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REGULAR ARTICLES

Several enteropathogens are circulating in suckling and newly weaned piglets suffering from diarrhea in the province of Villa Clara, Cuba

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Abstract Intestinal contents of suckling (n=45) and newly weaned (n=45) piglets, suffering from diarrhea in the province of Villa Clara in Cuba, were tested for viral, bacterial, and parasitic enteropathogens from May to June 2008. At least one enteropathogen was identified in 53.3 % of piglets and enterotoxigenic *Escherichia coli* (ETEC; 25.6 %) was the major pathogen; mostly STa^{+}/STb^{+} or $F4^{+}/STa^{+}/STb^{+}$ ETEC were isolated. The overall occurrence of the rest of pathogens was 10 % for transmissible gastroenteritis virus (TGEV) and *Cryptosporidium parvum*, 6.7 % for rotavirus A and *Isospora suis*, 5.6 % for α-toxigenic *Clostridium perfringens*, 3.3 % for verotoxigenic *E. coli* (VTEC), and 2.2 % for *Salmonella enterica* subspecies *enterica* serovar Newport. TGEV and α-toxigenic *C. perfringens* were only identified in suckling piglets, while *Salmonella* Newport and VTEC were only detected in weaned pigs. Porcine epidemic diarrhea virus (PEDV), β-toxigenic *C. perfringens*, *Eimeria* spp., and helminths were not identified. Eight kinds of mixed infections were detected in 25 % of enteropathogen positive piglets. ETEC was present in 10 of 12 mixed infections, and TGEV infections were never combined. This survey demonstrates that several enteropathogens are circulating in piggeries located in the province of Villa Clara in Cuba, and that is necessary to improve surveillance, prevention, and control of enteric infections in order to increase production efficiency.

Keywords Cuba · Diarrhea · Enteropathogens · Newly weaned piglet · Suckling piglet

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Introduction

In 2008, the Cuban Institute for Swine Research reported that gastroenteric diseases caused 31 % and 37 % of the total piglet's mortality during the pre- and post-weaning periods, respectively (Cabrera and García 2009). However, there is scarce information on the epidemiology of enteropathogens because in most of the diarrhea outbreaks diagnosis is restricted to clinical and macro-pathological examinations (Cabrera and García 2009), which are tentative rather than specific diagnosis tools (Elicker et al. 2010). Also, limited research on porcine enteropathogens has been performed during the last 20 years in Cuban piggeries (Fuentes et al. 2001; Barrera et al. 2005; Blanco et al. 2006).

Worldwide, despite the achieved improvement on pig health and management, diverse enteropathogens still circulating in swine production systems as potential agents of pre- and post-weaning diarrhea, which negatively affect performance of piglets and production efficiency (Straw et al. 2006). That's the case of recently reported ETEC in Zimbabwe and China (Madoroba et al. 2009; Wang et al. 2011), rotaviruses in

Argentina (Parra et al. 2008), *Cryptosporidium* in Turkey (Uysal et al. 2009), *Isospora* in Greece (Skampardonis et al. 2010) as well as *Clostridium perfringens* type A, *Clostridium difficile*, ETEC, *Isospora*, and rotaviruses in Brazil (Cruz et al. 2010). Straw et al. (2006) stated that the differential diagnosis of piglet's diarrhea as a multi-factorial disease has to be routinely performed in order to plan and execute an efficient disease control.

The epidemiology of any syndrome is a wide matter to deal with, specifically when previous information is almost lacking as happens with infectious diarrhea of young pigs in Cuba. Therefore, this study undertook the differential identification of enteropathogens in piglets suffering from pre- or post-weaning diarrhea in piggeries located in the province of Villa Clara.

Materials and methods

Survey design

In the period of May to June 2008, the veterinary authorities of the company of pork production in the province of Villa Clara, where most pork is produced in Cuba (González et al. 2007), agreed to sample contents from the large intestines of 90 piglets suffering from diarrhea in 6 of the 8 largest piggeries free of hog cholera outbreaks; otherwise, sampling is forbidden because of biosafety rules. The herd of the 6 piggeries together comprised 5,252 sows and 7,455 suckling piglets; the number of newly weaned piglets was not specifically controlled on the herd book. All piggeries followed a similar continuous flow management (farrow-to-finish) without straw bedding; they had similar facilities, used the same antibiotics, and shared breeding stock animals routinely. Pregnant sows were housed individually, and at the end of gestation, they were moved up to elevated farrowing crates with completely perforated plastic flooring. After weaning, piglets were housed in groups of 10 to 20 in elevated pens with perforated floors.

On the appointed day to sample in every piggery, the responsible veterinarian was asked to select in early morning inspection 6–9 litters (2 to 25-day-old) and 6–9 newly weaned pens (26 to 48-day-old) showing signs of watery diarrhea in order to complete 15 sampling places spread over the farrowing and weaning houses. Then, one piglet per litter or per pen was randomly caught and selected if it had signs of diarrhea in the perineum. Piglets and sows were not under antibiotic therapy or vaccinated against enteropathogens.

Before euthanasia, blood was collected and serum was obtained as previously described by de la Fé Rodríguez et al. (2011). For the identification of pathogens in which commercial diagnostic kits were used, intestinal contents and sera were aliquoted and stored at −20 °C until the kit application. For the rest of identification procedures intestinal contents started to be processed quickly.

Identification of bacteria

For identification of *Escherichia coli*, intestinal contents were streaked out on McConkey agar plates, and aerobically incubated for 18 h at 37 °C; thereafter three to five lactose-fermenting colonies were separately re-plated on the same medium and biochemically confirmed as *E. coli*. Intestinal contents were also plated onto 5 % sheep blood agar and a maximum of three hemolytic colonies were further processed as described above. One hemolytic isolate per piglet was preferred for being tested for fimbriae (F4, F5, F6, F18, and F41) and toxins (STa, STb, LT, and STx2e) encoding genes as described by Bruggeman et al. (2008).

For identification of *Salmonella* spp., intestinal contents were inoculated on brilliant green agar (BGA) and in 5 ml of Rappaport-Vassiliadis broth for 18 h at 37 °C in aerobic atmosphere followed by re-inoculation on BGA. Non-lactose-fermenting colonies were further biochemically identified, thereafter a slide agglutination was carried out using polyvalent (A+B+C1+C2+D+E1+E2+E4+F groups) and monovalent (A, B, C1, and C2 groups) *Salmonella* antisera (Finlay Institute, Cuba). Positive isolates were tested by the multiplex *Salmonella* DNA typing Premi®Test (CODA-CERVA, Brussels, Belgium; Wattiau et al. 2008).

The semi-quantitative detection of *C. perfringens* as well as the identification of α- and β-toxins were performed on intestinal contents by DAS-ELISA (BIO K 095, Bio-X Diagnostics Inc.). A sample was considered positive if it contained the bacterium and one toxin at least.

Identification of viruses

TGEV and PEDV were detected in intestinal contents by a qualitative chromatographic immunoassay kit (Anigen Rapid TGE/PED Ag test, Animal Genetics Inc.). Complementary, sera of suckling piglets were tested for antibodies against TGEV and porcine respiratory coronavirus (PRCV) by a differentiating blocking ELISA kit (SVANOVIR® TGEV/PRCV-Ab, Svanova Biotech). Rotavirus A was tested in intestinal contents by a DAS-ELISA kit (1.1.RT.K2, Ingenasa).

Identification of parasites

Eimeriidae and helminths were identified by light microscopy using direct smear and Sheather's sugar flotation. Oocyst sporulation was induced in *Eimeriidae* positive samples to allow the differential identification of *Eimeria* spp. and *Isospora suis* (Karamon et al. 2007). *Cryptosporidium parvum* antigen detection was performed by a DAS-ELISA kit (BIO K 070, Bio-X Diagnostics Inc.).

Statistical analysis

The proportions of piglets infected by every enteropathogen in every age group (suckling piglets, newly weaned piglets, 2 to 13, 14 to 25, 26 to 36, and 37 to 48-day-old piglets) as well as in the overall sample were compared by the chi-square test in StatGraphics Plus Version 5.0. Differences were considered significant at $p<0.05$ and comparisons between groups or piggeries were not performed.

Results

At least one enteropathogen was identified in 64.4 % of the 45 suckling piglets and in 42.2 % of the 45 newly weaned piglets (Table 1). The color of scours varied from milky to dark yellowish, and bloody diarrhea was not present. PEDV, β-toxigenic *C. perfringens*, *Eimeria* spp., and helminths were not detected.

ETEC was the most common enteropathogen except in the youngest age group (Table 1), and the combination of genes encoding STa and STb was often detected (Table 2). The only isolated *Salmonella* spp. was *Salmonella enterica* subspecies *enterica* serovar Newport (serogroup C2) in 2 weaned pigs (Table 1). Also, 66.7 % of suckling piglets was infected by *C. perfringens*, but only 16.7 % of them was positive for α-toxin; the α-toxin-positive samples contained a higher bacterium concentration (showed by a high ELISA signal: average $OD_{450\ nm}$=1.679) than the toxin-negative ones (average $OD_{450\ nm}$=0.990). Different from suckling piglets, 26.7 % of weaned piglets was infected by *C. perfringens* and toxins were not identified in this group.

TGEV was identified in 29 % of 2–13-day-old piglets. Most of suckling piglets (77.7 %) were sero-positive for TGEV, which was excreted by 40 % of sero-negative piglets as well as by 14 % of sero-positive ones. One piglet was sero-positive for PRCV and sero-negative for TGEV, and three were sero-negative for both coronaviruses.

It seemed as if the 14–25-day-old piglets were more susceptible to enteropathogens than the others; this age group showed the highest occurrence of rotavirus A, ETEC, α-toxigenic *C. perfringens*, *I. suis*, mixed infections, as well as the second highest of *C. parvum*. However, it should be noted that the number of tested and therefore also the number of positive animals in this age group were low. Furthermore, the percentage of piglets showing enteric infections was as high in the 2–13-day-old group.

Mixed infections were detected in 25 % of enteropathogen positive pigs and they were slightly more common in suckling piglets (7/12) than in weaned piglets (5/12; Table 3). Most of infections by *I. suis* (5/6), rotavirus A (4/6) and *C. parvum* (5/9) liked to be combined with other pathogens. Alpha-toxigenic *C. perfringens* infections were

Table 1 Percentage of diarrheic piglets infected by enteropathogens in six piggeries located in the province of Villa Clara in Cuba, from May to June 2008

Age groups	No. of pigs	Enteropathogens																*p* value[b]	Enteric mixed infections		Total[a]	
		Rotavirus A		TGEV		ETEC		VTEC		*Salmonella* Newport		α-Toxigenic *C. perfringens*		*I. suis*		*C. parvum*						
	N	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%		*n*	%	*n*	%
2–13-d.	31	2	6.5	9	29[b]	7	22.6	0	0	0	0	3	9.7	2	6.5	1	3.2	0.0002	4	12.9	20	64.5
14–25-d.	14	2	14.3	0	0	6	42.9[b]	0	0	0	0	2	14.3	2	14.3	2	14.3	0.0121	3	21.4	9	64.2
26–36-d.	24	1	4.2	0	0	5	20.8	3	12.5	2	8.3	0	0	1	4.2	2	8.3	0.1014	3	12.5	10	41.7
37–48-d.	21	1	4.8	0	0	5	23.8[b]	0	0	0	0	0	0	1	4.8	4	19	0.0029	2	9.5	9	42.9
Suckling	45	4	8.9	9	20	13	28.9[b]	0	0	0	0	5	11.1	4	8.9	3	6.7	0.0000	7	15.6	29	64.4
Weaned	45	2	4.4	0	0	10	22.2[b]	3	6.7	2	4.4	0	0	2	4.4	6	13.3	0.0003	5	11.1	19	42.2
Total	90	6	6.7	9	10	23	25.6[b]	3	3.3	2	2.2	5	5.6	6	6.7	9	10	0.0000	12	13.3	48	53.3

d. day-old

[a] Total of enteropathogen positive piglets per age group, pigs carrying mixed infections are counted as one

[b] Percentage value significantly different ($p<0.05$) from the rest in every group of piglets

Table 2 Fimbrial and toxin encoding genes identified among 26 pathogenic *E. coli* isolated from intestinal contents of 45 suckling (s) and 45 newly weaned (w) piglets suffering from diarrhea in six piggeries located in the province of Villa Clara in Cuba, from May to June 2008

Toxins	STa		STb		STa/STb		STb/LT		STx2e		None		Total (%)
Fimbriae	s	w	s	w	s	w	s	w	s	w	s	w	
F4	–	–	–	–	3	2	–	–	–	–	–	1	6 (23)
F6	–	1	–	–	–	–	–	–	–	–	–	–	1 (4)
F18	–	–	–	–	–	–	–	2	–	3	–	–	5 (19)
F5/F41	1	–	–	–	–	–	–	–	–	–	–	–	1 (4)
None	1	–	1	2	6	2	1	–	–	–	–	–	13 (50)
Total (%)	3 (12)		3 (12)		13 (50)		3 (12)		3 (12)		1 (4)		26 (100)

only mixed with *C. parvum* or *I. suis*. TGEV infections were not mixed, while ETEC was present in 10 of 12 combined infections.

Discussion

Nowadays, policies of the Cuban Ministry of Agriculture are to improve and increase swine production as pork is the most consumed meat in the country. In 2005, 1,980,000 pigs were slaughtered and 5 years later already 3,266,600 (O.N.E. 2011). Recently, the vice-director of the Cuban National Institute of Veterinary Medicine recommended an improvement on swine management and on the diagnosis of enteric diseases in order to decrease mortality due to diarrhea in young pigs (Ricardo 2008).

In this survey, 25.6 % of piglets was infected by ETEC which was the most common enteropathogen. In Cuban piggeries, vaccination against ETEC is not applied and enteropathogenic *E. coli* is resistant to most of the routinely administered antibiotics (de la Fé Rodríguez et al. 2012). For Cuba, this study is the first that demonstrates ST encoding genes in $F4^+$, $F5^+$, and $F41^+$ ETEC as previously these fimbriae were not identified (Blanco et al. 2006); also, is the first to report $F18^+/LT^+/STb^+$ ETEC corresponding with findings in USA (Zhang et al. 2007). The LT encoding gene was not commonly detected; nevertheless, most of ETEC carried ST encoding genes, which are sufficient for causing diarrhea in young pigs (Erume et al. 2008). All $F4^+$ ETEC were negative for LT; in contrast, a strong association between F4 and LT was reported by Zhang et al. (2007).

Table 3 Enteric mixed infections detected in intestinal contents of 45 suckling and 45 newly weaned piglets suffering from diarrhea in six piggeries located in Villa Clara, province of Cuba, from May to June 2008

Piglets	Age (days)	Enteric mixed infections
Suckling	3	*C. parvum* + α-toxigenic *C. perfringens*
	5	Rotavirus A + *E. coli* STb^+
	11	*I. suis* + α-toxigenic *C. perfringens*
	13	Rotavirus A + *E. coli* STa^+/STb^+
	14	*C. parvum* + *I. suis* + *E. coli* $F4^+/STa^+/STb^+$
	15	*I. suis* + Rotavirus A + *E. coli* $F4^+/STa^+/STb^+$
	16	*C. parvum* + *E. coli* Sta^+/STb^+
Weaned	34	Rotavirus A + *E. coli* $F4^+/STa^+/STb^+$
	34	*I. suis* + *E. coli* $F4^+/STa^+/STb^+$
	36	*C. parvum* + *Salmonella* Newport + *E. coli* STb^+
	41	*I. suis* + *E. coli* STa^+/STb^+
	46	*C. parvum* + *E. coli* $F4^+$

Eleven of 26 pathogenic *E. coli* carried genes encoding either F4 or F18. A recent survey that also included the six piggeries sampled in this study reported a high seroprevalence of specific antibodies against F4 and F18 in Cuban swine (de la Fé Rodríguez et al. 2011). It supports that $F4^+$ and $F18^+$ ETEC/VTEC isolated and characterized in this study are highly prevalent in Cuban piggeries and that vaccination against pathogenic *E. coli* might be advantageous. Results of this survey and of de la Fé Rodríguez et al. (2011, 2012) suggest that F4 and F18 fimbriae as well as enterotoxins are good candidates to be considered for this preventive strategy.

Salmonella spp. was not as commonly isolated as ETEC, and there is no information on the presence of the serovar Newport isolated in this survey in Cuban piggeries. Additionally, there are not reports demonstrating its role in the pathogenesis of piglet's diarrhea. Fever, diarrhea, dehydration, and enteritis have been observed in calves, cows, and horses from which *Salmonella* Newport was isolated (Poppe et al. 2006).

C. perfringens toxins are not currently surveyed in the Cuban Veterinary Diagnostic Laboratories. The higher concentration of *C. perfringens* in α-toxin-positive intestinal contents than in the negative ones, suggest the presence of active *C. perfringens* type A infections as reported by Das et al. (2009) in India. Mixed infections of α-toxigenic *C. perfringens* with *C. parvum* or *I. suis* were seen in two piglets, Songer and Uzal (2005) stated that preceding lesions provoked by other enteropathogens can enhance enteric colonization by *Clostridium*. Beta-toxigenic *C. perfringens*

was not identified, supporting the absence of reports on necrotic enteritis in the sampled piggeries which might be due to a β-toxin effect.

TGEV occurred as a single infection, and the associated diarrhea was not profuse and epidemic. From 2003 on, epidemic TGE have been reported in Cuba (Barrera et al. 2005), but the milder behavior found in the present study, and the high amount of TGEV-sero-positive piglets, indicates a possible change to the endemic form. Additionally, antibodies against PRCV were for the first time reported for Cuba. So the presence of these antibodies could explain milder TGE since an infection with PRCV induces antibodies able to neutralize TGEV (Usami et al. 2008).

Four out of six rotavirus A infections were mixed with ETEC. This association is often seen (Kim et al. 2010) and cause more severe enteritis than a rotavirus infection alone in young piglets (Neog et al. 2011).

In suckling piglets, *I. suis* occurred with lower percentage (8.9 %) than previously reported (44.7 %) in the province of Havana by Koudela et al. (1989); the housing improvement carried out in Cuban piggeries during last years could contribute to this difference. The 66.6 % of *I. suis* positive pigs was also infected with ETEC, Choi et al. (2003) stated that isosporosis could promote intestinal colonization by ETEC due to an increase of glycoconjugates on the jejunal enterocytes.

The 10 % occurrence of *C. parvum* found in this study is higher than the 2.1 % reported in the province of Havana, Cuba, by Cabrera and García (1985). However, this higher prevalence could be due to the high sensitivity of DAS-ELISA compared with flotation and staining methods. Probably, *C. parvum* acts often in concert with other enteropathogens to induce or exacerbate diarrhea (Enemark et al. 2003); in this study, 55.5 % of *C. parvum* infections was found to be combined with other enteropathogens.

Eight kinds of mixed infections were detected in 25 % of enteropathogen positive pigs. Ushida et al. (2009) closely associated mixed infections with piglet's diarrhea. However, some studies assessing the implication of certain enteropathogens on swine diarrhea did not perform a differential identification (Aliaga-Leyton et al. 2011).

In 46.7 % of pigs, enteropathogens were not detected and this could be due to (a) tests sensitivity; (b) non-infectious factors (Straw et al. 2006); (c) not-tested enteropathogens like other groups of rotaviruses (Kim et al. 2010) or *C. difficile* (Cruz et al. 2010); (d) not-tested virulence factors (i.e., EAST-I, AIDA-I, PAA; Lee et al. 2008); (e) insufficient recovery of the intestinal mucosa, such as the villous structure, after pathogen elimination; and (f) during the prepatent period, parasites are not yet detectable by the general parasitological methods employed in this survey (Karamon et al. 2007).

In conclusion, this study demonstrates that several enteropathogens are circulating in piglets suffering from diarrhea in the province of Villa Clara in Cuba. A better efficiency of pork production in Cuba might be achieved by the improvement of surveillance, prevention, and control programs of infectious diarrhea in young pigs, particularly colibacillosis.

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Ethical standards The handling of piglets during sampling complied with recommendations of the Cuban Veterinary authorities in all sampled piggeries.

Conflict of interest The authors declare that they have no conflicts of interests.

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