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Enteropathogens in Holstein calves with diarrhea during the first five weeks of age in México

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ABSTRACT

The aim was to assess the prevalence of etiologic agents of diarrhea in single and mixed infections and their relationship with age in Holstein calves. Fecal diarrheic samples (n=90) were taken to determine the causative agent (*E. coli* (K99), *Salmonella* spp., *Cryptosporidium* spp., Coronavirus, and Rotavirus). Samples tested were positive at least to one pathogen (87.7%), most of the positive cases were mixed infections (62%; $P<0.01$); from these, affected by two (59%) and by three or more agents (41%; $P=0.07$). *Cryptosporidium* spp. and Rotavirus were the most common agents and most of infections occurred during the first two weeks of age.

Key words: Dairy calf, Diarrheal stool, Intestinal pathogens, Neonate, Prevalence.

INTRODUCTION

Dairy cattle production is one of the main economic activities in the livestock sector in northern Mexico (Reta-Sánchez *et al.*, 2015). The Comarca Lagunera generates 20% of total milk volume in Mexico (~10 million t per year), produced by several dairy herds throughout an inventory of 443,526 heads, mainly conformed by Holstein dairy breed, managed under some of the most highly intensified production systems in Latin America (Chirino-Enoel *et al.*, 2012). Most of the times, these highly intensified production systems, have remarkable disadvantages because of their high animal densities, inadequate sanitary conditions and animal discomfort and stress, an scenario that favors the rapid spread of infectious diseases (Gulliksen *et al.*, 2009).

Early death of calves is a factor that results in the loss of heifer replacement (Mushtaq *et al.*, 2013), most of such deaths occurs because of neonatal diarrhea, a complex multifactorial syndrome where infectious, environmental, nutritional and immunological factors are involved (Cho and Yoon, 2014; Kabu *et al.*, 2015). This condition affects newborn calves worldwide causing great economic losses because of its increased morbidity and mortality (Schroeder *et al.*, 2012). Indeed, almost 70% of death cases observed in the dairy industry have been linked to the presence of diarrhea, especially during the first three weeks of age (Żychliñska-Buczek *et al.*, 2015). Neonatal and preweaning diarrhea has a complex etiopathogenesis, and *Cryptosporidium* spp., Rotavirus, Coronavirus, *E. coli* (K99) and *Salmonella* spp. are the pathogens most commonly associated (Bartels *et al.*, 2010). Nonetheless, during clinical field inspection, identification of etiologic agents is not possible, requiring laboratory studies, using varied number of techniques (Blanchard, 2012). The aim of this study was to determine the prevalence of *E. coli* (K99), *Salmonella* spp., *Cryptosporidium* spp., Coronavirus or Rotavirus as agents of diarrhea in calves under intensive production systems across the first weeks of age of calves in the Comarca Lagunera, México.

MATERIALS AND METHODS

The research was conducted from December 2007 to December 2008 in dairy herds (n=9) from the Comarca Lagunera, México (102°22′ and 104°47′ W and 24°22′ and 26°23′ N), at an altitude of 1,139 masl, with a semi-desert climate and an average annual temperature of 21°C (variations of 40°C from May-August to 6°C from December-January). The annual average rainfall is 266 mm (range of 163-504 mm), the dry season is from November to May. The study considered Holstein calves between 1-60 days old housed in individual calf hutches, feeding with milk replacer and supplemented with a concentrate based on corn and oats, and water *ad libitum*. All procedures were done according to the directions for animal welfare in the Mexican Legislation of Animal Health (DOF-07-06-2012).

Feces samples (15-20 g) were directly taken from the rectum of 90 calves presenting clinical signs of diarrhea, placed in sterile plastic containers properly labeled, and stored (4 to 8 °C) until processed in the Diagnostic Unit of the Universidad Autónoma Agraria Antonio Narro - Unidad Laguna.

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The diagnosis for *Cryptosporidium* spp. was made in extended fresh stool. Smears made from each non-concentrated fecal sample on glass slides, air-dried and stained using the modified Ziehl-Neelsen technique, examined by light microscopy (40X) lens for verifying the presence of *Cryptosporidium* oocysts, and interpreted as positive when bright red characteristic structures of oocysts of 4-6 µm on a light blue background were observed.

The culture of *Salmonella* spp. was made by seeding about 1g of stool added to 9mL of tetrathionate broth, and incubated for 24h at 37°C. The samples were seeded in *Salmonella-Shigella* Agar, and incubated for 24h at 37°C. The isolates were classified according to the characteristics of the colony and Gramm stain, and the lactose negative colonies were tested on TSI and urea media, to recognize visually suspected colonies. The identification of *Rotavirus, Coronavirus* and *E. coli* F5 (K99), was performed considered the ELISA BIO-X EASY-DIGEST BIO K 151 (Bio-X Diagnostics; Imre *et al*., 2007).

The total prevalence, single and mixed infections, by pathogen agent and age group in weeks are expressed in percentages. To determine the statistical difference in the prevalence of infection in single or mixed presentation by pathogen and age group (weeks), the chi-square test (P<0.05) was used. The analyses were performed using the statistical package MYSTAT 12, Version 12.02.00-2007, while assessment of significant differences considered a P≤0.05 (I_{95}).

RESULTS AND DISCUSSION

From the analyzed samples, 87.7% (79/90) were positive for at least one pathogen (Table 1). The five agents tested were present in the analyzed samples, being *Cryptosporidium* spp. and Rotavirus the most common and *E. coli* (K99) the least common (P<0.05; Table 1). Similar prevalences have been observed with 93% (Içen *et al*., 2013) and 86% (Pardo and Oliver, 2012), this highlights the role that such etiopathogens exert upon most diarrhea cases in calves under intensive dairy production systems. Bartels *et al*. (2010) reported that in 28.1% of the studied samples, no pathogens were detected, while in our study, no pathogens were detected only in 11 samples (12.2%). Cho and Yoon (2014) reported that the incidence of diarrhea cases where other enteric pathogens such as viruses, *C. perfringens* type C or *Giardia* spp. In such 12.2% of non-detected pathogens, some other scenarios could had been involved, generating the presence of diarrhea caused by management or nutritional disorders.

Table 1: Prevalence of causative agents (n/%) of diarrhea in Holstein calves on the Comarca Lagunera.

Pathogen	Total		Mixed infection	
Cryptosporidium spp.	48/90	53.3[a]	34/48	70.8[a]
Rotavirus	42/90	46.7[a]	39/42	92.9[b]
Coronavirus	25/90	27.8[b]	22/25	88.0[a,b]
Salmonella spp.	26/90	28.9[b]	18/26	69.2[a]
E. coli	14/90	15.5[c]	12/14	85.7[a,b]

Different literals between rows have statistical difference (P<0.05).

The more prevalent pathogenic agent in our study was *Cryptosporidium* spp., which was found in 50% of the cases. According to Thakre *et al*. (2017) *C. parvum, C, andersoni* and *C. bovis* are the most important species affecting bovines; however, we do not know which of the species are implicated in the prevalence in our study, and need further research. Içen *et al*. (2013) reported lower *Cryptosporidium* spp. levels such as 21.8%. Other authors reported prevalences of 63.3% and 79.9%, (Emre and Fidanci, 1998; Izzo *et al*., 2011). This suggest the importance of *Cryptosporidium* as primary agent of diarrhea because 30% of samples were confirmed as *Cryptosporidium* as the sole agent found (Trotz-Williams *et al*., 2005). This high prevalence may be due to contamination by fecal-oral transmission among animals or through water and food contaminated with *Cryptosporidium* oocysts.

Rotavirus was the second most prevalent agent with 47% of the cases. Gillhuber *et al*. (2014), reported a decreased prevalence (37.8%) compared with our results, but Izzo *et al*. (2011), reported a prevalence of 79.9%, above that observed in our study. The increased presence of Rotavirus could be due to that this pathogen is present in the environment and may have been increased because conditions such as poor hygiene, confinement, and passive immunity imbalance which can cause disease.

The third agent with the highest number of cases (prevalence of 28.9%) in our study was *Salmonella* spp. This finding is consistent with that reported in other studies, where this agent was also the third highest pathogen causing diarrhea, but with a prevalence of 13% (Almawly *et al*., 2013) and 23.8% (Izzo *et al*., 2011). However, it is above the 1.8% prevalence reported by de la Fuente *et al*. (1999). The presence of *Salmonella* spp. may be due to carrier status of animals as well as environmental pollution of water and food.

Regarding Coronavirus, we obtained a prevalence of 27.8% which is close to the 21.6% reported by Izzo *et al*. (2011), but well above (0.8%) of that reported by Pardo and

Table 2: Mixed infection by enteropathogens in Holstein calves on the Comarca Lagunera.

Agent	*Cryptosporidium spp.*	Rotavirus	Coronavirus	*Salmonella spp.*	*E. coli*
Cryptosporidium spp.	-	26 (28.9%)	13 (14.4%)	14 (15.6%)	5 (5.6%)
Rotavirus	-	-	19 (21.1%)	10 (11.1%)	11 (12.2%)
Coronavirus	-	-	-	3 (3.3%)	9 (10.0%)
Salmonella spp.	-	-	-	-	2 (2.2%)
E. coli	-	-	-	-	-

Oliver (2012), as well as the 3.4%, 5.2% and 11.1% reported in other studies (de la Fuente *et al.*, 1999; Almawly *et al.*, 2013; Gillhuber *et al.*, 2014). Meanwhile, *E. coli* (K99) was the least prevalent identified etiopathogen in our study, in line with other studies (Gillhuber *et al.*, 2014). However, the observed prevalence was lower compared to that reported (26.0% and 27.8%), in its respective studies (de la Fuente *et al.*, 1999; Içen *et al.*, 2013). As mentioned by Blanchard (2012), *E. coli* (K99) is an agent that causes diarrhea during the first week of age; however, in our study the most of cases were observed during the first two weeks of age. Coronavirus and *E. coli* are pathogens generally present in dairy herds; *E. coli* can be found in contaminated food and water due to elimination of the bacteria by healthy carrier animals or human fecal waste (Fairbrother and Nadeau, 2006). Also, Coronavirus is an agent that causes enteritis in adult cattle, which plays an important role as carriers contributing to the spread of the disease (Park *et al.*, 2006).

The highest number of positive cases were diagnosed as mixed infections (62%; $P<0.01$), of which 29 (59.2%) were affected by two pathogenic agents and 20 (40.8%) considered three or more agents ($P=0.07$). *Cryptosporidium* spp. and *Rotavirus* (29%) were the agents with the greatest number of cases, followed by Rotavirus with Coronavirus (21%) and less common *E. coli* (K99) with *Salmonella* spp. (2%; $P<0.001$; Table 2 and 3). Our prevalence of multiple infections is similar to the 63.3% (Emre *et al.*, 1998), yet lower than 71% (Izzo *et al.*, 2011) and 92.7% (Içen *et al.*, 2013). Another study mention that over 50% of calves with diarrhea were infected by more than one pathogen (Cho *et al.*, 2013). Our results agree with Içen *et al.* (2013), where reported that the diarrheal syndrome has a complex etiopathogenesis, environmental influences, management, physiological and immunological conditions and nutritional factors; indeed most pathogens studied had interaction with one or more pathogens (> 70%).

Cryptosporidium spp. and Rotavirus were the agents with the highest prevalence (53.3% and 46.7%, respectively), which could explain the high prevalence in mixed infections. This result is consistent with those reported in other studies (Pardo and Oliver 2012; Içen *et al.*, 2013; Gillhuber *et al.*, 2014). *Cryptosporidium* spp. with Rotavirus was the more common mixed infection, with an overall prevalence of 28.9%; this is in line with the reported by Izzo *et al.* (2011), where was observed that 24.8% of calves excreted both *Cryptosporidium* spp. with Rotavirus, while Gillhuber *et al.* (2014) obtained a lower prevalence (15.1% and 17%, respectively). These agents combined play a determinant role in neonatal calf diarrhea; however, the relative predominance of both pathogens remains unclear (Gillhuber *et al.*, 2014).

For all pathogens, the highest prevalence was observed during the first 14 days post-calving (34% and 39% during the first and second week, respectively; $P<0.05$). The

Table 3: Combination of enteropathogens on mixed infections in Holstein calves on the Comarca Lagunera.

Enteropathogen	n = 90	
	n	%
Cryptosporidium spp., Rotavirus, Coronavirus, *Salmonella* spp., *E. coli*	$2^{b,c}$	2.2
Cryptosporidium spp., Rotavirus, Coronavirus, *E. coli*	$3^{a,b,c}$	3.3
Cryptosporidium spp., Rotavirus, Coronavirus	$7^{a,b}$	7.7
Cryptosporidium spp., Rotavirus, *Salmonella spp.*	$5^{a,b,c}$	5.5
Rotavirus, Coronavirus, *E. coli*	$3^{a,b,c}$	3.3
Cryptosporidium spp., Rotavirus	9^{a}	10.0
Cryptosporidium spp., *Salmonella* spp.	$7^{a,b}$	7.7
Rotavirus, Coronavirus	$4^{a,b,c}$	4.4
Rotavirus, *Salmonella* spp.	$3^{a,b,c}$	3.3
Rotavirus, *E. coli*	$3^{a,b,c}$	3.3
Cryptosporidium spp., Coronavirus	1^{c}	1.1
Coronavirus, *Salmonella* spp.	1^{c}	1.1
Coronavirus, *E. coli*	1^{c}	1.1
Total	4^{9}	54.4

Different letters between rows indicate the statistic difference (P<0.05).

Table 4: Prevalence (n/%) of enteropathogens causing diarrhea in Holstein calves by age group on the Comarca Lagunera.

Age	*Cryptosporidium* spp.	Rotavirus	Coronavirus	*Salmonella* spp.	*E. coli*
1-7	14^{a} $(29.2)^{x,y}$	15^{a} $(35.7)^{x}$	8^{a} $(32.0)^{x,y}$	8^{a} $(30.8)^{x}$	6^{a} $(42.9)^{x}$
8-14	21^{a} $(43.8)^{x}$	15^{a} $(35.7)^{x}$	10^{a} $(40.0)^{x}$	9^{a} $(34.6)^{x}$	6^{a} $(42.9)^{x}$
15-21	10^{a} $(20.8)^{y}$	6^{a} $(14.3)^{y}$	3^{a} $(12.0)^{y}$	6^{a} $(23.1)^{x,y}$	2^{a} $(14.3)^{x,y}$
22-28	1^{a} $(2.1)^{z}$	3^{a} $(7.1)^{y}$	0^{a} $(0.0)^{z}$	3^{a} $(11.5)^{y,z}$	0^{a} $(0.0)^{y}$
29-35	2^{a} $(4.2)^{z}$	3^{a} $(7.1)^{y}$	4^{a} $(16.0)^{y}$	0^{a} $(0.0)^{z}$	0^{a} $(0.0)^{y}$
Total	48 (100)	42 (100)	25 (100)	26 (100)	14 (100)

[a]Different letters between rows indicate statistic difference (P<0.05).

[x,y,z]Different letters between columns indicate statistic difference (P<0.05).

number of diarrhea cases tended to decline, observing 17% (*P*<0.05) in the third week, and another decline to less than 4% and 5% in the fourth and fifth weeks, respectively (*P*<0.05; Table 4). The high percentage of diseases with multiple etiological agents during the first two weeks of life suggests that animals may have been fed either with poor quality colostrum or in small quantities. It is important to consider cleaning the housing cages, the proximal areas to the sick animals or other conditions that increase the risk of spread factors and contamination of newborn animals. Ingestion of good quality colostrum during the period close to birth is crucial for protection against pathogens (Singh *et al*., 2011). The last is particularly true, since the calves immune system is deficient, making them vulnerable to infections, especially when situations that trigger stress decrease the ability of the defense system to act against enteric infections, where multiple etiologies can be present (Muktar *et al*., 2015).

CONCLUSION

This study evaluated the prevalence of the main diarrhea's etiopathogens in Holstein calves in the Comarca Lagunera, Mexico. Our results showed that most calves (>60%) with signs of diarrhea have a combined infection. Our results confirm the presence of *Cryptosporidium* spp. and Rotavirus as the most common etiopathogens with the largest number of infections occurring during the first two weeks of life. Development of effective diagnostic systems and optimization of preventive and control programs emphasized to reduce the risk of infections. The large impact that such diverse etiopathogens has over animal production and public health warrants special attention.

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