-PCR) assays were tested for simultaneous detection and identification of bacterial agents associated with porcine proliferative enteritis, porcine intestinal spirochetosis, swine dysentery, and salmonellosis in diarrheic fecal samples. The DNA obtained from pure cultures of each bacterial agent or mixed in different combinations and concentrations was amplified by using *Lawsonia intracellularis* and *Salmonella*, or *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* specific M-PCR assays. After electrophoresis in agarose gel and staining, the amplification products indicated the presence of individual or simultaneous amplification of *L. intracellularis* and *Salmonella or B. pilosicoli* and *B. hyodysenteriae* specific DNA sequences. After standardization, the M-PCR tests were used to test 541 swine diarrheic fecal samples obtained from different regions in Brazil. The most frequently detected pathogen was *Lawsonia intracellularis* (13%), followed by *Salmonella* (4.8%), *B. hyodysenteriae* (1.4%), *B. pilosicoli* (1%) and their various associations. Results from this study suggest that the two M-PCR assays can be used for specific detection and identification of four important enteric bacterial pathogens alone or in combination.

**Key words:** enteritis, PCR, pig, bacteria, diagnosis.

## INTRODUCTION

It is necessary to accurately detect and identify porcine pathogens (5) in order to devise proper treatment and prevention programs. Porcine proliferative enteritis (PPE) caused by *Lawsonia intracellularis* (16), porcine intestinal spirochetosis (PIS) caused by *Brachyspira pilosicoli* (24), swine dysentery (SD) caused by *Brachyspira hyodysenteria*e (11) and porcine salmonellosis (PS) caused by *Salmonella* (25) are the most common diarrhea-causing bacterial diseases affecting pigs during the growing finishing stages of production. The economic impact of these diseases on pig production results in mortality, growth rate losses and substantial antibiotic costs (16).

The diseases caused by *L. intracellularis*, *B. pilosicoli*, *B. hyodysenteriae*, and *Salmonella* affect a similar age groups during the production cycle. These agents are transmitted by the fecal-oral route and their isolation and identification can take-up to several days or weeks and the results are not always definitive (8,9). Diagnosis of enteric diseases has been made on basis of clinical signs, isolation of this etiologic agents for PIS, SD and PS and detection of *L. intracellularis* by PCR for PPE (12,25).

Amplification of pathogen-specific DNA sequences using PCR is considered to be the future in diagnostics, because it increases sensitivity, specificity, besides allowing for a faster diagnosis (5). It has been shown that the detection of *L.*

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*intracellularis*, *B. hyodysenteriae*, *B. pilosicoli* and *Salmonella* using specific DNA sequences (primers) and PCR amplification is more sensitive and specific than standard culture techniques for diagnosis of SD, PIS and PS and histopathological analysis for PPE (1,12,18,22).

Currently, the use of PCR in veterinary medicine is still limited to a small number of diagnostic laboratories, the introduction of M-PCR can change this picture, since it allows for reducing the costs and time spent in the diagnosing enteric diseases maintaining however, the sensitivity and specificity of the original tests.

In Brazil, the incidence of the above-mentioned enteric pathogens is poorly studied, although diarrhea is commonly seen during growing and finishing. Therefore, this study aimed at standardizing the M-PCR by using different PCR tests previously described for diagnosing PPE, PIS, SD and PS, and to apply the M-PCR in the differential diagnosis of enteritis in pigs coming from different regions in Brazil.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*Brachyspira hyodysenteriae* strain ATCC 49527 and *Brachyspira pilosicoli* strain ATCC 51139 were grown up to the late exponential phase (approximately $10^8$ cells $mL^{-1}$) in pre-reduced Trypticase soy broth (PRAS), anaerobically sterilized, as described previously (15, 19). Broth cultures were constantly stirred at 37ºC in a 10% hydrogen, 10% carbon dioxide, and 80% nitrogen atmosphere.

*Salmonella* Typhimurium ATCC 6994 was grown aerobically in Luria-Bertani broth at 37ºC. Purified DNA from *L. intracellularis* was isolated from organisms grown in cell culture as described previously (12).

### Fecal samples

Five hundred and forty one fecal samples were obtained from seventy swine herds from the main pig producing regions in Brazil, including the states of São Paulo, Santa Catarina, Paraná, Rio Grande do Sul, Minas Gerais, Mato Grosso do Sul, Goiás. The diarrheic animals sampled were between 60 and 140 days old (growing – finishing stages).

### DNA extraction

The procedure based on the binding of DNA to silicates in the presence of high guanidine thiocyanate (GuSCN) concentrations of was modified and used to extract total DNA of bacterial strains and fecal samples (4).

A sample of 0.3 g of feces or 200 mL of bacterial culture was added to a microtube and with a lytic guanidine thiocyanate solution and a diatom suspension. Then the tube was vortexed for 30 s and centrifuged (12,000 x g) for 15 s and the supernatant discarded. The pellet was washed twice with another guanidine thiocyanate solution, and twice with ethanol 70%. Finally the pellet was washed with acetone and dried. A elution buffer was added to the pellet and it was heated to 56ºC for 10 min. The tube was centrifuged 12,000 x g for 5 min and the supernatant containing the DNA was used to M-PCR

### Primers and M- PCR reactions

Primers for specific amplification of *L. intracellularis*, *B. pilosicoli*, *B. hyodysenteriae* and *Salmonella* are described in Table 1. The primers were commercially synthesized (Life Technologies- Grand Island, NY) and tested for multiplex PCR with DNA obtained from bacterial strains alone and in different combinations.

Under our conditions, the use of the M-PCR as described by Elder *et al.* (8) resulted in preferential amplification of *Salmonella*, in detriment of *B. hyodysenteriae* detection (data not shown). In our laboratory, we were not able to amplify the DNA of *L. intracellularis* using the program described by these authors, nor include the *B. pilosicoli* primers. Based on the obtained results, we decided try other combinations of *B. hyodysenteriae* and *B. pilosicoli* primers. The primers pairs showing higher sensitivity and considered to be compatible to be used in one test tube reactions were selected for detection of *B. pilosicoli* and *B. hyodysenteriae* by M-PCR.

Two PCR amplification mixtures (50 ml) were used, M-PCR1 consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM each dATP, dCTP, dGTP, dTTP, 50 pmol of each primer (12,22), and 1.0 U of *Taq* DNA polymerase (Life Technologies-Grand Island, NY), autoclaved, ultrapure water and 10 µL of DNA template. M-PCR2 consisted of 1X PCR bufer, 3 mM $MgCl_2$, 200 mM each dATP, dCTP, dGTP, dTTP, 20 pmol of each primer (1,13), and 1.0 U of *Taq* DNA polymerase (Life Technologies- Grand Island, NY), autoclaved, ultrapure water and 10 µL of DNA template. Amplification was carried out in a DNA thermocycler (Model MJ Research 200). Different annealing temperatures were tested and two programs were selected for use in the M-PCR assays (Table 2). The amplified products were separated by electrophoresis in 2.0% agarose gel and stained with ethidium bromide. The 100 bp DNA ladder (Life Technologies- Grand Island, NY) was used as a molecular size marker.

In order to evaluate the effect of the number of bacterial cells on the sensitivity and specificity of the M-PCR assay, purified DNA from *L. intracellularis*, *B. pilosicoli*, *B. hyodysenteriae* and *Salmonella* were tested combined and alone in various dilutions to simulate concentrations among 1 to $10^9$ bacterial cells.

## RESULTS

### M-PCR assays

Different primers described for *B. pilosicoli* and *B. hyodysenteriae* detection were tested with various DNA concentrations and the pairs showing higher sensitivity were

**Table 1.** Primers tested for multiplex PCR amplification of specific DNA sequences of *L. intracellularis, B. pilosicoli, B. hyodysenteriae, Salmonella.*

| Agent | Primers 5 -3 (sense e antisense) | Expected Product | Reference |
|---|---|---|---|
| *L. intracellularis*[a] | TATGGCTGTCAAACACTCCG TGAAGGTATTGGTATTCTCC | 319 pb | 12 |
| *Salmonella*[a] | TGCCTACAAGCATGAAATGG AAACTGGACCACGGTGACAA | 457 pb | 22 |
| *B. hyodysenteriae* | GGTACAGGCGGAAACAGAC TCCTAT TCTCTGACCTACTG | 1550 pb | 7 |
| *B. hyodysenteriae*[b] | TTA AAA CAA GAA GGA ACT CTA ATA AAC GTC TGC TGC | 821 pb | 1 |
| *B. hyodysenteriae* | ACC TAT TAT TTA ATT AAA CTT ATC GAA AAT ACT AAT AAA AGA CGG | 561pb | 10 |
| *B. pilosicoli* | TACGGCTACCTTGTACGACTT AGAGGAAAGTTTTTTCGCTTC | 1300 pb | 20 |
| *B. pilosicoli*[b] | TCCGCCTACTCACCCTTTAC AGAGGAAAGTTTTTTCGCTTC | 361 pb | 18 |

[a] selected primers for utilization in M-PCR1 assay;
[b] selected primers for utilization in M-PCR2 assay.

**Table 2.** Programs used for M-PCR DNA amplifications.

| Program 1 | | | Program 2 | | |
|---|---|---|---|---|---|
| Temperature | Steps | | Temperature | Steps | |
| 95ºC | 5 min | 1 cycle | 96ºC | 3 min | 1 cycle |
| 95ºC | 1 min | | 96ºC | 1 min | |
| 55ºC | 1 min | 38 cycles | 60ºC | 1 min | 38 cycle |
| 72ºC | 1,5 min | | 72ºC | 1,5 min | |
| 72ºC | 5 min | 1 cycle | 72ºC | 3 min | 1 cycle |

selected for M-PCR assay (data not shown). M-PCR amplification of purified DNA from *L. intracellularis*, *B. pilosicoli, B. hyodysenteriae* and *Salmonella* in combination has not yielded the expected results. The expected products were obtained when *L. intracellularis* (319 bp) and *Salmonella* (457 bp) were amplified alone or in combination starting from various concentrations of DNA using the program 1 (annealing temperature of 55ºC). Amplification of *L. intracellularis* DNA was not possible with annealing temperature of 60ºC.

Amplification of *B. pilosicoli* (361bp) and *B. hyodysenteriae* (821bp) DNA products was obtained alone or in combination from different concentrations of DNA using program 2 (annealing temperature of 60ºC). Amplification of *B. pilosicoli* with annealing temperatures of 55ºC produced nonspecific bands in fecal samples. When DNA of *Salmonella* were submitted to M-PCR with DNA of *Brachyspira*, a preferential amplification of DNA of *Salmonella* was observed.

The optimal $MgCl_2$ concentration for reactions with *L. intracellularis* and *Salmonella* was 1.5 mM, while the optimal $MgCl_2$ concentration for reaction of *Brachyspira* with selected primers was 3.0 mM. The use of 3.0 mM $MgCl_2$ concentration in *L. intracellularis* and *Salmonella* resulted in nonspecific bands. Lowered concentration of this salt in reactions with *Brachyspira* gave poor results. Based on these observations, two programs (Table 2) and two different reactions M-PCR1 (*L. intracellularis* and *Salmonella*) and M-PCR2 (*B. pilosicoli* or *B. hyodysenteriae*) were used as described. These reactions yielded the expected results in detecting bacterial strains in different concentrations, with a sensitivity similar to previously described (1,12,18,22). The sensibility values of PCR and M-PCR for *L. intracellularis*, *B. pilosicoli*, *B. hyodysenteriae* and *Salmonella* were $10^3$, $10^3$, $10^2$, and $10^2$ bacterial cells respectively.

**Analysis of fecal samples**

The five hundred and forty one fecal samples tested resulted in 13% (70/541) samples positives for *L. intracellularis*, 4.8% (26/541) positives for *Salmonella*, 1% (5/541) positive for *B. pilosicoli* and 1.4% (8/541) positives for *B. hyodysenteriae*. The infections occurred either alone singly or in a variety of combinations, as shown in Table 3.

**Table 3.** Results obtained from detection of *L. intracellularis, B. pilosicoli, B. hyodysenteriae* and *Salmonella* in 541 fecal samples.

| Agent | N | % |
|---|---|---|
| *L. intracellularis* | 70 | 13 |
| *B. pilosicoli* | 5 | 1,0 |
| *B. hyodysenteriae* | 8 | 1,4 |
| *Salmonella* | 26 | 4,8 |
| *L. intracellularis and B. Pilosicoli* | 16 | 3,0 |
| *L. intracellularis and Salmonella* | 4 | 1,0 |
| *B. hyodysenteriae and Salmonella* | 2 | 0,4 |
| *L. Intracellularis, B. pilosicoli and Salmonella* | 2 | 0,4 |
| Negatives | 408 | 75 |
| Total | 541 | 100 |

## DISCUSSION

The amplification of purified DNA obtained from four different pathogens, in different dilutions, using two M-PCR assays, confirmed previous reports with individual PCR assay (1,12,18,22). Amplification of specific 319-bp and 457-bp products were observed with M-PCR1 assays for *L. intracellularis* and

*Salmonella* respectively. In addition, amplification of specific 361-bp and 821-bp products were obtained with M-PCR2 assay for *B. pilosicoli* and *B. hyodysenteriae* respectively. Under the described conditions, nonspecific amplification products were not detected in the M-PCR1 and M-PCR2 tests with purified bacterial DNA or with fecal samples.

The importance of *L. intracellularis* in Brazilian herds was previously described by Moreno *et al.* (17), reporting 15% of positive fecal samples (146/971). Similar results were obtained by Takahashi *et al.* (23), in Japan (14.9% - 33/221). Chang *et al.* (6) in Taiwan (5.5% - 31/560), Jordan *et al.*, (13) in United States (5,6% - 35/621) and Kim *et al.* (14) in Korea (3,3% - 16/490) reported lower incidences than those observed in Brazil. Occurrence of healthy pigs carrying *L. intracellularis* and eliminating the agent intermittently in feces has been reported (16).

Though the fact that 6.6% of the pigs were shedding *Salmonella*, this did not mean that they had clinical PS, since they were not showing signs of septicemia. These results means that the animals are actually carrier, and pose risks for human health. During slaughter, the carcass may be contaminated by animal's feces containing *Salmonella* (25).

The incidence of *B. hyodysenteriae* detected in the present study was lower that those described by Scarcelli *et al.* (21) and Barcellos *et al.* (2). In different regions in Brazil, these authors reported a incidence of 9.1% and 13.5%, respectively. The incidence of *B. pilosicoli* was also lower than the prevalence of 7.2% (157206) reported by Barcellos *et al.* (2). The difference between our findings and those reported by Barcellos *et al.* (2) and Scarcelli *et al.* (21) can be explained by regional differences, as well as by the use of antimicrobials in the studied herds. It has been reported that the antibiotic use can reduce the isolation and detection rates of Brachyspira from swine feces (2). The isolation of *B. hyodysenteriae* from feces of pigs without clinical signs of diarrhea is infrequently, but *B. pilosicoli* can be present in these cases (11).

The detection and identification of porcine intestinal spiroquetes by PCR- restriction fragment length polymorpism analysis of ribosomal DNA encoding 23S rRNA was described by Barcellos *et al.* (3). This technique was shown to be useful for identification of pathogenic spiroquetes, as *B. pilosicoli* and *B. hyodysenteriae*, but was more time-consuming, labor-intensive and expensive than M-PCR.

The studied combination of agents reinforces the importance of using the M-PCR assay for the diagnosis of enteric diseases in pigs. The use of M-PCR technique included in a diagnostic panel provides a practical and cost-effective method for rapid detection of pathogens associated with swine diseases.

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## RESUMO

### Identificação de agentes bacterianos de doenças entéricas em suínos por PCR-multiplex

A enterite proliferativa suína, a espiroquetose colônica, a disenteria suína e a salmonelose são as doenças entéricas mais freqüentes em suínos das fases de crescimento e terminação no Brasil. O diagnóstico destas doenças através de técnicas bacteriológicas tradicionais é difícil, lento e no caso da enterite proliferativa suína, impossível. A detecção destes agentes através da reação de polimerase em cadeia é altamente sensível e específica e está se tornando uma ferramenta muito útil no diagnóstico em Medicina Veterinária. No presente estudo foram testadas duas técnicas de PCR sob a forma de "multiplex" para detecção e identificação simultânea dos agentes bacterianos envolvidos na enterite proliferativa suína, na espiroquetose colônica, na disenteria suína e na salmonelose em amostras de fezes diarreicas. O DNA obtido de culturas puras de cada agente sozinho ou misturado em diferentes combinações e concentrações foram amplificados utilizando a Multiplex PCR específica para *Lawsonia intracellularis* e *Salmonella*, ou *Brachispyra pilosicoli* e *Brachispyra hyodysenteriae*. Após a padronização, a M-PCR foi aplicada na detecção dos quatro agentes em 541 amostras de fezes diarreicas de suínos provenientes de diferentes Estados do Brasil. O agente mais freqüente foi *Lawsonia intracellularis* (13%), seguido pela *Salmonella* spp. (4,8%), *B. hyodysenteriae* (1,4%), *B. pilosicoli* (1%) e suas diferentes associações. Os resultados obtidos indicam que as duas reações testadas permitem a detecção destes quatro importantes patógenos entéricos de maneira rápida e específica.

**Palavras-chave:** enterite, PCR, suíno, bactéria, diagnóstico.

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