Veterinary Microbiology 166 (2013) 375–385

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Case–control study of microbiological etiology associated with calf diarrhea

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ARTICLE INFO

Article history:
Received 20 July 2012
Received in revised form 27 June 2013
Accepted 1 July 2013

Keywords:
Calf diarrhea
Case–control study
Enteric pathogens
Multiplex PCR detection

ABSTRACT

Calf diarrhea is a major economic burden for the US cattle industry. A variety of infectious agents are implicated in calf diarrhea and co-infection of multiple pathogens is not uncommon in diarrheic calves. A case–control study was conducted to assess infectious etiologies associated with calf diarrhea in Midwest cattle farms. A total of 199 and 245 fecal samples were obtained from diarrheic and healthy calves, respectively, from 165 cattle farms. Samples were tested by a panel of multiplex PCR assays for 11 enteric pathogens: bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), bovine enterovirus (BEV), bovine norovirus (BNoV), Nebovirus, bovine torovirus (BToV) *Salmonella* spp. (*Salmonella*), *Escherichia coli* (*E. coli*) K99$^+$, *Clostridium perfringens* with β toxin gene and *Cryptosporidium parvum* (*C. parvum*). The association between diarrhea and detection of each pathogen was analyzed using a multivariate logistic regression model. More than a half of the fecal samples from the diarrheic calves had multiple pathogens. Statistically, BRV-A, BCoV, BNoV, Nebovirus, *Salmonella*, *E. coli* K99$^+$, and *C. parvum* were significantly associated with calf diarrhea ($p < 0.05$). Among them, *C. parvum* and BRV-A were considered to be the most common enteric pathogens for calf diarrhea with high detection frequency (33.7% and 27.1%) and strong odds ratio (173 and 79.9). Unexpectedly BNoV (OR = 2.0) and Nebovirus (OR = 16.7) were identified with high frequency in diarrheic calves, suggesting these viruses may have a significant contribution to calf diarrhea.

1. Introduction

Calf diarrhea is a major cause of economic loss with high morbidity and mortality in the cattle industry worldwide (Bartels et al., 2010; de la Fuente et al., 1999; Kelling et al., 2002; Uhde et al., 2008; United, 2007). Many factors are known to contribute to calf diarrhea. Historically, calf diarrhea has been commonly attributed to bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), *Salmonella* spp. (*Salmonella*), *Escherichia coli* (*E. coli*) K99$^+$, and *Clostridium perfringens* (*C. perfringens*) type C and *Cryptosporidium parvum* (*C. parvum*) (Acha et al., 2004; Reynolds et al., 1986; Saif and Smith, 1985; Snodgrass et al., 1986). The specific etiology of many field cases of calf diarrhea still remain undiagnosed (Milnes et al., 2007). Recently, bovine norovirus (BNoV), Nebovirus, bovine enterovirus (BEV) and

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http://dx.doi.org/10.1016/j.vetmic.2013.07.001

bovine torovirus (BToV) have been identified as potential causes of calf diarrhea (Blas-Machado et al., 2007; Haschek et al., 2006; Hoet et al., 2003a; Kaplon et al., 2011; Otto et al., 2011; Park et al., 2007, 2008a,b). Some of these agents (i.e., BNoV, BEV and BToV) have also been found in feces from clinically healthy calves (Haschek et al., 2006; Jimenez-Clavero et al., 2005; Mijovski et al., 2010; Shanks et al., 2008) and many of previous epidemiological studies for BNoV and BToV have been focused only on diarrheic calves (Hoet et al., 2003b; Milnes et al., 2007; Park et al., 2007, 2008b). Their role in calf diarrhea still remains to be evaluated.

Various laboratory methods have been applied for the detection of infectious agents in feces. Historically, virus isolation, electron microscopy, enzyme-linked immunosorbent assay, latex agglutination test, bacterial culture, direct microscopy of fecal smear (acid-fast stain), and/or fecal flotation have been commonly used to test fecal samples for enteric pathogens (Cho et al., 2010). These procedures are reliable; however, they are time-consuming and require specialized knowledge. Recently, nucleic acid based tests, such as polymerase chain reaction (PCR) assays, have become popular for rapid and sensitive detection of infectious agents (Albini et al., 2008; Cho et al., 2010). Multiplex real-time PCR panels have been proven to be a useful diagnostic tool for concurrent detection of several target enteric pathogens with high sensitivity and specificity (Albini et al., 2008; Cho et al., 2010), which decreases bias in diagnostic outcome due to testing method.

The following case–control study was conducted to: (a) assess the prevalence of 11 infectious agents consisting of 7 common [BRV-A, BCoV, BVDV, *Salmonella*, *E. coli* K99$^+$, *C. perfringens* with β toxin gene (Cpt β) and *C. parvum*] and 4 emerging enteric pathogens (BNoV, Nebovirus, BEV and BToV) in fecal samples from healthy and diarrheic calves in the Midwest by using a panel of PCR assays; and (b) determine their association with diarrhea as well as investigate their potential interactions in expression of disease.

2. Materials and methods

2.1. Animals and samples

All fecal samples used in the study were originated from clinically diarrheic and healthy calves during year 2010–2011. A total of 199 fecal samples from diarrheic calves were procured from submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) and used as cases. The samples were from 140 cattle farms with the most of the samples (99%) originated in the Midwest [Iowa (78%), Minnesota (8%), Wisconsin (4%), Missouri (3%), Ohio (3%), Illinois (1%), South Dakota (1%) and Nebraska (1%)]. No more than 4 samples were randomly selected from the same farm if a large number of samples were submitted. A vast majority of the samples tested were from sick animals before treatment begun according to referring veterinarians. Approximately 41% and 42% of the samples were from dairy and beef breeds, respectively. The remaining 18.5% of the samples were submitted without breed identification. Physical appearance of first 99 of the 199 fecal samples was recorded as 'watery' or 'semi-solid' upon receiving as fresh samples were available to the investigators before freezing.

A total of 245 fecal samples were collected from clinically healthy (i.e., no diarrhea) calves in 25 different beef or dairy farms which were evenly distributed across the State of Iowa and used as controls. These farms were pre-selected to be part of other field-based study in which on-going health monitoring was required including use of any medication. Samples were collected twice from each farm at approximately 2-week intervals with continuous monitoring of health status to ensure lack of diarrhea among animals on each farm. At each time of sample collection, 5 calves were randomly selected for sampling.

Most of the source farms were similar in overall farm management, including vaccination and medication, and nutritional status. Most (96.4%) of the calves tested were less than 6 months old in age. Two third of the control calves were less than 3 months of age while 80% of the case calves were less than 3 months of age. Only 1and 7 cases were submitted from a 7-month-old diarrheic calf and clinically healthy yearlings or older cattle, respectively.

2.2. Detection of pathogens

All fecal samples were examined for 11 different microorganisms (i.e., BRV-A, BCoV, BVDV, BEV, BNoV, BToV, Nebovirus, *Salmonella*, *E. coli* K99$^+$, *C. parvum* and Cpt β) using a panel of polymerase chain reaction (PCR) based assays. All except BEV have been reported as pathogens implicated in calf diarrhea.

Before PCR testing, each fecal sample was suspended in 0.01 M phosphate-buffered saline (pH 7.4) to make 30% fecal homogenates and then centrifuged for 1 min at $100 \times g$ for clarification as previously described (Cho et al., 2010). The supernatant was then used for viral and bacterial nucleic acid extraction using MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Austin, TX) according to the manufacturer's instruction. The extraction procedure was performed using Kingfisher® 96 Magnetic Particle Processor (Thermo Fisher Scientific Inc., Waltham, MA). All extracts were stored at −80 °C until tested.

Probe-based real-time PCR (rtPCR) assays for all pathogens except BToV and Nebovirus were performed in a duplex or singleplex PCR format with Path-ID™ Multiplex One-Step RT-PCR Kit (Applied Biosystems, Austin, TX) and AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Austin, TX), respectively. For BToV, a SYBR Green rtPCR assay was used with QuantiTest™ SYBR® Green PCR Kit (QIAGEN, Valencia, CA).

For rtPCR set-up, 7 μl of template and 18 μl of the reaction mixture for the duplex PCRs (Table 1, real-time PCR set 1, 2, 5 and 6) and 5 μl of template and 20 μl of the reaction mixture for singleplex PCRs (Table 1, real-time PCR set 3 and 4) were used. All reaction mixtures contained 400 nM of each primer, 120 nM of the probe except BToV, RT-PCR buffer, RT-PCR enzyme mix, and nuclease-free water. The volume of each reagent added to a reaction mixture was as per manufacturer's instruction. The sequence information of primers and probes used for