The prevalence of swine enteropathogens in Brazilian grower and finish herds

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Abstract

Diarrhoea among growing and finishing pigs is an important problem in many herds. The prevalence of L. intracellularis, B. pilosicoli, B. hyodysenteriae, Salmonella spp., enterotoxigenic E. coli, Trichurus suis and the occurrence of mixed infection were investigated. Fecal samples for forty-six herds with diarrhea or a history of diarrhea were randomly collected in Minas Gerais state, Brazil. The enteric pathogens were detected by culture (E. coli and Salmonella sp.), PCR (L. intracellularis and Brachyspira spp.) and eggs counts (T. suis). The overall herd prevalence of L. intracellularis, Salmonella enterica serotype Typhimurium and enterotoxigenic E. coli were 19.56%, 6.52%, 10.86% respectively. Mixed infection was diagnosed in 30.43% of herds, and L. intracellularis and Salmonella enterica serotype Typhimurium are main pathogens association (10.87%). B. pilosicoli was diagnosed only in two herds, always associated with mixed infections. B. hyodysenteriae and T. suis were not demonstrated in any sample. These pathogens have been reported world-wide but studies regarding epidemiology in Brazil are few. This study contributes to establish of prevention programs for the control enteropathogens in grower finish herds in Brazil.

Key words: swine, enteropathogens, grower finish herds, prevalence, Brazil.

Introduction

According to research, Brazil is the fourth largest producer of pigs, behind China, the European Union and United States. The state of Minas Gerais is the fourth largest producer of pork in Brazil, and the growth of pig production in the state has been increasing year by year (Abipesc 2009). Because of this it is necessary to accurately detect and identify porcine pathogens in order to devise proper treatments and prevention programs (Baccaro et al., 2003).

The enteric infections are among the most frequent diseases in pig production, being responsible for significant losses and significant economic impact on industry. The damages are represented primarily by reducing weight gain, mortality and expenses with antibiotics (McOrist 2005). Further, some of these diseases can be transferred to humans (Weneger and Bager, 1997). Several agents have been suggested as possible causes of diarrhea in growing/finishing pigs. Infection by L. intracellularis causing porcine proliferative enteritis, swine salmonellosis caused by Salmonella enterica serotype Typhimurium, porcine intestinal espirochetosis caused by B. pilosicoli, swine dysentery caused by B. hyodysenteriae, and Trichurus suis causing intestinal trichuriasis are among the most prevalent at this age (Batte et al., 1977; Thomson et al., 1998; Stege et al., 2000; Baccaro et al., 2003). Enterotoxigenic strains of Escherichia coli may also be present among growing pigs weighing 30-50 kg with or without symptoms (Stege et al., 2000).

The risk of diarrhea increases when the herd is infected by two or more pathogens simultaneously (Stege et al., 2000; Suh and Song, 2005). Usually the pigs with mixed infection have lesions more pronounced with lower development of the herd. Suh and Song (2005) analyzing 462 swine fecal samples observed the occurrence of mixed infections, represented by 3% of the samples simulta-
neously infected with *Salmonella* sp. and *B. hyodysenteriae*, 2.2% with *L. intracellularis* and *Salmo-
nella enterica* spp. and 1.3% with *L. intracellularis* and *B. hyodysenteriae*.

These pathogens have been reported in world-wide but (Thomson et al., 1998; Stege et al., 2000; Jacobson et al., 2005; Suh and Song, 2005; La et al., 2006) the importance and involvement of some of these organisms in clinical disease still remains to be clarified in the Brazilian swine herds. The purpose of this study was to determine the prevalence of the enteric pathogens *L. intracellularis*, *B. pilosicoli*, *B. hyodysenteriae*, *Salmonella enterica*, enterotoxigenic *E. coli*, *T. suis* and the occurrence of mixed infections in Brazilian growing/finishing pigs from swine farms with diarrhea or a history of diarrhea in the state of Minas Gerais, Brazil.

### Materials and Methods

#### Herds and animals

Farrow-to-finish herds with diarrhea or a history of diarrhea were randomly selected from four different geographical regions of the state of Minas Gerais, Brazil [South and southwest (SSW), metropolitan area of Belo Horizonte (MBH), Zona da Mata (ZM) and Triângulo Mineiro e Alto Paranaiba (TMHP)]. The sample size was calculated at two levels, property and animals. The feces were collected between January 2008 and February 2009. To determine the number of herds, simple sampling was used with an estimated prevalence of 50% confidence level selected for serotyping at the Institute Oswaldo Cruz, Rio de Janeiro, Brazil.

Aliquots of the feces mixed in PBS were plated in MacConkey agar (37 °C/24 h) for the isolation of lactose positive colonies (Lac+ colonies). These colonies were frozen in glycerol at -80 °C for later PCR multiplex for enterotoxigenic *E. coli*.

#### Detection of *L. intracellularis*, *B. hyodysenteriae* and *B. pilosicoli*

The methods use for the diagnostic of *L. intracellularis*, *B. hyodysenteriae* and *B. pilosicoli* and the sensitivity of detection are a multiplex –PCR for rapid detection (La et al., 2006). The positive controls were kindly provided by department of Veterinary and Biomedical Sciences at University of Minnesota, EUA.

#### Extraction of DNA and multiplex PCR primers

DNA was extracted from all faeces using the QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer’s instructions. The respective primer sequences were:

- H1 (5’-ACTAAAGATCTGATGATTGTG-3’) and H2 (5’-CTAATAACGTCTGCG-3’) targeting a 354 bp region on the nicotinamide adenine dionucleotide hybrane (NADH) oxidase (nox) gene of *B. hyodysenteriae*;
- P1 (5’-AGAGGAAAGTTTTTTCGCTC-3’) and P2 (5’-GCACCTATGTTAAGCCTCGT-3’) targeting a 823 bp region of the 16S rRNA gene of *B. pilosicoli*; and
- Lint-146F (5’-GATAATCTACCTTCGAGACGG-3’) and Lint-745R (5’-TGACCTCAGTGTCAGTTATC-3’) targeting a 655 bp region of 16S rRNA gene of *L. intracellularis*.

#### Multiplex PCR

The DNA was amplified in 25 μL total volume. Amplifications mixtures consisted of 1 X PCR buffer (containing 1, 5 mmol L-1 of MgCl2), 1.25 U of Taq DNA polymerase (Cenbiot, Porto Alegre, Brazil), 0.1 mmol L-1 of each dNTP (Invitrogen, Carlsbad, CA), 0.2 mmol L-1 of each primer pair (H1 and H2, P1 and P2, Lint-745R) (Prodinol, Wisconsin, USA) and 2.5 μL chromosomal template DNA. Cycling conditions involved an initial 5 min Taq DNA polymerase activation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 90 s, and a primer extension step at 68 °C for 2 min. A final 10 min extension step at 68 °C. The PCR products were subjected to electrophoresis in 1% (w/v) agarose gels in 1 x TAE buffer, stained with ethidium bromide and viewed over UV light. The sensitivity of detection of the multiplex PCR was estimated (La et al., 2003).

#### Multiplex PCR for detection of *Escherichia coli* enterotoxigenic virulent genes

For the diagnosis of enterotoxigenic *E. coli* genes a multiplex-PCR was used (Macedo et al., 2007). As positive
controls for the factors of virulence, four reference samples of *E. coli* were kindly provided by the Veterinary Diagnostic Laboratory at the University of Minnesota.

**Extraction of DNA**

After thawing the Lac + colonies were plated on MacConkey agar. Isolated colonies were resuspended in microtubeh containing 500 µL sterile PBS. The bacteria were centrifuged at 7000 rpm for 10 min for sedimentation. Sediment was added 270 µL lysis solution (Tris HCl 1.0 M, pH 8.0; EDTA 0.5 M; NaCl 4.5 M; SDS 10%) and 200 µL of High TE (Tris HCl 1.0 M, pH 8.0; EDTA 0.5 M). After mixing and vortex for 20 to 30 seconds, the mixture was incubated at 55 °C for at least 3 h. The lysate containing the bacterial DNA was subjected to DNA extracting by phenol-chloroform (Sambrook *et al.*, 1989).

**Multiplex PCR**

The PCR technique used four pairs of primers for the toxins Stb, StaP, LT and Stx2e and five pairs for the fimbriae K99, F18, 987P, K88 and F41. Briefly the DNA was amplified in 10 µL total volume containing 10 ng of bacterial DNA. Amplifications mixtures consisted of 0.2 mmol L⁻¹ of each dNTP (Prodimol, Wisconsin, USA), 1.5 mmol L⁻¹ of MgCl₂ (Cenbiot, Porto Alegre, Brazil), 0.5 mmol L⁻¹ of each primer (Invitrogen, Carlsbad, CA) and 5 U of TAQ polymerase (Cenbiot, Porto Alegre, Brazil). Cycling conditions involved an initial 1 min Taq DNA polymerase activation step at 94 °C, followed by 25 cycles of denaturation at 94 °C for 1 min, at 55 °C for 1 min, and a primer extension step at 70 °C for 2 min. A final 10 min extension step at 72 °C. The PCR products were subjected to electrophoresis in 6% (w/v) polyacrylamide gels in 1 x TBE buffer, stained with silver.

**Parasitological exam**

For detection of *T. suis* eggs in swine feces, fecal samples were individually examined by flotation technique (Willis-Mollay) with a hyper saturate solution of NaCl (Hoffmann 1987). The infection intensity was scored according the number of eggs per gram of feces: 0, none; 1-5 mild; 5-10, moderate; and more than 10 severe.

**Statistics**

For statistical analyses Fischer’s exact test (SAS Institute, Cary, North Caroline, USA 1999) was used to determine the association between herd size and pathogen prevalence.

**Results**

The study comprised 46 farrow-to-finish pig herds. Forty six farms were analyzed, 31 with 101-500 sows and 15 with > 500 sows. The number of herds collected from each region and is in Table 1. A total of 512 faecal samples were examined. The analysis by the Fisher exact test showed no significant association between the pathogen and the size of the farm.

**Salmonella spp.**

The only subspecies of *Salmonella spp.* found, potentially pathogenic for pigs, was *Salmonella enterica* subspecies Typhimurium. The overall herd prevalence of *Salmonella enterica* subspecies Typhimurium was 6.52%. *Salmonella enterica* subspecies Typhimurium was diagnosed in 3 herds and was detected in 31 (6.05%) samples. One herd with 101-500 sows and two herds with more than 500 sows were positive for *Salmonella enterica* subspecies Typhimurium (Table 2).

Others subspecies of *Salmonella enterica*, not pathogenic for pigs were found in seven herds (15.2%). *Salmonella enterica* subspecies Agona was observed in one herd (2.17%) and two samples (0.4%) in a MBH herd with 101-500 sows. The *Salmonella enterica* subspecies Panama was diagnosed in three herds (6.52%) and 13 samples (2.54%). All herds were localized in ZM, two with 101-500 sows and one with > 500 sows. The *Salmonella enterica* subspecies Schwarzenground was observed in one herd (2.17%) and two samples (0.4%) in a MBH herd with > 500 sows. *Salmonella enterica* untypable was observed in two herds (4.34%) and two samples (0.4%). The herds were located in MBH one with 101-500 sows and one with > 500 sows.

*L. intracellularis*, *B. pilosicoli* and *B. hyodysenteriae*

The limit of detection obtained in PCR was 103 cells per gram of faeces for *B. pilosicoli* and 10⁴ for *L. intracellularis* and *B. hyodysenteriae*. The 10⁴ cells per gram of feces were obtained when the three species were applied together in equal number in the fecal samples.

*B. hyodysenteriae* was not isolated from any of the samples from the 46 piglet-producing herds. The overall herd prevalence of *B. pilosicoli* was 4.36%. This pathogen was demonstrated in two herds and in nine (1.75%) samples, always in mixed infections (Table 2). *L. intracellularis* was diagnosed in 9 herds (19.56%) and was detected in 59 samples (11.52%). This bacteria was more prevalent in ZM (8.69%) and TMHP (6.52%) regions (Table 2).

| Table 1 - Number of properties samples and herd size in each region of Minas Gerais. |
|-----------------------------------------------|
| Regions | ZM | TMHP | MBH | SSW | Total |
|--------|----|------|-----|-----|-------|
| Herd size |    |      |     |     |       |
| 101-500 | 11 | 5    | 8   | 7   | 31    |
| > 500   | 7  | 5    | 2   | 1   | 15    |
| Total   | 18 | 10   | 10  | 8   | 46    |

aZona da Mata” (ZM), bTriângulo Mineiro e Alto Paranaíba (TMHP), cMetropolitan area of Belo Horizonte (MBH), dSouth and southwest (SSW).
The overall prevalence of enterotoxigenic *E. coli* was 10.87%. The number of positive herds and samples was 5 and 26 (5.49%) respectively. The 512 stool collected only 473 samples showed growth of positive lactose colonies in MacConkey’s agar. The enterotoxigenic *E. coli* bacteria was more prevalent in ZM (6.52%) (Table 2).

The enterotoxigenic genotype more observed was the profile StaP-Stb-F18 with 8/26 (30.76%) positive samples, followed by genotype StaP-Stb-987P with 6/26 (23.07%) positive samples. Other genetic profiles found were: LT-K88, StaP-Stb-K99, Sbt-987p-K99, StaP-987p all with two samples each and K99-LT, STx2E-987p, LT-K88-STx2E and 987p-LT all with one positive sample detected.

Trichuris suis

*T. suis* eggs were not diagnosed in any of the samples from the 46 piglet-producing herds.

Mixed Infection

The overall herd prevalence of mixed infection was high 30.43%. Mixed infections were diagnosed in 14 herds
and 67 samples (13.08%). Nine herds were positive for two agents (19.56%), and five herds were positive for three agents (10.87%). There was a higher frequency of mix infection in herds with 101-500 sows, 10 in a total, compared with herds with more than 500 sows four (Table 2). The MBH region had the highest occurrence of mix infection, five herds. The most common mixed infection in herds was Salmonella enterica subspecies Typhimurium associated with L. intracellularis present in 5 herds (10.87%) and 28 fecal samples (5.47%), following by L. intracellularis associated with enterotoxigenic E. coli and Salmonella enterica subspecies Typhimurium present in 4 properties (8.69%) and 20 samples (3.9%). The concomitant detection of enterotoxigenic E. coli and L. intracellularis was diagnosed in 3 herds (6.52%) and 12 samples (2.34%). B. pilosicoli was detected concomitantly with L. intracellularis in 1 herd (2.18%) corresponding a four samples (0.78%), and with both L. intracellularis and Salmonella enterica subspecies Typhimurium, one herd (2.18%) and three samples (0.58%) (Table 2).

Discussion
Generally, studies on enteric disease have focused on a specific pathogen (Fellstrom et al., 1996), decreasing the chances of a correct diagnosis of the sanitary problems that affect the herd. The prevalence of six enteropathogens with pathogenic potential to grower/finisher pigs was studied and this prevalence was associated with herd size. It was been reported that outbreaks of L. intracellularis and Brachyspira spp. occur more often in the large production units (Holyoake et al., 1996), indicating an association between herd size and disease outbreak. However, the results in this study did not show this.

The prevalence of hers infected with L. intracellularis was 19.56%. This result is in agree with previously reported in other countries, where the prevalence of L. intracellularis varies between 15% and 93.7% (Thompson et al., 1998; Stege et al., 2000; Jensen et al., 2006; Biksi et al., 2007) and in Brazil. In Brazil works reported a prevalence of 15% of positive faecal samples (146/971), and 30% of 203 herds from seven different regions (Moreno et al., 2002; Baccaro et al., 2003). The differences among the prevalence numbers observed in this and in others works may be due to regional variations, different swine breeding systems, use of antibiotics and sampled population (sick or healthy animals) and detection assay used.

Using detection techniques, the prevalence of B. pilosicoli and B. hyodysenteriae in different geographic regions has been estimated. In a study in Danish B. pilosicoli was isolated from 19% from 79 herds and B. hyodysenteriae in 2.5% (Stege et al., 2000). In a survey conducted in U. K. between 1992 and 1996 that involved 85 pig farms B. pilosicoli was identified on 21 (25%) units, and B. hyodysenteriae on 6 (7%) units (Thomson et al., 1998). A recent study from Sweden revealed that the presence of B. pilosicoli in 34 herds of 105 studied and the absence of B. hyodysenteriae, the presence of B. pilosicoli was significantly associated with herds having poor performance in growing pigs and with mix infection (Thomson et al., 1998; Jacobson et al., 2005). In our survey the presence of B. pilosicoli was associated with mix infection and only in two herds. None positive samples for B. hyodysenteriae were detected. This lower prevalence may be explained by the use of additives in the feed, in many countries, including Brazil, the prevalence of Brachyspira sp. might be concealed by the use of antimicrobial feed additives (Hampson 2000). In a study conducted in Brazil 38 farms were randomly selected and bacteriologically examined (22 from farms using medicated feed and 16 with non-medicated feed), B. hyodysenteriae and B. pilosicoli were isolated respectively from 0% and 6.25% in medicated herds and from 31.8% and 45.5% from non-medicared farms (Barcellos et al., 2003)

In addition, variations related to PCR techniques could be observed. There is showed that variations like the use of pooled samples and the DNA extraction protocol may interfere negatively with the sensitivity of the test (Chiriboga et al., 1999) resulting in false negative results. The practical limit of detection of the multiplex PCR when the three species were applied together in the faecal samples was 10^3 cells per gram of feces. These levels of detection are consistent with than those previously reported (Jones et al., 1993; Møller et al., 1998; La et al., 2003). PCR inhibition is also a problem in the diagnosis of faecal samples prepared directly for PCR. Hence, inhibition might be a problem in the detection of clinical and subclinical carriers (Jacobson et al., 2004). It is known that bacterial isolation for Brachyspira sp. is more sensitive than the PCR technique (Komarek et al., 2009), and this fact may have contributed to the low numbers observed.

The overall prevalence for Salmonella enterica subspecies Typhimurium (6.52%) and others subspecies of Salmonella enterica (15.2%) was high when compared with results of Salmonella enterica in others countries like Danish (10.1%) and U.K (13%) (Thomson et al., 1998; Stege et al., 2000). This variation in prevalence can be caused by differences in pig housing (asymptomatic carriers) or feeding regimen (meat meal) which might affect the degree of transmission (Hampson 2000). Salmonella spp. were diagnosed as the primary agent in both ZM and MBH regions, it is believed that the highest density of pigs in such areas associated with older facilities have contributed to this finding. Salmonella enterica subspecies Typhimurium can cause diarrhea in pigs, but even more important is that Salmonella spp. is a zoonotic agent (Weneger and Bager, 1997; Stege et al., 2000). Salmonella spp. is a key player in the contamination of pig meat and its derivatives (Weiss et al., 1999).

Pathogenic E. coli was previously identified in 49 of 54 pigs from poor performance herds (Jacobson et al.,
A survey observed 76.8% of 72 herds infected by hemolytic E. coli and other work demonstrated 24.1% of the herds with pathogenic E. coli (MFøller et al., 1998; Stege et al., 2000). Despite all these results the significance of the presence of E. coli in grower/finish herds has not been well clarified. Enterotoxigenic E. coli has been associated with damage to small intestinal villi, loss of villi epithelium and diarrhea in weaned pigs (Macedo et al., 2007). However, this enteric pathogen may alter the ecological balance of the gut flora or change the environment conditions in the gut, which would favor some bacteria (Jacobson et al., 2003). Normally the affected herds had case history of previous problems with post-weaning diarrhea, this may indicate that these herds suffer from a high pathogen load, or that the post-weaning diarrhea might predispose to outbreaks of others enteric diseases (MFøller et al., 1998; Thompson et al., 1998; Stege et al., 2000; Jacobson et al., 2005). An uncharacteristic presentation of F18-positive E. coli enteritis in 11-weekold pigs, and according to this author, strains of E. coli positive for the F18 pilus, enterotoxins, and Stx2e endotoxin should be added to the list of differential diseases that cause severe diarrhea and vomiting in grower-finisher pigs (Pittman 2010). Of the 512 samples tested 39 no showed growth for Lac + colo-
ies, it is believed that the overuse of antibiotics in the growing and finishing phase may have inhibited the growth of these bacteria on MacConkey agar.

Interactions between pathogens may occur and it is often observed in herds that have or had history of severe diarrhea with dead loss of pigs, usually in this cases there is an increase of lesions in the intestine (Jacobson et al., 2005; Suh and Song, 2005). Mixed infection was present in 30.43% of herds, and the main combination observed was Salmonella Typhimurium associated with L. intracellularis. This association was already reported (Suh and Song, 2005) 2.2% of the 462 samples positive for Salmonella spp and L. intracellularis. Other associations have already been reported in Sweden 58% of 320 fecal samples were concomitant infected by B. pilosicoli and L. intracellularis (Jacobson et al., 2005).

T. suis once relatively common in swine industry (Batte et al., 1977), was not detected in any of the samples. The control in recent years, associated with technological improvements in the swine industry, have resulted in the reduced incidence of helminth parasites.

In conclusion, L. intracellularis is the main swine enteropathogen observed in herds in the state of Minas Gerais following by enterotoxigenic E. coli and Salmonella enterica subspecies Typhimurium. There was a high occurrence of mixed infections in herds with a close relationship of L. intracellularis combined with other agents. The occurrence of a large number of herds positive for E. coli, suggest that this bacteria may be the cause of diarrhea in growing and finishing pigs.

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