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Neonatal piglets mesocolon edema and colitis due to *Clostridium difficile* infection: prevalence, clinical disease and pathological studies

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Abstract

Clostridium difficile is a Gram-positive opportunistic anaerobic bacterium found in the soil, water as well as the digestive tract of several species of mammals. Currently in high-health herds and not related with antibiotic treatment, *C. difficile* has been emerged in association with neonatal catarrhal, fibrinous or purulent colitis in 2 to 7 day-old piglets. Mesocolon edema and colitis with a "volcano" lesion are pathological key marks of *C. difficile* infection. In farm 1, the prevalence of neonatal piglet mesocolon edema and colitis was evaluated in an extensive study of postmortem preweaning mortality. Lectinhistochemistry pattern of normal colon and colon with edema and colitis were analyzed in order to provide more accurate information related with pathogenesis of *C.difficile* infection. In farm 2, a clinical description of an outbreak of neonatal colitis in piglets due to *C. difficile* infection was reported. A total 820 piglets were post-mortem examined, from them, 8 cases were classified as suspected of *C. difficile* infection (0.1%). Age of affected piglets varied between 3 to 14 days. In all of them, the key mark was the severe mesocolon edema and 2 cases were characterized by focal necrosis and loss of epithelial cells associated with a focal infiltration of neutrophils and macrophages in the lamina propria and lumen like erupted volcano. Lectins SBA and DBA that has affinity to α Gal epitope were negative in normal samples but reacted strongly positive at the glycocalix of the villi enterocytes of the affected samples indicating a change in the carbohydrates pattern of the cells surfaces that might favor the binding of *C. difficile* toxin A (TcdA). In farm 2, from 11 examined piglets, 6 of them (54%) showed different degree of mesocolon edema and in only 2 of them; volcano lesions were the main histopathological findings. Rectal swab from 8 diarrheic and 5 postmortem examined piglets were surveyed for *eltA estI estII* and stx_{2e} virulence gens of *E. coli* and all samples were negative. *Clostridium difficile* toxins A and B were identified only in the sample with severe gross and microscopic changes. Isolation of *C. difficile* was unsuccessfully.

Infection of *C. difficile* is present in Argentinean pig farms. Diagnosis of subclinical infection might be negligent if post-mortem studies of preweaning mortality at weekly intervals are not performed. However, when clinical disease appeared, mortality might be high as it was reported in the farm 2. Comparative lectinhistochemistry studies from field cases added further information of the carbohydrates present on glycocalix of villi enterocytes related with toxin receptors.

Key Words: Mesocolon edema, colitis, clinical disease, *Clostridium difficile*, pathological studies, neonatal piglets.

Introduction

Clostridium difficile is a Gram-positive opportunistic anaerobic bacterium found in the soil, water as well as the digestive tract of several mammal´s species, birds and reptiles (9, 15, 16). In mammals, the infection by *C. difficile* has a common a clinical signs of diarrhoea that follow a change of the normal intestinal flora by antibiotics. This protective effect of the normal microbiota is referred to as "colonization resistance" (1, 6). According with the affected species, *C. difficile* causes pseudomembranous colitis in human, enterocolitis in foals, typhlocolitis in adult horses, and typhitis in hamsters and guinea pigs (5, 15, 16). Currently they are renamed as *C. difficile*-associated disease (CDAD) (6, 7, 15).

Clostridium difficile infection in pigs was first reported in 1980 in association with swine dysentery due to *Brachyspira hyodysenteriae* (8). When *C. difficile* was inoculated in gnotobiotic pigs a reduction of feed intake and a mucous to mucohaemorrhagic feces were seen (8), however when *C. difficile* toxins was applied in colonic loops of 25-60 kg body weight conventional pigs, it failed to produce pathological changes (14). Beside, *C. difficile* was isolated from 2 months old pigs with showed retarded growth, diarrhea and fibrinonecrotic enterocolitis (5). Currently in high-health herds and not related with antibiotic treatment, *C. difficile* has been emerged in association with neonatal catarrhal, fibrinous or purulent colitis in 2 to 7 day-old piglets (9, 15, 16, 17). At this age group, complex gut microbiota is poor development to exclude *C. difficile* colonization (1).

Lectins have the ability to bind to specific carbohydrate moieties and have been used as a tool in the identification of specific sugar residues on the gut goblet cell of newborn, suckling and weaned pigs (4) and cell type-related changes in both the epithelial cell glycocalix and goblet cell mucins in the small intestine (2). Besides, its application in formalin fixed paraffin embedded tissues has provided a more accurate recognition of the cellular deviation in pathological condition (3, 11) as well as new information related with pathogenesis of CDAD. Presumptive diagnosis of *C. difficile* includes the relationship between: clinical signs, age of affected piglets and histopathological findings. Detection of toxin A (TcdA) and toxin B (TcdB) on affected gut contents are necessary to arrive at final diagnosis (15, 16, 20).

The aims of this work were: a) to determine the prevalence of neonatal piglet mesocolon edema and colitis on an extensive study of postmortem preweaning mortality**,** b) to compare the lectinhistochemical pattern of normal colon and colon with edema and colitis and c) to describe an outbreak of neonatal colitis in piglets due to *C. difficile* infection.

Material and methods

The prevalence of neonatal piglet mesocolon edema and colitis study was performed in farm 1, a farrow-to-finish operation of 1000 sows. The farm was free of Aujeszky and TGE virus. Attempts to control *Escherichia. coli* diarrhea was made by the sow vaccination twice at 30 and 15 days before farrowing. Five farrowing houses with AIAO flow system kept piglets from farrowing to weaning at 21 days. No treatment with antibiotic was applied except after signs of diarrhea. The study comprised visits at weekly intervals during 6 months and necropsy of all dead and kept refrigerated piglets. According with the gross findings causes of preweaning mortality (PWM) were classified in 8 categories (12). Some categories were subdivided according with the occurrence of specific lesions or findings. From cases of mesolocon edema, samples of colon were taken for complementary studies such as histopathology, parasitology and bacteriology. Light microscopic studies comprised: histochemistry (Gram, Groccot and Warthin Starry stains) and lectinhistochemistry (7 lectins, Table 1). The later was performed according with a previously study (11).

The farm 2 was a mixed out-door/in-door farrow-to-finish operation of 160 sows'. It reported since 3 months ago an increased of PWM from 15-18% to 25-30% and even, in some weeks, 50%. Piglets 1 to 7 days-old were affected with diarrhea, dehidratation and death. Almost 50 to 70% of the litters were affected and signs tend to persisting even after the treatment with antibiotics. Both gilts and sows parities were affected and no clinical signs in other categories were reported. Eleven piglets 1 to 5 days-old were post-mortem examined and samples were taken for complementary studies. It comprised bacteriology from death and diarrheic piglets, antibiotic resistance, PCR for *E. coli* virulence gens: *eltA, est, estII* and stx_{2e} and histopathology. From 3 samples with different degree of mesocolon edema kept -70°C, detection of toxins A/B of *C. difficile* was performed by a rapid commercial optic immunoassay (BioStar OIA CdTox AB, Inverness Medical-BioStar Inc. CO, USA) according with the instruction of the company.

Results

In the farm 1 a total 820 piglets were post-mortem examined, from them, 8 cases were classified as suspected of *C. difficile* infection (0.1%). Age of affected piglets varied between 3 to 14 days. In all of them, the key mark was the severe mesocolon edema (Fig. 1) associated or not with ascites, hydrothorax or peritonitis. The colon contents were yellow to tan yellow with mucous to fibrinopurulent or caseous exudates. No specific signs associated with this condition were reported by farrowing personal, however diarrhea mainly associated with *Isospora suis* infection was highly prevalent on this farm.

Microscopic findings showed a marked edema of the colonic mesothelium, some of them with perivascular

cuffing of neutrophils and mononuclear cells. When small lymphoid cell aggregates were included in the mesothelium, it appeared enlarged and reactive. In 3 cases the colon lumen were filled by a mucous to fibrinopurulent exudates with cellular debris, colonies of cocobacillus and large numbers of rod-shaped bacteria Gram and Warthin Starry positive were seen. Beside, in one case numerous ovals or round budding (blastospores) silver stain positive (Grocott stain) cells resembling *Candida* spp were observed associated with bacteria. The colonic epithelia were generally not affected at all or with slight changes such as edema in the lamina propria in the apical portion of the villi with mononuclear cells infiltration. However, 2 cases with severe lesions were characterized by focal necrosis and loss of epithelial cells (Fig. 2) associated with a focal infiltration of neutrophils and macrophages in the lamina propria and lumen like erupted volcano, "volcano lesion" (Fig. 3).

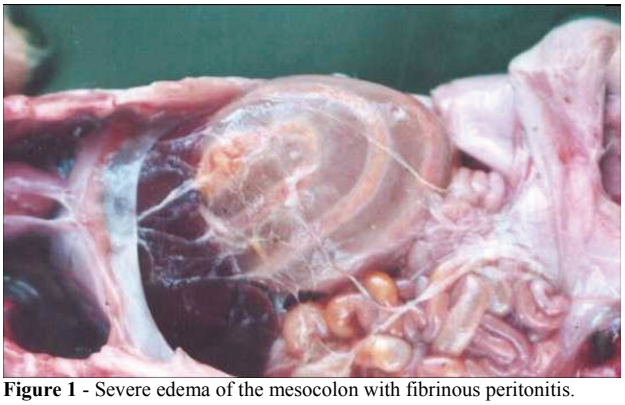

Figure 1 - Severe edema of the mesocolon with fibrinous peritonitis.

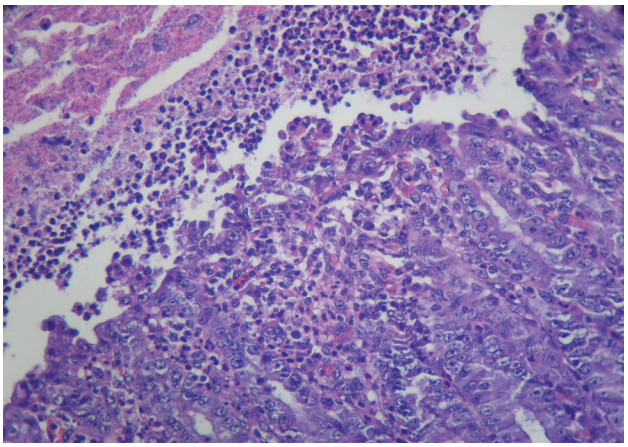

Figure 2 - Colon. Loss of the superficial epithelium with neutrophils into the lumen. HE, obj. 40x.

Control colonic mucosal enterocytes were non-reactive to lectins UEA, SBA, DBA and Con A, while cryptal cells showed weak to moderate affinity in the apical pole to PNA and both cryptal and villi localization were strong positive to WGA and RCA (Table 2). However, in pathological samples, these cells showed different reactivity pattern with lectins UEA, RCA, PNA, SBA, DBA and Con A. Particularly villi enterocytes showed strong affinity for SBA and DNA at the glycocalix (Table 2). In normal samples, goblet cells were weak to moderate reactive to PNA and strong to WGA while in affected one had strong binding to lectins UEA, SBA and DBA. Bacteriological studies for Gram negative aerobic pathogenic bacteria were negative and no attempt of *C. difficile* isolation was performed.

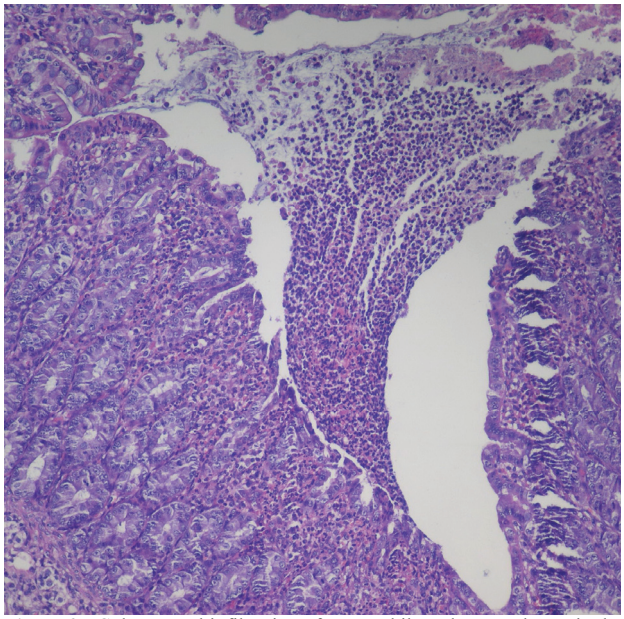

Figure 3 - Colon. Focal infiltration of neutrophils and macrophages in the lamina propria and lumen like erupted volcano, "volcano lesion". HE, obj. 20x.

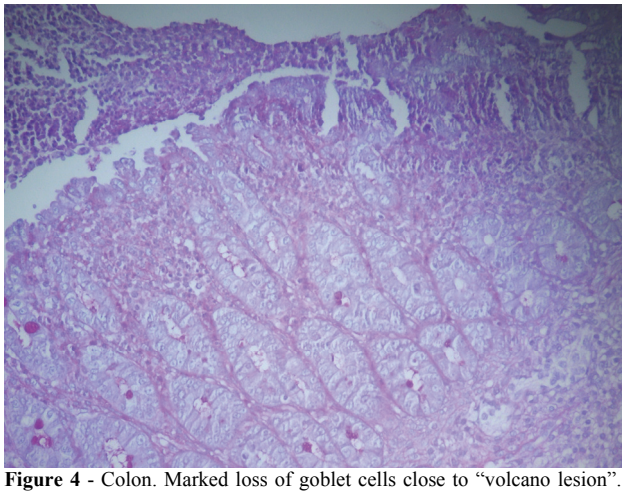

Figure 4 - Colon. Marked loss of goblet cells close to "volcano lesion". PAS, obj. 40x.

In farm 2, from 11 examined piglets, 6 of them (54%) showed different degree of mesocolon edema and in only 2 of them, loss of absorptive epithelia, reduction of goblet cells and neutrophils infiltration on lamina propria ejecting into the lumen like "volcano" were the main histopathological findings. Besides, a reduction of the

Table 1 - Names of lectins, abbreviations, sugar specificities and concentrations

Lectins	Abbreviation	Carbohidrate specificity	Concentration
Concanavalina ensiformes	Con A	α D-Man; α -D- Glc	30 μm/mlA
Ulex europeus I	UEA-1	α L- Fuc	30 μm/ml
Glycine maximus	SBA	α D- GalNAc, α D-Gal	30 μm/ml
Dolichus biflorus	DBA	α D-GalNAc	30 μm/ml
Arachis hypogaea	PNA	β D-Gal (β1-3)-D-GalNAc	30 μm/ml
Ricinus communis	RCA-1	β D-Gal	30 μm/ml
Tricticum vulgaris	WGA	α D-GlcNAc, NANA	10 μm/ml

Table 2 - Lectins reactivitiy of normal colon (N) and colon with mesocolon edema and colitis (L).

Cells	UEA		RCA		PNA		WGA		SBA		DBA		ConA	
Goblet	N	L	N	L	N	L	N	L	N	L	N	L	N	L
Cripta	0	3	0	0	1-2	0	3	3	0	3	0	2	0	0
Villi	0	3	0	0	0	0	3	3	0	3	0	3	0	0
Enterocyte														
Cripta	0	0	3^{1}	1	$1\text{-}2^{2}$	0	3	3	0	0	0	0	0	1^{2}
Villi	0	$3^{2,3}$	3^{1}	1	0	3^{1}	3	3	0	$3^{1;3}$	0	3^{1}	0	1^{2}
Endothelia	0	0	0	0	0	0	0	0	0	0	3	3	0	0
Neutrophil	0	0	0	0	0	0	0	0	0	0	3	3	0	0
Macrophage	0	0	0	0	0	0	0	0	0	0	0	0	0	3

(0)= negative; (1)= weak positive; (2)= moderate positive; (3)= strong positive, [1]= positive reactivity in the glycocalix; [2] = positive reactivity at the apical pole; [3] = positive reactivity at the supranuclear region.

goblet cells was observed (Fig. 4) Strains of *E. coli* isolated from small and large intestine were sensitive to florfenicol and gentamicine and resistant to others 10 antibiotics. However, rectal swab from 8 diarrheic and 5 postmortem examined piglets were surveyed for *eltA estI estII* and stx_{2e} virulence gens of *E. coli.* All samples were negative. *Clostridium difficile* toxins A and B were identified only in the sample with severe gross and microscopic changes. Isolation of *C. difficile* was unsuccessfully.

Discussion

Overall post-mortem studies of the causes of PWM during the weeks in which neonatal colitis was registered showed that diarrhea was the second cause of mortality, however the values founded were not as high as those previously reported (12). Clinical signs associated with *C. difficile* infection include respiratory distress, abdominal distension, scrotal edema and diarrhea beginning shortly after birth (15). Morbidity oscillated from 10% to 90% with a mean of 20% and mortality rate up to 50% (17). In our both studies the aforementioned signs not were observed by the stockman except the diarrhea. Previous studies on farm 1 showed that diarrhea found in the first week was due to *E. coli* infection and those found in the second and third week was mainly due to *I. suis* alone or in association with *C. perfringens* type A (13). The age of the affected piglets, between 1 to 14 days and, the severe mesocolon edema with mucous or paste yellowish colonic contents are considered the features for a presumptive diagnosis of *C. difficile* infection (9, 15, 16, 17, 19) and were the reason for taken samples for complementary studies. However, the positive predictive value of mesocolon edema for *C. difficile* enteritis was only 42% (19). Histopathological studies showed multifocal erosions, mucous or fibrinopurulent exudates in the colonic lumen with neutrophils and macrophages infiltration in the lamina propria with a "volcano" fashion (9, 15, 17). The presence of Gram-positive bacteria closed to the villi and the reduced number of goblet cells in those areas added further evidence of related infection (20). In both studies, the above histopathological changes were only observed in 4 cases. A previous study showed that they are more commonly observed in piglets with high levels of toxins A or B (17). These changes are uncommon in suckling piglets associated with others enteric pathogens related with this age group (19).

In human being and hamster, *C. difficile* infection is an establish cause of antibiotic-associated or dietary changes diarrhea (1, 6), because normal stable gut microbiota has to be disrupted before *C. difficile* can became establish and produce toxins (1). In new born piglets, like neonates, complex gut microbiota takes more than 5 days to be established in order to exclude *C. difficile* (1,19). The use of antibiotics early in life as it was reported in farm 2 might retard it. However, piglet's outbreaks with non exposure to antimicrobials have been reported (15, 16). In farm 1, the findings in one pig with lesions of *C. difficile* and concurrent infection with *Candida* spp favour

the idea that growth of both agents was associated after treatment with broad-spectrum antimicrobials. *Candida* spp appeared to colonize debilitated mucous surface, e.g. post-antibiotic enteritis, where developed pseudohyphal invasion of the superficial layers of the epithelium (10).

In *C. difficile* several virulence factors had been described some of them related with the colonization such as fimbriae, capsule, chemotaxis, flagellin and hydrolytic enzymes (1). Moreover, the production of the so-called "large clostridial toxins" seems to be essential for the damage of the colonic mucosa. TcdA is a protein of 308 kDa of molecular weight that act as enterotoxin which cause fluid accumulation in the intestine due to increased vascular permeability and haemorrhages (1) and TcdB, that is a 269 kDa cytotoxin for cultured cells (1, 16) but has little activity in vivo unless there is prior damage of enterocytes (6). Both toxins appeared to act synergically and are internalized by enterocytes in which cause disruption of cytoskeleton, alteration in the proteins synthesis, disturbances in the cell division with cells detachment with the consequent apoptosis of the isolated cells (1, 6, 16). Besides, Tad A stimulated macrophage cytokine production, TNFα, IL 1β and leukotrienes, which favours the neutrophils chemotaxis which in turn increased the tissue damage with the characteristic histopathological findings (1). A small percentage of *C. difficile* strains produce a binary toxin named CDT formed by separated polypeptides that is toxic to eukaryotic cells (6). A prospective study focused on toxin detection and several clinical and pathological parameters found that 58% of samples had normal colon contents, 59% had mesocolon edema, 75% had colitis and 72% typhlitis, the later two associations were statistically significant (19, 20).

It was showed that *C. difficile* requires a fermentable carbohydrate such as glucose or monosaccharides e.g. D-GalNAc or NANA present in the secretory colonic mucin as a carbon source (18). However, *C. difficile* lack of enzymatic machinery to cleave those monosaccharides from oligosaccharide side chains (18). Colonic bacteria are able to eliminate *C. difficile* through competition for these growth-limiting substrates even-though the above bacteria were the first organism established (18). Lectinhistochemistry study showed not changes between normal and affected samples related with D-GalNAc and NANA. However, in our study, affected villi enterocytes showed a different sugar patter in relation with the normal one, with strong reactivity for α L-Fuc (UEA I lectin), α D-GalNAc (PNA lectin), and α D-Man and α -D- Glc (Con A lectin). Some of those carbohydrates located in the brush border of enterocytes might acts as receptors for A toxin (6). Immunohistochemical study showed specific binding to TcdA, but not TcdB, to the epithelium of the small and large intestine of neonatal pigs not related with the carbohydrate Galα1-3β1-4GlcNAc-R (α galactosyl or α Gal epitope) (7). In accordance, lectins SBA and DBA that has affinity to α Gal epitope were negative in normal samples but reacted strongly positive at the glycocalix of the villi enterocytes of the affected samples indicating a change in the carbohydrates pattern of the cells surfaces that might favor the binding of TcdA.

In conclusion *C. difficile* alone or in mixed infection is present in Argentinean pig farms. Diagnosis of subclinical infection might be negligent if post-mortem studies of PWM at weekly intervals are not performed. However, when clinical disease appeared, mortality might be high as it was reported in the farm 2. Treatment of piglets with broad-spectrum antibiotics early after farrowing failed to control the diarrhoea and seems to be the main cause of persistence of infection Results of the comparative lectinhistochemistry study from field cases CDAD agree with those obtained using immunohistochemisty related with the carbohydrates of the surface of villi enterocytes.

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References

1. BORRIELLO SP. Pathogenesis of *Clostridium difficile* infection. *J. Antimicrob.Chemother*. (*Suppl C*)., 1998, 41,13-19.
2. CHAE CH., LEE Y. Age-related lectin histochemical changes in the porcine small intestine. *J. Vet. Med. Sci*., 1995, 57, 883-89.
3. COOPER H. Lectins as probes in histochemistry and immunohistochemistry: the peneaut (*Arachis hypogaea*) lectin. *Human Pathol*., 1984, 15, 904-06.
4. JAEGER LA., LAMAR CH., TUREK JJ. Lectin binding to small intestinal goblet cells of newborn, sucking and weaned pigs. *Am. J. Vet. Res*.,1989, 50, 1984-87.
5. JONES MA., HUNTER D. Isolation of *Clostridium difficile* from pigs. *Vet. Rec*., 1983, 112, 253.
6. KEEL MK, SONGER JG. The comparative pathology of *Clostridium difficile*-associated diseases *Vet.Pathol*., 2006, 43, 225-40.
7. KEEL MK., SONGER JL. The distribution and density of *Clostridium difficile* toxin receptors on the intestinal mucosa of neonatal pigs. *Vet.Pathol.* 2007, 44, 814-22.
8. LYSONS RJ., HALL GA., LEMCKE RM., BEW J., LUTHER PD. Studies of organisms possibly implicated in swine dysentery. *Proceedings 8th International Pigs Veterinary Society Congress* (*IPVS*). Copenhagen, Denmark., 1980, pp. 231.
9. POST KW., SONGER JG., JOST BH., GLOCK RD., HOLTCAMP A. The emergence of *Clostridium difficile* as a cause of porcine neonatal enteritis. *32nd*

Annual Meeting of AASV., 2001., Nashville, Tennessee. USA pp, 373-75.

10. TAYLOR DJ. Miscellaneous bacterial infections. STRAW, BE., D´ALLAIRE, S., MENGELING, WL., TAYLOR, DJ. Eds *Diseases of Swine*. Ames, Iowa:The Iowa State University Press., 1999, 627-30.
11. RIGANTI JG., MASSONE AR., QUIROGA MA., ARMOCIDA AD., PERFUMO CJ. Lectin histochemical binding profile of ileum samples from pigs affected with proliferative enteropathy. *Proceedings 15th International Pig Veterinary Society Congress (IPVS)*, Birmingham, England, 1998, 123.
12. SANZ M., SERNIA C., VIALE G., BUSTOS G., SANGUINETTI H., RISSO M., VENTURINI L., IDIART J., PERFUMO C. Why should piglets dead at the pre-weaning period be post-mortem examinated and statistically analyzed at weekly intervals? *Proceedings 32nd Annual Meeting American Association of Swine Veterinarians,* Nashville, Tennessee, 2001, 69-73.
13. SANZ M., VENTURINI L., ASSIS RA., UZAL F., RISSO MA., IDIART JR., PERFUMO CJ. Fibrinonecrotic enteritis of piglets in a commercial farm: a postmortem study of the prevalence and the role of lesion associated agents *Isospora suis* and *Clostridium perfringens*. *Pesq. Vet. Bras*. 2007, 27, 297-300.
14. SISK DB., COLE JR., PURSELL AR. *Clostridium difficile* toxin in the piglet. *Proceedings 7th International Pigs Veterinary Society Congress (IPVS)*, Mexico, 1982, 67.
15. SONGER JG., POST KW., LARSON DJ., JOST BH., GLOCK RD. Infection of neonatal swine with *Clostridium difficile. J. Swine Health Prod*., 2000, 6, 185-89.
16. SONGER JG., ANDERSON MA. *Clostridium difficile*: An important pathogen of food animals. *Anaerobe.,* 2006, 12, 1-4.
17. WATERS EH., ORR JR., CLARK EG., SCHAUFELE, CM. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. *J. Vet. Diagn. Invest*., 1998, 10, 104-08.
18. WILSON KH., PERINI F. Role of competition for nutrients in suppression of *Clostridium difficile* by colonic mucosa. *Infect.Immun*.,1988, 56, 2610-14.
19. YAEGER M., FUNK N., HOFFMAN L. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J. Vet. Diag. Invest.,* 2002 14, 281-87.
20. YAEGER MJ., KINDON JM., SONGER JG. A prospective, case control study evaluating the association between *Clostridium difficile* toxins in the colon of neonatal swine and gross and microscopic lesions. *J. Vet. Diagn. Invest*., 2007, 19, 52-5