RESEARCH ARTICLE

Case–control study of pathogens involved in piglet diarrhea

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Abstract

Background: Diarrhea in piglets directly affects commercial swine production. The disease results from the interaction of pathogens with the host immune system and is also affected by management procedures. Several pathogenic agents such as *Campylobacter* spp., *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., group A rotavirus (RV-A), coronaviruses (transmissible gastroenteritis virus; porcine epidemic diarrhea virus), as well as nematode and protozoan parasites, can be associated with disease cases.

Results: All bacterial, viral, protozoan, and parasitic agents here investigated, with the exception of *Salmonella* spp. as well as both coronaviruses, were detected in varying proportions in piglet fecal samples, and positive animals were equally distributed between case and control groups. A statistically significant difference between case and control groups was found only for *Cystoisospora suis* (p = 0.034) and *Eimeria* spp. (p = 0.047). When co-infections were evaluated, a statistically significant difference was found only for *C. perfringens* β2 and *C. suis* (p = 0.014).

Conclusions: The presence of pathogens in piglets alone does not determine the occurrence of diarrhea episodes. Thus, the indiscriminate use of antibiotic and anthelminthic medication should be re-evaluated. This study also reinforces the importance of laboratory diagnosis and correct interpretation of results as well as the relevance of control and prophylactic measures.

Keywords: Case–control study, Diarrhea, Piglets

Background

Diarrhea in piglets represents one of the major health problems affecting swine production farms. In fact, enteric infections have become one of the main causes of morbidity and mortality in neonatal farm pigs, resulting in economic losses especially when suckling and weaned piglets are affected. The disease has a multifactorial etiology influenced by environmental, management and physiological factors that include interaction of pathogens, farm procedures, and host immunity [1].

Diarrhea in piglets can be caused by several pathogenic agents, including *Campylobacter* spp., *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., group A rotavirus (RV-A), coronaviruses (transmissible gastroenteritis virus—TGEV; porcine epidemic diarrhea virus—PEDV), as well as by nematode and protozoan parasites. However, most studies have focused on a few or only one agent and consequently our understanding of the relative importance of pathogens and other factors may have strong biases [2].

The present case–control study was carried out with piglets under field conditions in the state of São Paulo, Brazil, in order to evaluate the relative significance of pathogens in the development of intestinal disorders. It integrates microbiologic and epidemiologic data through the investigation of pathogenic agents and virulence factors in case and control animals.

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Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 2 of 7

Methods

Study design, case definition and sampling

This field-based case–control study was conducted in the state of São Paulo, in the southeastern region of Brazil, between September 2010 and July 2012. The sampling unit was a swine pen, which was defined as a group of piglets born from the same sow. Piglets with clinical signs of diarrhea represented cases, whereas piglets without clinical manifestations represented controls. The two groups were from the same farm and of similar age, but were not from the same pen.

To detect an odds ratio of 3.5 for control group exposures of 25 % or greater, with a confidence level of 95 % and a power of 80 %, the required sample size was 42 cases.

Individual fecal samples from 184 piglets (1 day to 4 weeks old) were collected from 88 pens at 16 farrow-to-finish pig farms. Among these pens, 43 represented case groups and 45 were controls.

This research was approved by an animal ethics committee subordinated to the National Council for Animal Experimentation of Brazilian Ministry of Science, Technology and Innovation (CETEA-IB 93/10).

Laboratory methods

As summarized in Table 1, bacterial isolation, characterization of virulence and pathogenicity factors, RNA detection of viruses by RT-PCR, and coproparasitologic exams for the detection of nematode eggs as well as of protozoan cysts and oocysts were performed on the samples. Discrimination between *Cystoisospora* spp. and *Eimeria*

spp. was achieved by the modified sugar flotation technique (Sheather's sugar solution) performed after the feces with 2.5 % potassium dichromate were incubated for 5–12 days at 37 °C in a biological oxygen demand (BOD) incubator [3].

Statistical analysis

The distribution of positive samples between cases and controls was statistically evaluated through Pearson's Chi square or Fisher's exact test, using the Minitab® 16.1.0 software.

Results

Bacterial, viral, protozoan, and parasitic agents that cause diarrhea, except for *Salmonella* spp. and both coronaviruses, were detected in varying proportions in the 184 examined animals (Table 2).

When individuals were clustered into 88 groups (case, n = 43; control, n = 45), statistically significant differences were found only for protozoans (Table 3). Animals positive for bacterial and viral agents were equally distributed between case and control groups (p > 0.05) as shown in Table 3.

Co-infections were analyzed, and a statistically significant difference between groups was found only for *C. perfringens* β2 and *C. suis* co-infection (p = 0.014).

Table 1 Diagnostic tests performed on piglet fecal samples

Agent	Test	References
Bacteria		
Campylobacter spp.	Isolation	[4]
C. coli	Biochemical methods	[4]
C. perfringens	Isolation	[5]
C. perfringens	PCR α, β, ε e ι and cpb2 genes	[6, 7]
E. coli	Isolation	[8]
E. coli	PCR StaP, Stb e LT genes	[9]
Salmonella spp.	Isolation	[8]
Parasite		
Nematodes	Flotation test	[10]
Protozoa	Flotation test	[3, 11]
Virus		
Coronaviruses (PEDv and TGEv)	RT-PCR	[12]
Group A rotavirus	RT-nested-PCR	[13]

Table 2 Distribution of bacterial, viral, and parasitic agents of diarrhea in individual fecal samples from case and control piglets

Agent	Case	Control	Positive samples (%)	Positive farms
Bacteria				
C. coli	43	28	71 (38.59)	13
C. perfringens type A	26	16	42 (22.83)	10
C. perfringens β2	24	15	39 (21.19)	10
E. coli Sta toxin	5	1	6 (3.26)	4
E. coli Stb toxin	9	7	16 (8.69)	7
E. coli LT toxin	0	0	0 (0)	0
Salmonella spp.	0	0	0 (0)	0
Parasites				
C. suis	48	16	64 (34.78)	14
Eimeria spp.	13	2	15 (8.15)	6
Gastrointestinal Strongyles	4	3	7 (3.80)	2
Virus				
Coronavirus	0	0	0 (0)	0
Group A rotavirus	51	21	72 (39.13)	12
Total samples	123	61	184	16

Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 3 of 7

Table 3 Distribution of diarrheal agents between case and control groups

Agent	Case group (n=43)	Control group (n=45)	Pearson Chi-square	p value	Fisher's exact test (p)
Bacteria					
C. coli	23	23	0.050	0.823	–
C. perfringens type A	19	15	1.092	0.296	–
C. perfringens β2	17	14	0.684	0.408	–
E. coli Sta toxin	4	1	2.057	–	0.197
E. coli Stb toxin	6	7	0.045	0.832	–
E. coli LT toxin	0	0	–	–	–
Salmonella spp.	0	0	–	–	–
Parasite					
C. suis	23[a]	14[a]	4.519	*0.034[a]*	–
Eimeria spp.	8[a]	2[a]	4.377	–	*0.047[a]*
Gastrointestinal Strongyles	2	1	0.394	–	0.612
Virus					
Coronaviruses	0	0	–	–	–
Group A rotavirus	16	15	0.145	0.704	–

[a] Statistical significant difference between case and control groups. Italic values indicate *p* < 0.05

Discussion

Except for *Salmonella* spp. and both coronaviruses, all other agents commonly associated with diarrhea in pigs were detected in varying proportions in the 184 animals examined in the present study. One to six different agents were found at each farm, and one to four pathogens were detected in stool samples of infected animals.

Most studies of diarrhea in pigs have focused on a single agent, which can result in a biased view of the relevance to the disease of a particular pathogen. Calderaro et al. [14], however, studied 21 swine herds in the state of São Paulo, Brazil, from 1996 to 1997 and determined the frequency of bacterial, viral, and protozoan agents in the feces of piglets with clinical signs of diarrhea. Among the 174 samples tested in their study, 40.2 % were positive for *E. coli*, 31.6 % for *C. suis*, 10.9 % for rotavirus, and 1.2 % for *Cryptosporidium parvum*, with some samples having more than one pathogen present. Interestingly, 32.8 % of the samples tested negative for any agent. More recently, a matched case–control study evaluated the frequency of rotavirus, haemolytic *E. coli*, *C. difficile*, *C. perfringens* types A and C, *Eimeria* spp., *Cystoisospora* spp., and *Cryptosporidium* spp. associated with neonatal mild diarrhea in piglets. The study was carried out in litters of 1- to 7-day-old piglets from 28 pig farms in the state of Rio Grande do Sul, Brazil. Despite a wide range of frequencies of the different agents in case and control groups, no agent was significantly associated with diarrhea in case litters when compared to controls. Thus, the authors stressed the need for caution when interpreting laboratory diagnosis of mild diarrhea, as the detection of a single agent does not necessarily indicate that it causes the problem [15].

Fecal samples from suckling (n = 205) and weaned piglets (n = 82) with diarrhea from 24 farms in Southern Germany were examined. *C. suis* was diagnosed in 26.9 % and *C. parvum* in 1.4 % of the piglets investigated. It was found that 17.6 % of the animals were infected with enterotoxigenic *E. coli* and 4 % were positive for rotavirus. The occurrence of the pathogens was significantly associated with the age of the animals examined [16].

Rotaviruses represent one of the most frequently detected viral agents associated with diarrhea in swine worldwide, especially in 1- to 4-week-old pigs [17, 18]. In 75 % of the visited farms, almost 40 % of stool samples tested were positive for RV-A, indicating the high frequency of this viral infection among piglets in Brazil. Nevertheless, this viral agent was equally distributed between case and control groups. According to Svensmark et al. [19], rotaviruses are more frequently detected in semiliquid and loose stools than in normal or watery stools. However, when rotavirus infection was studied in 1090 litters from 26 intensively managed Danish sow herds, an association between virus detection and diarrhea could not be demonstrated [19]. On the other hand, a significant difference has been reported regarding the frequency of RV-A in diarrheic and non-diarrheic fecal samples [20]. These previous results, together with ours, indicate that in spite of the wide distribution of rotaviruses, additional factors may be involved in the development of clinical cases.

Negative RT-PCR results obtained in this study for coronaviruses confirm previous reports of the absence of serological evidence of these infections in Brazilian pig herds [14, 21, 22].

Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 4 of 7

Although campylobacteriosis is one of the most common causes of diarrhea in humans, the role of *Campylobacter* spp. in swine gastrointestinal disorders is still controversial. In 2005, a study suggested that pigs represent an important *C. coli* reservoir in Germany. However, the clinical relevance of this finding was not evaluated, because this broad study aimed at monitoring foodborne pathogens [23]. An experimental infection conducted to evaluate the colonization and translocation ability of a porcine strain of *C. coli* showed that all ten infected animals remained in very good health, although overall fecal consistency, rated on a five-point scale, decreased from 4.0 to 3.5 over 4 days [24]. In another study, no statistically significant difference was found in the number of pigs with *Campylobacter* spp. between diarrheic and healthy animals. However, CFU counts were significantly different in the two groups, suggesting that *Campylobacter* spp. may play a role as a cofactor in pig diarrhea [25]. Despite the fact that *C. coli* was the most frequent bacteria found in the present study, with almost 40 % of samples positive and 81.25 % of farms positive, no difference in frequency was found between case and control groups, which is in agreement with previous reports. Nevertheless, in one industrialized well-managed indoor farm, we found that all animals that were positive only for *C. coli* had severe diarrhea, while control animals were negative for all pathogens tested. Altogether, these results suggest that *C. coli* may play a role in pathogenesis, although it is important to consider other agents or factors not tested in this work.

Clostridium perfringens type A was found in almost 23 % of diarrheic and non-diarrheic samples from 62.5 % of the farms, yet again there was no statistical difference between case and control groups, even when the subgroup of *C. perfringens* carrying the cpb2 gene was investigated (21.2 % positive samples). Chan et al. [26] identified *C. perfringens* as the causal agent of gastrointestinal tract illness in 28 of 237 studied cases, and genotyping of 17 strains showed that they belonged to toxinotype A and had the cpb2 gene. In another study, intestinal positivity for *C. perfringens* was detected in 73 % of diarrheic and 78 % of healthy piglets. Those bacteria were mostly present in the intestinal lumen. In 20 % of diarrheic and 30 % of healthy animals, bacteria were found within the mucus layer and in direct contact with the intestinal epithelium. However, presence and location of *C. perfringens* in the intestinal tissue did not significantly correlate with histological lesions [27]. Other authors necropsied and took intestinal samples from 46 piglets from 10 farms with a consistent history of type-A *C. perfringens* neonatal diarrhea. Samples were compared to those from an unaffected cohort of piglets. Based on the number of intestinal bacteria, presence of consensus

cpb2 in *C. perfringens* isolates, expression of cpb2 in piglet intestines, and known or unknown causes of diarrhea, these investigators were unable to distinguish between healthy and diarrheic piglets [28]. The role of cpb2-harboring *C. perfringens* in the development of diarrhea was also investigated through the assessment of cytotoxicity to porcine IPI-21 and human Caco-2 cell-lines. Supernatants of cpb2-harboring *C. perfringens* were cytotoxic to both cells to variable extents. However, toxin removal by anti-beta 2 toxin antibodies or degradation by trypsin did not reduce the cytotoxic effect of supernatants [29]. These results indicate the need for further studies focused on elucidating the role of cpb2-positive *C. perfringens* type A in neonatal diarrhea.

Neonatal intestinal infection with *E. coli* causes severe diarrhea and frequently kills piglets [30]. Different strains are described as responsible for clinical conditions, especially strains that produce enterotoxins such as the heat-labile enterotoxin (LT) and the heat-stable enterotoxin (ST) [31, 32]. In the present study, less than 12 % of examined samples were positive for ST (STa or STb), and no statistically significant difference between case and control groups was found. In Canada, from 2001 to 2010, 31 % of 237 samples submitted for gastrointestinal disease laboratory diagnoses had enterotoxigenic *E. coli* (ETEC) infection, and ETEC was less likely to be recovered when *C. difficile*, *C. perfringens* or rotavirus were detected (p < 0.05) [26]. In four commercial Danish swine herds, intestinal positivity for *E. coli* was found in 88 % and 80 % of the small intestines of diarrheic and non-diarrheic piglets, respectively. Nevertheless, diarrheic piglets had large numbers of *E. coli* more frequently than non-diarrheic piglets [27]. Our results showed that 25 and 43.75 % of the farms were positive for *E. coli* STa and STb toxins, respectively, which represents a risk of outbreaks and of selection of resistant pathogenic strains.

Salmonella spp. was not found in the examined samples. These findings were expected, because this agent is not usually found in such young piglets [33, 34].

The equal distribution of bacterial agents between groups may have resulted from the extensive use of antibiotics in Brazilian swine production. This finding reinforces the need for a reassessment of the use of antibiotics in food-producing livestock.

Based on parasitological analysis, only two farms and 3.8 % of samples were positive for nematode eggs, and no statistically significant difference between case and control groups was found. These results suggest that systematic use of anthelminthic drugs associated with indoor housing systems and hygiene procedures can control infection by breaking the chain of transmission. However, animal welfare concerns are leading to changes in management practices. During recent decades, the number of

Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 5 of 7

organic and "green" swine herds has increased, and this may be an indication that former risk factors could arise again [35].

The detection frequency of *Eimeria* spp. was 8.15 % among tested samples (37.5 % of farms), and there was a statistically significant difference between case (18.60 %) and control (4.44 %) groups (p = 0.047). Some authors consider *Eimeria* spp. infection in piglets an uncommon cause of clinical signs [36–39]. However, more recently, *Eimeria* spp. was identified in 13 % of fecal samples from suckling piglets with diarrhea [40].

C. suis was the most commonly detected coccidian agent, present in 34.78 % of samples and widespread in the studied farms (87.5 %). A significant difference was again observed between case (53.49 %) and control (31.11 %) groups (p = 0.034). The ability of *C. suis* to cause diarrhea in piglets is well documented [41, 42], as is its frequency of infection in young piglets: 17.3 % in the Republic of Korea [43], 53.8 % in Germany [44], 31.6 % in Brazil [14], 6.3 % in Canada [26], and 8.9 % in Cuba [45]. Our results differ from those of another study that was recently published in Brazil in which no statistical difference between case and control groups was found [15]. Methodological aspects of the two studies could explain the differing results. We collected samples from 1-day- to 4-week-old animals, while piglets between 1 and 7 days of age were sampled in the previous Brazilian study. Age of piglets seems to be crucial for the outcome [46]. *C. suis* infections in piglets undoubtedly have a high impact. However, encouraging the use of drugs to control this agent could lead to abuses similar to those seen with antibiotic use. Coccidian oocysts are generally regarded as relatively resistant to environmental factors and apt to survive for considerable periods. However, high temperature (25–30 °C) in combination with low relative humidity (53–62 %) rapidly reduces the viability of *C. suis* oocysts. This finding might point to a possible control mechanism requiring only some environmental control and proper management of farrowing pens, like by allowing a few extra days in-between litters or by increasing desiccation somehow, might be able to reduce the number of infective *C. suis* oocysts that has escaped pen cleaning [47].

According to Mengel et al. [48], newborn piglets exposed to natural *C. perfringens* type A infection and to low-level experimental infection with *C. suis* showed an increase in clinical disease, mortality, and metabolically active *C. perfringens* type A. In the present study, analyses of 28 possibilities of co-infection by two agents and 55 possibilities of co-infection by three agents identified a potential for worsening conditions only in the combination of *C. suis* and *C. perfringens* type A (cpb2 gene) (p = 0.014), corroborating the hypothesis that simultaneous infection with these agents soon after birth may lead to an increase in the severity of clinical disease in piglets [48].

Recently, a non-hemorrhagic diarrhea during the first week of life, with no detection of known infectious agents and characterized by a milk-filled stomach and flaccid intestines at necropsy was described. The syndrome is not related to starvation or infection by enterotoxigenic *E. coli*, *C. perfringens* type A or C, *C. difficile*, rotavirus, coronavirus, *Cryptosporidium* spp., *Giardia* spp., *C. suis* or *Strongyloides ransomi*. The existence of neonatal diarrhea with unspecific lesions and without known pathogens is not a new phenomenon [49], but this study also reinforces the importance of laboratory diagnosis and correct interpretation of results as well as the relevance of control and prophylactic measures.

Conclusions

The presence of known pathogens in piglets alone does not seem to determine the occurrence of diarrhea. The indiscriminate use of antibiotic and anthelminthic medication should be reassessed. The importance of laboratory diagnosis and correct interpretation of data as well as the relevance of control and prophylactic measures should be reinforced.

The aim of this case–control study was to assess the association between a variety of pathogens and the occurrence of diarrhea episodes in 1-day- to 4-week-old piglets. Statistically significant differences in pathogen frequency between animals in case and control groups were found for the protozoan agents *C. suis* and *Eimeria* spp., and *C. suis* and *C. perfringens* type A co-infection. This finding may indicate that coccidian agents should be independently considered in disease control and monitoring programs.

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Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 6 of 7

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Ruiz *et al. BMC Res Notes* (2016) 9:22

Page 7 of 7

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