

UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA



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ETIOLOGY OF NEONATAL DIARRHEA IN CALVES IN LOWER BAVARIA, GERMANY

BRUNA DANIELA PEREIRA MENDES

ORIENTADOR:
Doutor Virgílio da Silva Almeida
TUTOR:
Dr. Julian Bartels

2020

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BRUNA DANIELA PEREIRA MENDES

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ORIENTADOR:

Doutor Virgílio da Silva Almeida

TUTOR:

Dr. Julian Bartels

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Nome: Bruna Daniela Pereira Mendes

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Resumo

Etiologia da diarreia neonatal em vitelos na Bavaria de Baixo, Alemanha

A diarreia neonatal é uma das principais causas de perdas económicas para os produtores, afetando sobretudo vitelos no primeiro mês de vida. Trata-se de uma doença multifatorial, que resulta nas interações entre hospedeiro, ambiente, nutrição e vários agentes infecciosos. Entre estes, os mais frequentemente isolados são o *C. parvum*, o rotavírus, o coronavírus e a *E. coli* K99. A rápida identificação destes agentes infecciosos é crucial para uma intervenção eficaz.

Os objetivos do presente estudo foram identificar a frequência destes quatro agentes infecciosos, em vitelos com diarreia neonatal, em explorações leiteiras na Baviera de Baixo, recorrendo a um teste rápido de imunocromatografia (Fassisi® BoDia test), bem como avaliar a utilidade destes mesmos testes rápidos em campo.

Foram investigadas 18 amostras de fezes de vitelos com diarreia neonatal de 17 explorações pecuárias. O rotavírus (55,6%; n=10) e o *C. parvum* (33,3%; n=6) foram os microrganismos mais frequentes, seguidos pelo coronavírus (16,7%; n=3) e a *E. coli* (11,1%; n=2). As co-infecções corresponderam a 16,7% dos casos, associadas apenas ao rotavírus e ao *C. parvum*, sendo que se encontrou o *C. parvum* associado a co-infecções ($p < 0.05$).

O teste rápido de imunocromatografia foi positivo para pelo menos um agente etiológico em 88,9% dos casos investigados. Detetaram-se dois falsos-negativos para *E. coli* que refletem a menor sensibilidade do teste para a bactéria. Os resultados obtidos sugerem que os testes rápidos são muito úteis para esclarecer a etiologia da diarreia neonatal em vitelos, sendo uma ferramenta rápida e eficaz para o médico veterinário, ajudando a implementar medidas preventivas na manada, nomeadamente vacinação, e a ajustar o protocolo de tratamento, sobretudo em infeções por *C. parvum*. A taxa de sucesso do protocolo terapêutico utilizado durante o estudo foi de 83,0% (n=12).

Palavras-chave: diarreia neonatal, vitelo, rotavírus, *Cryptosporidium parvum*, *Escherichia coli*

Abstract

Etiology of neonatal diarrhea in calves in Lower Bavaria, Germany

The neonatal diarrhea is a major cause of economic losses to cattle producers, affecting frequently calves within the first month of life. It is a complex multi-factorial disease, resulting from an interaction between host, environment, nutrition and infectious agents. Among several organisms that cause neonatal calf scours, the four more frequently identified enteropathogens are *C. parvum*, rotavirus, coronavirus and *E. coli* K99. Accurate and rapid identification of the infectious agent(s) involved is crucial for an appropriate intervention and prevention measures.

The aims of this study were to quantify the presence of these four major enteric pathogens in neonatal calves' diarrhea in dairy farms from Lower Bavaria region by a commercially rapid immunochromatographic test (Fassisi® BoDia test), and to evaluate the suitability of this test for practical use in the field.

Eighteen fecal samples were collected from diarrheic calves from seventeen dairy herds for identification of the etiological agent. Rotavirus (55.6%; n=10) and *C. parvum* (33.3%; n=6) were the most frequent enteropathogens, followed by coronavirus (16.7%; n=3) and *E. coli* (11.1%; n=2). Co-infections were found in 16.7% of the cases, being only associated to rotavirus and *C. parvum*. A significant statistical association was found for *C. parvum* involvement in co-infections ($p < 0.05$). On the contrary, there was no statistical association of co-infections by rotavirus ($p > 0.05$). The rapid test was positive for at least one etiologic agent in 88.9% of the cases. There were two false negatives for *E. coli* due to the low sensitivity of this test. Despite that, the global results suggest that this test is of great value to establish the etiology of neonatal diarrhea in calves and it is a helpful, fast and effective tool for the veterinary practitioner. The success rate of the therapy protocol was 83.0% (n=12).

Keywords: neonatal diarrhoea, calves, rotavirus, *Cryptosporidium parvum*, *Escherichia coli*

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Abbreviations & Acronyms

AB – Antibiotic	RVA – Rotavirus group A
Ag-ELISA – Antigen-capturing enzyme-linked immunosorbent assay	S – Spike glycoprotein
BCoV – Bovine Coronavirus	ST – Heat-stable enterotoxin
cAMP – Cyclic adenylate monophosphate	STEC – Shiga Toxin-producing <i>E. coli</i>
cGK II – cGMP-dependent protein kinase II	UTI – Urinary Tract Infection
cGMP – Cyclic guanylyl monophosphate	VIBs – Virus inclusion bodies
CL ⁻ - Chloride ion	VP4 – Rotavirus outer capsid protein
DAEC – Diffusely Adherent <i>E. coli</i>	VP6 – Rotavirus intermediate capsid protein
<i>E. coli</i> – <i>Escherichia coli</i>	VP7 – Rotavirus outer capsid glycoprotein
EAEC – Enter aggregative <i>E. coli</i>	
EHEC – Enterohemorrhagic <i>E. coli</i>	
EIEC – Enteroinvasive <i>E. coli</i>	
ENS – Enteric nervous system	
EPEC – Enteropathogenic <i>E. coli</i>	
ETEC – Enterotoxigenic <i>E. coli</i>	
EU – European Union	
GCC – Guanylyl cyclase-C	
HCO ₃ ⁻ - Bicarbonate ion	
HE – Hemagglutinin-esterase glycoprotein	
IgG – Immunoglobulin G	
IV – Intravenous	
LAT – Latex agglutination test	
LPS – Lipopolysaccharide	
LT – Heat label-toxin	
NaCl – Sodium Chloride	
NaHCO ₃ – Sodium bicarbonate	
NCD – Neonatal calf diarrhea	
NSAID – Non- steroidal anti-inflammatory drug	
NSP4 – Nonstructural glycoprotein	
°C – Celsius	
PCR – Polymerase chain reaction	

I – Internships Report

1. Brief description of the Curricular Internship: Tierarztpraxis Dr. Bartels, Germany

The curricular internship for the Master's final semester took place at the Dr. Bartels Veterinary Clinic, under the supervision of Dr. Julian Bartels himself, in the state of Bayern in Germany, between the 1st of October 2018 and the 31st of January 2019. This clinic works with all animal species, from companion animals, horses, livestock and occasionally exotic animals and birds. Many services are provided, including medicine, vaccination programs, surgery, ultrasound diagnostics, nutrition care, dermatology, oncology among others, while also having a small laboratory for blood analysis, bacterial cultures, coprology exams and ectoparasites analysis.

During the internship, I worked with several practitioners and veterinary assistants that helped me to consolidate and expand my knowledge and teach me different methods of practice as a vet.

As the clinic is located in a small town in a rural area, there were many bovine clinical cases to attend, making possible this study. There were multiple interventions during these four months, which included prophylactic vaccinations, estrus synchronization of cows and heifers, artificial insemination, gestation diagnosis by rectal palpation and by rectal echography, birth assistance, cesareans, diarrhea and pneumonia treatments in calves, retained placenta, uterine prolapse, mastitis, castration, dehorning, euthanasia and other tasks. In companion animals, daily routines included consultations and follow-ups, wound disinfections, castrations and to a rabbit, monitorization of anesthesia and recovery.

2. Brief description of the Professional Internship: Virbac Headquarters, France

The second internship took place at the Virbac Headquarters in Carros, France, during the period of 25th of March 2019 to 17th of May 2019, where I got the opportunity to cross contact with many veterinary researchers from all departments, in what is one of the biggest pharmaceutical companies dedicated to animal care and welfare worldwide. I integrated the Global Medical Department, under the supervision of Dr. Christelle Fontaine, actively participating in a research program on GnRH agonists in companion animals, having also closely followed the R&D and Marketing teams, where some of my tasks involved literature review, data compilation and presentation to support future projects in reproduction and oncology areas. Despite this second internship was to short, it was a big experience. I understood the great importance to seek new and better ways to perform veterinary practice. Innovate and keep improving existing products and search for new solutions with one goal: to improve the health and welfare of animals and those who foster them.

II – Introduction

Diarrhea is defined as an increase in fecal water loss due to an imbalance between absorption and secretion of water and electrolytes (Scott et al. 2004; Kumaseran et al. 2012). The neonatal calf diarrhea (or calf scours) is defined as a complex multi-factorial disease, resulting from an interaction between host, environment, nutrition and infectious agents. The diarrhea can be white, yellow, grey or blood-stained, and is often foul-smelling. It is the most observed clinical sign of illness in young calves and occurs mainly in the first month of life, due to its poor immune response capacity (Uhde et al. 2008; Kumaresan et al. 2012). The younger the calf, the greater is the risk of developing diarrhea and death usually results from progressive, severe dehydration, acidosis, and loss of electrolytes (Scott et al. 2004; Izzo et al. 2011). It is of enormous importance to understand the dynamics between different factors to control calf scours in a herd efficient. It is also a serious welfare problem and a major cause of economic loss to cattle producers due to poor growth, mortality and treatment costs (Scott et al. 2004; Luginbühl et al. 2005; Cho and Yoon 2014).

Many studies worldwide identified the major enteric pathogens associated with neonatal diarrhea in dairy calves (Bartels et al. 2010; Izzo et al. 2011). Nowadays, the four major enteropathogens associated with neonatal calf scours are *Cryptosporidium parvum*, bovine rotavirus, bovine coronavirus (BCoV) and enterotoxigenic *Escherichia coli* (E coli F5) (Uhde et al. 2008; Blanchard 2012; Içen et al. 2013).

As the clinical signs induced by the different microorganisms are similar and do not allow a safe conclusion on the etiology, laboratory diagnosis cannot be waived (Luginbühl et al. 2005; Uhde et al. 2008).

Accurate and rapid identification of the infectious agent(s) is essential for an appropriate intervention and planning preventive measures, such as vaccination or identification of sources of infection in the herd (Uhde et al. 2008; Izzo et al. 2011; Içen et al. 2013). It should also be borne in mind serious control of the non-infectious factors, like assurance of colostrum intake, hygiene, reduction of population density, or modified components of the calving system, as is part of the overall program (Mukhtar et al. 2016).

III - Literature review

1. Characterization of the Lower Bavaria region

Lower Bavaria, or Niederbayern in German, is one of the seven regions of Bavaria state in Germany. Located in the east side of the state, and limited to the northeast by the Czech Republic, and to the south-east by Austria, the name Lower Bavaria results from the relative position of the Danube. With a total area of 10,329.91 km² and 1.2 million inhabitants (Dia-Niederbayern 2017), the administrative seat of the district is Landshut. This part of Bavaria includes great national parks, nature reserves, bird sanctuaries, lots of geotopes, involving the largest protection area of the Bavarian Forest landscape, a well-known tourist destination. The agriculture plays an important role at the Lower Bavaria economy as well as the automotive industry.

2. Dairy farming in Bavaria and the Vet's role

With more than 4.1 million dairy cows, Germany is at the top of the milk production in the European Union (EU), and the fifth globally. Dairy cattle are the backbone of livestock farms in Bavaria and an important source of livelihood for Bavarian agriculture. Almost half of Germany's milk producers are in Bavaria state (28 988 dairy farmers) (VMB 2019), supplying close to 25% of the total German milk production with the 1.2 million dairy cows. At 2018, Lower Bavaria region had 4,044 dairy farmers registered with a total of 143,641 dairy cows (VMB 2019). The dairy farms in Bavaria are traditionally small to medium-sized, rural family businesses, with around 39 cows per farm (VMB 2019). Yet, there is also some variety of stock sizes, due to modern and industrialized large-scale farms and cooperatives.

There are different forms of facilities and husbandry of animals in Bavaria dairy farms. The tie-stall, a stable for livestock, in which the animals are tethered or tie-up in one place all year round. Common in this region, these stables meet the legal requirements and there is a close bond between owners and cows, and a responsible handling of the animals. However, the number of farms with this kind of stall tend to decline over time. As an alternative, many farmers switch from tethering to playpen keeping. This allows a more appropriate social behavior, increases animal comfort and is more efficient for larger herds. There are different forms of pens, where the animals can move freely in the barn. Furthermore, it has labor benefits and it is expected to increase milk yield (Thünen 2019).

Currently, more than 70% of the Bavarian cows are kept in stables (VMB 2019), but some farmers combine the confined stock with grazing according to the seasons.

The most frequent breeds of dairy cows in Bavaria are Simmental (Fleckvieh), followed by Brown Swiss, Holstein Friesian and other breeds like German Gelbvieh, Murnau-

-Werdenfels and Pinzgauer (Figure 1) (VMB 2019). Their tolerance to climatic conditions makes these breeds well adapted to mountain regions and border areas. A Bavarian dairy cow produces on average 6,965 kg of milk per year (VMB 2019). Since milking is the most time-consuming job in dairy farms, they use the automatic milking system. The animals in surplus needed for the dairy herd replacement are fattened. Although the beef industry is strong in German with the dairy farming intertwined, a suckler cow husbandry plays a comparatively minor role (BMEL 2019).

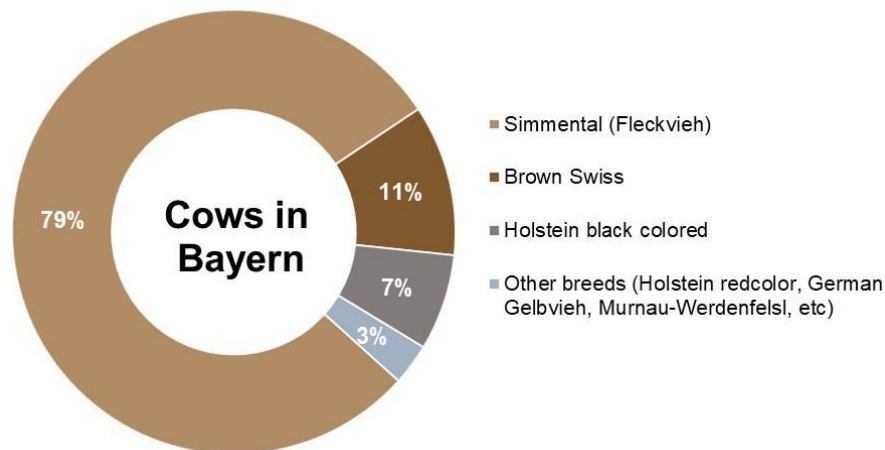


Figure 1. Breeds of dairy cattle in Bavaria (Verband der Milchzeuger Bayern 2019).

To meet the demands of consumers, the competitiveness of milk production is therefore of great importance in Bavaria. Beyond regular milk production, where the dairy cattle have a conventional handling, there is an increasing production of organic milk by bio farms, over the last few years (EU Agricultural Markets Briefs 2019). The Bavarian milk is used for direct consumption (830,833 t), yogurts products (222.286 t), different sorts of cheese, including processed cheese (969,740 t), butter (93.932 t) as well other milk and cream products (108.210 t) (LfL 2011).

Because the feeding of dairy livestock is extremely important to ensure that the animals are well-performing, it is used a feed mix of forage and concentrated feed. Some farmers even have a mechanization of feed extraction and presentation to meet the nutritional needs of the cows individually (LfL 2011). Forage base is both, high-water feeds such as green fodder, as well as dry roughage like hay and straw. The basic fodder is usually own production and the supplemented protein and mineral feed (additives) purchased (VMB 2019). Organic dairy cows are fed with organic food, have more stricter guidelines access to outdoor grazing, and their milk production while taking antibiotics, is kept out of the milk supply for a longer period (beyond the regulated withdrawal period).

The general farm medicine focuses on the basic medical care of livestock herds. From birth to lactation and/or meat production, the veterinarian is responsible to establish a balance between animal health and the economic success of the farming production. The veterinary practice approaches individual therapy plans to herd diseases prevention and control plans. When needed, the vet should also help in herd management, evaluate the (potential) diseases caused by housing conditions and give nutrition consulting. In addition, the veterinarian is often recruited by the district institution to perform official public health inspections to the farms.

The social importance of small dairy farms for landscape management and for the management of grassland is huge. It is precisely these farms that cultivate and conserve the grassland areas, pinewoods and other ecologically valuable points and thus ensure the protection of the soils as well as the preservation of biodiversity. Sadly, over the past few years, the number of Bavaria dairy farmers halved (VMB 2019), due to difficulties in rearing cattle, namely increasing land prices and deficit of qualified workers.

3. Major infectious etiologies of diarrhea

3.1. Rotavirus

Bovine rotavirus belongs to the genus Rotavirus within the family Reoviridae. It is a non-enveloped virion, with three concentric protein layers that surround the viral genome of 11 double-stranded RNA segments, giving a wheel-like appearance to the virion (Scott et al. 2004; Greenberg and Estes 2009). Rotaviruses are assigned to seven serogroups (A through G) based on antigenic and genetics similarities of the intermediate capsid protein (VP6) (Papp et al. 2013; Rocha et al. 2017). Most bovine rotavirus belong to group A, the most prevalent group, although groups B and C rotaviruses have also been identified in field cases, but outbreaks remain unclear (Tsunemitsu et al. 1992; Chinsangaram et al. 1995; Ghosh et al. 2007; Papp et al. 2013). Group A rotaviruses encode 6 structural (VP1–VP4, VP6, and VP7) and 6 nonstructural (NSP1–NSP5/6) proteins (Greenberg and Estes 2009). Rotavirus Group A (RVA) strains are antigenically heterogeneous and are classified in multiple G and P types defined by the VP7 (glycoprotein or G-type antigen) and VP4 (protease-sensitive protein or P-type antigen) proteins, respectively (Fritzen et al. 2019a). The VP7 genes encode the outer capsid protein shell of the virion whereas VP4 forms spikes that emanate over the outer capsid. Currently, 36G and 51P genotypes are known, being G6, G8 and G10 together with P[1], P[5], and P[11] genotypes, respectively, the most prevalent in cattle (Rocha et al. 2017; Fritzen et al. 2019b).

While VP4, VP6 and VP7 play a major role in maintaining viral structure, virus attachment and antigenicity, nonstructural glycoprotein 4 (NSP4) holds a special role as a viral enterotoxin, which will be discussed with more details below (Cho and Yoon 2014).

3.1.1. Epidemiology

Rotavirus was one of the first identified viral causes of diarrhea in calves, initially known as neonatal calf diarrhea virus. Bovine rotavirus is ubiquitous and susceptible calves became infected after ingesting a few virions from fecal contamination of the environment by diarrheic calves or healthy carriers (Pastoret et al. 1984). The virus typically affects calves less than 3 weeks old with a peak incidence at 6 days of age and an incubation period within 12 hours to 24 hours (Foster and Smith 2009). Affected calves develop watery diarrhea, with colors that varies from pale yellow to green, with a clinical cure of uncomplicated cases in 2 days. In more severe cases bovine rotavirus may be fatal (mortality rate around 50%) (Kumaresan et al. 2012). Unlike many enteropathogenic bacteria, rotaviruses subsist in temperate and tropical climates. In temperate climates, like Europe, seasonal peaks of the infection can vary broadly from autumn to spring, but most common is in the cooler, dryer months, if the temperature does not get near freezing (Cook et al. 1990). Furthermore, the management, milk uptake and immune status are also considered as risk factors for increased incidence of rotaviruses. Finding of rotaviruses in both diarrheic and healthy calves suggests that infections could be symptomatic or asymptomatic (Freitas et al. 2011). An eventual concomitant infection either other enteric organisms, might influence the course of the disease and should not be excluded (Pastoret et al. 1984; Chinsangaram et al. 1995).

Rotaviruses are highly distributed in cattle as well as in most other mammals (like horses, pigs and dogs) and has been identified as a significant pathogen of children (Martella et al. 2010; Otto et al. 2015). Moreover, rotaviruses are considered zoonotic agents. Human RVA strains have a high degree of genetic homology with animal strains and direct animal-to-human transmission can occur, particularly in low-income countries (Martella et al. 2010; Crawford et al. 2017).

3.1.2. Replication

Rotavirus infect and replicate in the mature, non-dividing enterocytes, located on the surface of the villi (Ramig 2004). The rotavirus attachment (by VP4 though VP7) and entry into cells constitute a multistep process. Binding is mediated by sequential interaction with a series of sialoglycans (such as gangliosides GM1 and GD1a) and histo-blood group antigens (HBGAs) on the host cells (Crawford et al. 2017). The virus enters by an unknown mechanism, with the outer capsid component's loss, activating the virion-associated

transcriptase (Lundgren and Svensson 2001; ICTV 2011; Arias et al. 2014). Viral proteins and mRNA are extruded from the icosahedral apices of the virion and concentrate in cytoplasmic inclusions called viroplasms or virus inclusion bodies (VIBs) (Ramig 2004; ICTV 2011). Rotavirus mRNAs serve as templates for both protein synthesis and genome replication (Arnold et al. 2013). It is in these inclusions that viral RNAs are packaged, replicated and double-layered particles are assembled (Trask et al. 2012). Progeny double-layered particles mature into triple-layered virions by budding into the endoplasmic reticulum (Arnold et al. 2013). Intracellular events, probably involving NSP4, causes an intracellular increase of calcium (Ca_2^+) concentration that regulate rotavirus replication and triggers several cellular processes, including disruption of the microvillar cytoskeletal network and consequently necrosis (Greenberg and Estes 2009). NSP4 appears also to be released specifically by a Ca_2^+ -dependent, non-classical secretion pathway prior to cell lysis (Ramig 2004). Newly produced rotaviruses are released from the cells through that cell lysis or by a Golgi-independent vesicular transport (exocytosis) (Trask et al. 2012; Crawford et al. 2017). In short, these events promoted by rotavirus infection lead to a malabsorptive diarrhea through destruction of mature enterocytes in the villi, secretion of a viral enterotoxin as NSP4 and activation of the enteric nervous system by vasoactive components from the damaged cells (Cho and Yoon 2014).

3.1.3. Pathophysiology

The severity and location of rotavirus intestinal infection vary among animal species, but usually causes villus atrophy and affects the caudal part of the small intestine (Ramig 2004; Cho and Yoon 2014). Rotavirus-induced diarrhea has two proposed mechanisms (Crawford et al. 2017). An osmotic diarrhea due to malabsorption, secondary to mature enterocytes loss and their replacement with immature enterocytes containing less lactase, which reduces the ability to absorb the glucose and galactose produced from the digestion of lactose (Scoot et al. 2004; Foster and Smith 2009). Thus, lactose accumulates in the large intestine and due to its hypertonicity, decreases the absorption of water from the intestinal tract (Scott et al. 2004). Furthermore, crypt fluid secretion increases the amount of fluid in the lumen, contributing to diarrhea (Ramig 2004). However, the severity of clinical signs does not correlate with histologic damage sometimes, what leads to speculate that may be another mechanism (Foster and Smith 2009). Ball et al. (1996) demonstrated for the first time that NSP4 rotavirus enterotoxin could induce a dose- and age-dependent diarrhea clinically similar to rotavirus itself (Ball et al. 1996). Initially produced during intracellular viral replication, NSP4 causes several changes in the movement of nutrients and water across the epithelium. It decreases, with an indirect pathway, the ability to digest carbohydrates and

directly inhibits sodium glucose cotransporter SGLT1 that is critical for effective water absorption, contributing to the pathogenesis of rotaviral diarrhea (Foster and Smith 2009).

The other proposed mechanism is a secretory diarrhea due to the NSP4 enterotoxin. NSP4, secreted from cells infected with rotavirus, binds to intestinal epithelial cells and signals through phospholipase C (PLC) to increase cytoplasmic calcium levels, which activates calcium-dependent chloride channels (Figure 2) (Greenberg and Estes 2009; Crawford et al. 2017). Activation of these channels causes excessive secretion of chloride ions (Cl^-) into the intestinal lumen, creating an osmotic gradient that facilitates the transport of water into the lumen, leading to secretory diarrhea (Green and Estes 2009; Crawford et al. 2017). Yet, the importance of this finding is being increasingly questioned. The ENS appears to play a role in rotavirus-induced secretion, but the mechanism responsible for this effect is uncertain (Lundgren and Svensson 2001).

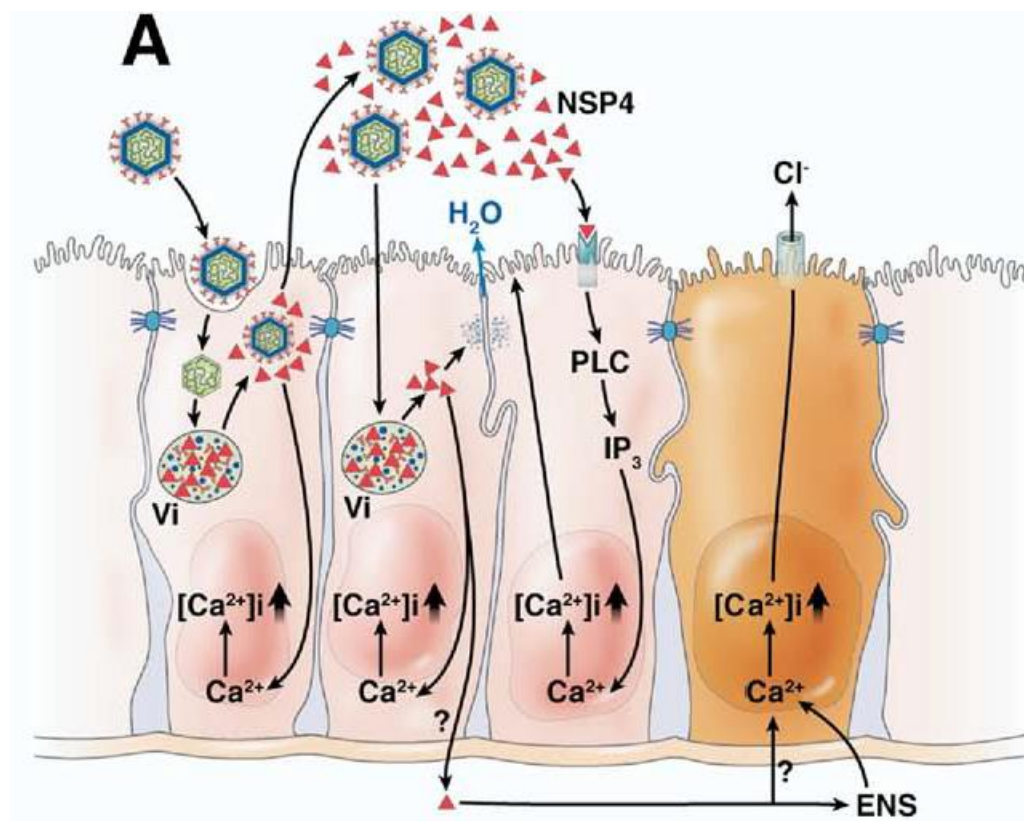


Figure 2. Mechanism by which rotaviruses cause secretory diarrhea due to the NSP4 enterotoxin after rotavirus infection of enterocytes. (adapted from Greenberg and Estes 2009, p. 1942).

3.2. Coronavirus

Bovine coronavirus (BCoV) is a member of the genus Coronavirus, within Coronaviridae family. It is an enveloped virus with a positive-sense, single-stranded RNA genome, with a pleomorphic spherical shape, covered by a corona of club-shaped surface projections (peplomers) (Decaro et al. 2008). Currently, animal coronaviruses are organized into three antigenic groups, according to its genetic and serological properties (Gomez and Weese 2017). BCoV, which belongs to Group 2, encodes five major structural proteins within the genomic RNA: spike (S) glycoprotein, membrane (M) protein, nucleocapsid (N) protein, hemagglutinin-esterase (HE) protein, absent in other groups, and small membrane protein. (Yoo et al. 1991; Liu et al. 2006).

3.2.1. Epidemiology

BCoV can commonly be found in both healthy and diarrheic calves, complicating the assessment of its role as a primary pathogen (Bartels et al., 2010). Higher prevalence rates of BCoV in diarrheic calves have been reported recently compared to those identified two to three decades ago (Lojkić et al. 2015). Further, new strains of BCoV have been described worldwide in the last decade (Gomez and Weese 2017). BCoV infection has a high morbidity but usually causes low mortality (mortality rate varies from 1 to 25%) with outbreaks typically in autumn and winter seasons (Kumaresan et al. 2012; Lojkić et al. 2015). Calves usually become infected with three weeks of age, and peak incidence happens between days 7 and 10, although disease may occur in calves up to three months of age (Foster and Smith 2009). Transmission is by the fecal–oral route and the incubation period is 20 to 30 hours and continues for 3 to 6 days (Clark 1993). Infected calves have a profuse diarrhea for several days that rapidly develops to dehydration and acidosis (Scoot et al., 2004; Kumaresan et al. 2012). The feces color varies from yellow to white, containing milk and blood clots in severe cases (Kumaresan et al. 2012; Gomez and Weese 2017).

Recently, BCoV have also been identified in wild ruminants, including several species of deer, waterbuck antelope, alpaca and giraffe (Decaro et al. 2008). Even more, the same virus strain could be responsible for simultaneous appearance of enteric and respiratory disease in the same calf, as well as been associated with winter dysentery and respiratory tract illness in cows (Liu et al. 2006; Decaro et al. 2008; Gomez and Weese 2017).

3.2.2. Replication

It seems likely that BCoV attaches to the enterocyte via spike (S) and hemagglutinin-esterase (HE) glycoproteins by an uncertain mechanism, which allow fusion of the viral envelope with the cell membrane or by endocytotic vesicles (Schultze et al. 1991; Yoo et al. 1991). Once inside the cell virus replication occurs entirely within the cell cytoplasm. The genomic RNA first attaches to ribosomes and directs the synthesis of RNA dependent RNA polymerase (Clark 1993). This enzyme directs transcription of a complementary negative (-) length RNA strand from the virion genomic RNA strand. The (-) RNA strand serves as a template for synthesis of full-length viral genomic RNA (Clark 1993). Viral RNA synthesis produces both genomic and sub-genomic RNAs. Sub-genomic RNAs serve as mRNAs for the structural and accessory genes (Fehr and Perlman 2015). Translation of the mRNAs coding for the non-structural and nucleocapsid (N) proteins occurs in the cell cytoplasm, whereas the membrane (M), spike (S) and hemagglutinin (HE) glycoproteins are synthesized on ribosomes at the rough endoplasmic reticulum (RER) (Fehr and Perlman 2015). The M glycoprotein is only glycosylated once it reaches the Golgi apparatus, whereas the S and HE glycoproteins are glycosylated at the RER during protein synthesis and subsequently modified during transport through the Golgi complex (de Haan and Rottier 2005). BCoV particles are assembled in the cytoplasm by a budding process through the RER (Scott et al. 2004). The N protein interacts with newly synthesized genomic RNA to form fragile nucleocapsids, which align on the cytoplasmic surface of the membranes of the RER and Golgi due to an interaction with the M glycoprotein (de Haan and Rottier 2005; Fehr and Perlman 2015). In these membranes the host cell proteins are replaced by viral glycoproteins and released from intact cells, using the normal cell secretory mechanisms, despite few particles are released by lysis (Clark 1993; Scott et al. 2004).

3.2.3. Pathophysiology

Diarrhea secondary to coronavirus is mainly caused by intestinal epithelial loss and malabsorption (Foster and Smith 2009). Viral infection starts in the proximal small intestine and usually spreads throughout the entire small intestine and colon (Clark 1993; Cho and Yoon 2014). Mature enterocytes located on the surface of villi are the primary target of BCoV, although crypt enterocytes are also affected, extending the duration of the clinical signs. Virus damage of the infected villi from small intestine and colonic crypt cells, results in replacement by immature enterocytes cells and atrophy of the colonic ridges (Clark 1993; Scott et al. 2004). Consequently, the decrease in digestive and absorptive capacities, with

severe lesions in areas of the intestine that absorb water (ileum, caecum and colon), may account for the waterier nature of the diarrhea seen in BCV infections (Scott et al. 2004).

3.3. *Escherichia coli*

The Gram-negative *Escherichia coli* (*E. coli*) bacteria is a facultative anaerobic, rod-shaped, coliform bacillus of the genus *Escherichia* within the *Enterobacteriaceae* family. It is a commensal inhabitant of the large intestine in mammals also found in the environment (Mukhtar et al. 2016). There have been many different serotypes of *E. coli* identified according to its antigen's variety. On the surface, it has a capsule (antigen K), composed by carbohydrates, a flagellum (antigen H) used for mobility, containing protein subunits called flagellins, and fimbriae or pili (antigen F), with three functional classes of specialized adhesins that allow cellular juxtaposition and the sharing of genetic material by conjugation (Gay 1965; Hacker 1992; Orskov et al. 1977). The flagellar antigen (H) is based on the different types of flagellins. Under the capsule, the cell wall is composed of three layers: an outer membrane, where somatic antigens (O) are located on lipopolysaccharides (LPS), and an inner membrane separated by peptidoglycans periplasm (Orskov et al. 1977). The O antigen is the O-specific polysaccharide of the cell wall lipopolysaccharide (LPS). Nevertheless, there are several *E. coli* clones that acquired specific virulence factors allowing them to cause three general clinical syndromes: enteric/diarrhea disease, urinary tract infections (UTIs) and sepsis/meningitis (Nataro and Kaper 1998). Among the intestinal *E. coli* pathogens there are six categories based on virulence scheme (Kaper et al. 2004): enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC).

Between these pathogroups, the most common cause of neonatal diarrhea is ETEC strains through adhesin antigens production, like k88 and K99 (also referred to as F5), and the action of heat-labile (LT) and heat-stable enterotoxins (ST) (Hadad and Gyles 1982; Nataro and Kaper 1998; Foster and Smith 2009; Kolenda et al. 2015).

3.3.1. Epidemiology

Cattle are primary reservoirs of human pathogenic *E. coli*. They can carry *E. coli* asymptomatically and shed it in their feces, causing diarrhea in childhood, immunosuppressed and travelers into developing world (Kaper et al. 2004; Cobbold et al.

2007; Muktar et al. 2016). Calves become exposed to pathogenic *E. coli* in the environment when other infected or carrier calves and cows shed the bacteria in the feces.

According to Bashahun and Amina (2017), there is a lack of epidemiological studies available from field outbreaks of colibacillosis to provide convincing evidence of the pathogenicity of certain strains of *E. coli* for calves. Yet, some studies say, neonatal calves are most susceptible to ETEC infection in the first 4 days after birth (typically less than 48 hours of age), developing watery diarrhea, which can range from mild, self-limiting to severe purging disease (Kaper et al. 2004; Foster and Smith 2009). Outbreaks have been associated with poor housing and hygienic measures as well a bad management at calving (Scott et al. 2004). In endemic areas, ETEC infections tend to be clustered in warm, wet months, when multiplication of the bacteria in food and water is more efficient (Nataro and Kaper 1998). ETEC organisms are ingested by calves and cause watery diarrhea by the presence of specific adhesins and toxins (Foster and Smith 2009). Equally, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are isolated from diarrheic and healthy calves, but their role in calf disease remains controversial (Kolenda et al. 2015; Muktar et al. 2016). It is also possible for calves to become infected via nasopharyngeal mucosa through inhalation, which can lead to meningitis (Kolenda et al. 2015). Variable prevalence of *E. coli* in calves has been reported by different studies. For instance, Bartels et al. (2010) in Dutch (2.6%), Izzo et al. (2011) in Australia (17.4%), El-Seedy et al. (2016) in Egypt (75.6%), Bendali et al. (1999) in south France (20.3%) and 63.6% by Osman et al. (2012). Because nonpathogenic *E. coli* are extremely common, the agent's isolation from each diarrheic sample is of little value, unless the presence of the virulence factors can be demonstrated by serological typing or when diagnosis other infectious agents of calf diarrhea by testing for rotavirus and coronavirus, *Salmonella spp.* and *Cryptosporidium spp.* (Foster and Smith 2009, Kolenda et al. 2015).

3.3.2. Pathophysiology

Following ingestion, ETEC strains from calves attaches the intestinal epithelium mediated by adhesin antigens, which is mainly the F5 (K99) and F41 fimbriae and multiplies in enterocytes of the intestinal villi (Kolenda et al. 2015; Foster and Smith 2009). The distal portion of the small intestine offers the most favorable environment for ETEC colonization due to the low pH (less than 6.5) (Cho & Yoon, 2014). After colonization, calf ETEC initiate their pathogenic actions by secretion of the heat-stable toxin (ST). On the other hand, the heat-labile enterotoxin (LT) is rarely produced by calf ETEC strains (Scott et al. 2004). STs are small, single-peptide toxins that include two unrelated classes (STa and STb), but only

Sta occurs in calf's diarrhea (Acres 1985; Kaper et al. 2004). The subunit A binds the brush border membrane enzyme, guanylyl cyclase-C (GCC) present throughout the villi and crypts, within enterocytes (Al-Majali et al. 2000). Binding of STa to GCC produce increased levels of intracellular cyclic guanylyl monophosphate (cGMP). In turn, cyclic GMP activates cGMP-dependent protein kinase II (cGKII), which leads to the up-regulation of chloride secretion into the gut (Scott et al. 2004, Foster and Smith 2009). This up-regulation pulls water (by osmosis) and several ions like sodium and potassium into the intestinal lumen, inhibiting the villous enterocytes ability absorption and leading to diarrhea. Alternative mechanisms involving prostaglandins, calcium and the enteric nervous system (ENS) have been proposed, but the evidence for the involvement of these factors is inconsistent (Nataro and Kaper 1998, Foster and Smith 2009).

At last, the pathophysiology of ETEC is dependent on several factors, leading to diarrhea culminating in dehydration, metabolic acidosis, and death in severe cases.

3.4. *Cryptosporidium parvum*

Cryptosporidium has been an enigma since it was first described by Edward Tyzzer in 1907 in the gastric glands of a mouse. Following the next seventy years from Tyzzer's description (1907), *Cryptosporidium* continued to be viewed as a curiosity. According to Plutzer and Karanis (2009), *Cryptosporidium* can be more accurately described as follows: *Cryptosporidium* spp. belongs to the phylum Apicomplexa (*Sporozoa*), whose members possess an apical complex; class *Sporozoa*, whose members reproduce by asexual and sexual cycles; subclass Coccidia, the life cycle of which involves merogony, gametogony, and sporogony; order *Eucoccidiida*, in which schizogony occurs; suborder Eimeriina, in which independent micro- and macrogamy develop; and family *Cryptosporidiidae*, whose members have four naked sporozoites within their oocysts. The most recent review listed 30 species of the ubiquitous protozoan parasite *Cryptosporidium* that infect a wide range of hosts including wild and domestic mammals, amphibians, fish and reptiles (Fayer et al. 2004; Thompson et al. 2016). *Cryptosporidium parvum*, *C. bovis*, *C. ryanae* and *C. andersoni*. have all been reported in calves (Santín et al. 2004; Blanchard 2012). *Cryptosporidium parvum* is an enteric coccidia commonly isolated from neonatal calf diarrhea and a potential zoonotic agent (Foster and Smith 2009; Chalmers et al. 2010).

3.4.1. Epidemiology

Several studies found that *Cryptosporidium* was the most common or second-most common pathogen in the feces of diarrheic calves and a noteworthy cause of diarrheal

disease in humans throughout the world (Blanchard 2012; Thompson et al. 2016). In a study, *C. parvum* constituted 85% of the *Cryptosporidium* infections in pre-weaned calves but declined in older cattle (Santín et al. 2004; Fayer et al. 2007). However, there are several factors that affect the prevalence of cryptosporidiosis including age, bedding type, hygiene, colostrum feeding, management practices and climate (Ogendo et al. 2017). Infection starts commonly via fecal-oral route, through contact with infective stages of the sporulated oocysts present in contaminated environment by feces of infected animals (Ayele et al. 2018). Cryptosporidiosis is seen in neonatal calves, usually when they are aged 1 to 2 weeks with the peak at 11 days old. Most calves become infected, but not all develop diarrhea (Scott et al. 2004). Diarrhea caused by *C. parvum* rarely occurs after 3 months of age (Fayer et al. 1998; de Graaf et al. 1999; Santín et al. 2004). After infection, diarrhea may be intermittent and last from 4 to 17 days with peak at 3 to 5 days (Fayer et al. 1998). Depression and anorexia follow the profuse yellow to brown diarrhea, which contains mucus and occasionally blood streaks (Kumaresan et al. 2012). Morbidity is usually high and mortality low, although some outbreaks have been associated with high mortality, especially in cases of acute and chronic diarrhea in young and weak animals (Scott et al. 2004; Kumaresan et al. 2012; Ayele et al. 2018). Studies show a close association between diarrhea occurrence and oocysts excretion of *C. parvum* (Fayer et al. 1998; Santín et al. 2004). *C. parvum* oocyst are shed in large numbers, 10⁷ oocyst per gram of feces, as early as 3 days of age, peaks at 2 weeks of age and can continue to occur in adult cattle (Fayer et al. 1998). These oocysts are already fully sporulated when excreted in feces and are therefore immediately infectious for both animals and humans (de Graaf et al. 1999). The oocysts can survive for more than a month in the environment under favorable conditions (e.g., high temperature and moisture with low UV radiation) and are resistant to most disinfectants (Tzipori and Ward 2002; O'Handley and Olson 2006). Calves appear to be resistant to subsequent infections after the initial episode of *C. parvum* diarrhea, but the severity and incidence of clinical signs in calves shedding oocysts can be variable depending also of the mixed infections with other pathogens that may occur (Kumerasan et al. 2012).

3.4.2. Life Cycle

Cryptosporidium species are monoxenous, completing their entire life cycle within a single host. Once *C. parvum* is ingested, the cycle starts with exposure to gastric acid and bile salts, leading to the oocyst excystation and release of four sporozoites (Figure 3) (Foster and Smith 2009). The sporozoites invade mostly the ileum, but they can infect from the abomasum to the colon, penetrating the plasma membrane of the enterocytes (parasitophorous vacuole), developing into trophozoites (Thompson et al. 2005). At this part,

a trophozoite involve both asexual (type I meront) and sexual (type II meront) developmental stages (Thompson et al. 2005). Trophozoites develop to type I meronts, containing eight type I merozoites, which are released to become trophozoites themselves and infect new enterocytes to form a second generation of merozoites (Scott et al. 2004). Otherwise, in the sexual phase, type I meronts develop into type II meronts, which release type II merozoites to produce macrogamonts and microgamonts (Scott et al. 2004; O'Handley and Olson 2006). The latter produces microgametes, which fertilize macrogamonts and give rise to zygotes and from oocysts (Scott et al. 2004). Sporulation occurs within the host, releasing thick walled oocysts into the environment and thin walled oocysts, which auto-infect the same host organism (Cho and Yoon 2014; Bones et al. 2019).

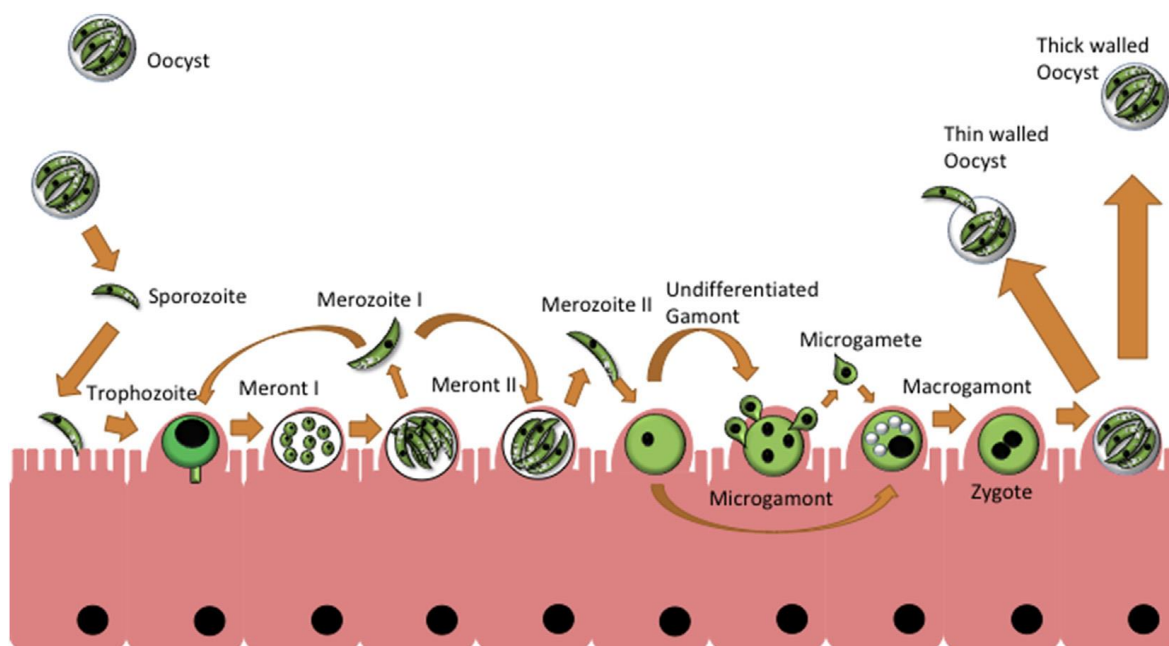


Figure 3. Outline of the *Cryptosporidium parvum* life cycle. (adapted from Bones et al. 2019, p. 29).

3.4.3. Pathophysiology

As said above, *Cryptosporidium* infections are mainly concentrated in the distal small intestine, but it is also found in the caecum and colon. Despite *Cryptosporidium* appears to cause diarrhea by destroying or accelerating the loss of epithelial cells, the pathogenic mechanism(s) is (are) still unknown (Tzipori and Ward 2002). The invasion of *C. parvum* into enterocytes induces changes in intestinal cytoskeleton structures, leading to villous atrophy in calves (Heine et al. 1984). Furthermore, crypt hyperplasia also occurs to replace the lost epithelial cells, however, disruption of the epithelial barrier can occur despite these efforts (Gookin et al. 2002). The population of mature enterocytes is reduced in size and numbers of immature cells are increased, which can be explained by two potential mechanisms. The first

is a direct cytotoxic effect of the organism on the intestinal epithelium, but this is not well supported by the current literature (Gookin et al. 2002; Foster and Smith 2009). The second mechanism for cell loss is apoptosis, because apoptotic cells are consistently found *in vitro* and *in vivo* model's infections. Although apoptosis can lead to death of the infected enterocyte, there is evidence that *Cryptosporidium* subverts this host attempt to eliminate infection (Gookin et al. 2002). With this, additional research is needed to elucidate: the organism, too maintain its intracellular habitat, or the host, to limit spread of infection (Foster and Smith 2009). Nonetheless, the combined loss of microvillus border and villus atrophy in addition to loss of membrane-bound digestive enzymes, contributes to fermentation of undigested milk in the intestinal lumen, promoting a malabsorptive diarrhea (Tzipori and Ward 2002; Nydam and Mohammed 2005). Some studies have also documented a prostaglandin-mediated anion secretion (Cl^- or HCO_3^-) and inhibition on neutral NaCl absorption to account for all the fluid loss seen in diarrhea caused by *C. parvum* (Foster and Smith 2009). Despite unclear, macrophages appear to be the most likely source of prostaglandins secretion from mesenchymal cells in infected tissue, leading to an increase in intracellular calcium and cAMP, which activates anion secretion and decreases sodium absorption (Gookin et al. 2002). This malabsorption and prostaglandin-mediated diarrhea ranges from mild to life-threatening, depending on parasitic doses and co-infections with other pathogens (Foster and Smith 2009).

4. Diagnosis of neonatal calf diarrhea

Various pathogens have been implicated in the development and rapid progression of NCD. Having said that, an etiological diagnosis should be performed as quickly as possible. Laboratory testing is necessary for an accurate diagnosis not only to confirm the cause at the individual level, but also to help clinicians and producers to implement appropriate interventions at herd level in a timely manner (Millemann 2009; Içen et al. 2013). Many factors such as sample size and sampling time, types and quality of the specimens as well as laboratory methods used can influence the diagnostic outcomes.

The diagnostic procedure starts by collecting clinical data such as age, vaccination record, clinical signs and farms history. For diagnostic testing, stool specimens are picked from acutely diarrheic animals prior to therapy with optional blood samples. Fresh or formalin-fixed gastrointestinal tissues (including the regional lymph nodes and liver) collected along with colonic contents from freshly corpses or euthanized calves are of great value for diagnosis during outbreaks (Reck 2009; Cho and Yoon 2014). Based on the type of specimen collected and the clinical history, the sample is subjected to a laboratory diagnosis. As many enteric pathogens are difficult to isolate from gastrointestinal environment, there are

numerous techniques when testing samples. Virus isolation is considered the gold standard for detecting viral pathogens in specimens, however, bovine rotavirus and coronavirus, are difficult to isolate or propagate in cell cultures (Duckmanton et al. 1998; Cho and Yoon 2014). Direct visualization with electron microscopy as well as new methods such as Antigen-capturing enzyme-linked immunosorbent assay (Ag-ELISA), Latex agglutination test (LAT) and Polymerase Chain Reaction (PCR) techniques have been widely used as excellent alternatives (Cho et al. 2010).

Fecal bacteria culturing is a commonly used laboratory method to isolate and identify bacterial pathogens. MacConkey agar plates are selectively used to culture Gram-negative bacilli like *E. coli* bacteria (O'Leary et al. 2009). In some cases, further immunological testing is required for the identification of bacteria (e.g. an agglutination test for *E. coli* K99+). Similarly, the LAT has been frequently performed to identify *E. coli* K99+ in calf diarrhea cases (Cho et al. 2010). Furthermore, PCR and Real-time PCR are especially useful for detecting bacteria that require a long time to grow.

Fecal flotation and direct microscopy are commonly used to diagnose *C. parvum* oocysts and others parasite eggs, beyond once more the Ag-ELISA and the PCR methods (Balatbat et al. 1996; Ballweber 2006).

More recently, an antigen-capturing ELISA assay has been commonly used for the rapid detection of a wide range of targets from bacteria, virus and parasites in specimens from diarrheic calves. For this method, antibody is attached to a solid surface such as glass, plastic or a membrane filter. The antibody captures target antigen (if present in the sample), triggering a cascade of colorimetric reactions that indicates an antigen-antibody reaction (Goryacheva 2016). While the microtiter plate method has been employed in diagnostic laboratory settings, the membrane-bound method using a lateral flow technique, such as strip test or rapid kits, are the most common platform for in-clinic or patient side-tests (Cho and Yoon 2014). Among several commercial Ag-ELISA kits that are available to detect rotavirus, coronavirus, *E. coli* K99+ and *C. parvum* in fecal samples, we used, for the present study, the Fassisi® BoDia kit test (Luginbühl et al. 2005; Uhde et al. 2008; Cho et al. 2012). The Ag-ELISAs are well known for rapid turnaround, high-throughput testing, plug-in-and-play capability and portability. However, analytic sensitivity of this method tends to be lower than that of isolation by culture or nucleic acid-based assays (PCR) (Cho et al. 2010). In some situations, the expense of a commercial kit may be also cost-prohibitive.

5. Treatment of neonatal calf diarrhea

Diarrhea can be fatal to neonatal calves due to the dehydration and metabolic acidosis, typically characterized by electrolyte imbalances (sodium and potassium) (Trefz et

al. 2015). Dehydration promotes the clinical signs of sunken eyes and ‘tenting’ of skin folds. The ears and mouth feel cold to touch and the body temperature tend to fall. Young calves with severe diarrhea have also a decrease in absorption of nutrients that, together with the low-fat reserves, leads to anorexia, weakness and lethargy, observed with minimal glycogen reserves that contribute to the hypoglycemia. It is also important to refer that metabolic acidosis develops from bicarbonate ions loss in feces and concomitant impairment of renal function due to low perfusion of the kidney blood vessels. Moreover, the increased hydrogen ions move into cells, forcing potassium ions to be lost, leading to a hyperkalemia that boost an eventually cardiac failure (Scott et al. 2004). As clinical signs in diarrheic neonatal calves develops rapidly, a successful treatment depends upon a rapidly medical intervention.

The highest priority in treating scours is to replace the water and electrolytes loss with fluid therapy. Oral administration solutions are most appropriate for scouring calves that are still able to stand and who are alert enough. There are electrolyte powders that can be mixed with water or milk, prepared by pharmaceutical manufacturers (like Diakur® Plus, Boehringer Ingelheim), providing the correct proportions of salts relative to water and helping in the restoration of fluid balance, electrolytes loss and glucose. When calves are too weak or too lethargic with moderate to severe dehydration and acidosis, the fluid therapy is given through intravenous catheter (IV) placed in the jugular or, for larger quantities given during a longer period, in the auricular veins (Meganck et al. 2014). There are crystalloid solutions commercially available for effective IV fluid therapy, including isotonic and hypertonic saline (NaCl), isotonic and hypertonic sodium bicarbonate (NaHCO_3), acetate or lactated Ringer’s solution, and concentrated solutions of dextrose (Radostits 1975; Spence 2006; Berchtold 2009). The quantity of replacement fluid in liters is calculated by multiplying the estimated dehydration in percentage with body weight in kilograms according to the following formula:

Equation 1. Replacement fluids.

$$\text{Replacement fluid [L]} = \text{dehydration [\%]} \times \text{bodyweight [Kg]}$$

Legend: Liters (L); Percentage (%); Kilograms (Kg).

A flow rate of 30 to 40 mL/kg/h is recommended to avoid risks of overhydration and pulmonary edema, yet a maximum rate of 80mL/kg/h for IV fluid therapy has been used without inducing significant overhydration and hypertension (Berchtold 2009). The IV volume to be given depends on the calf’s size and the severity of the dehydration (Figure 4).

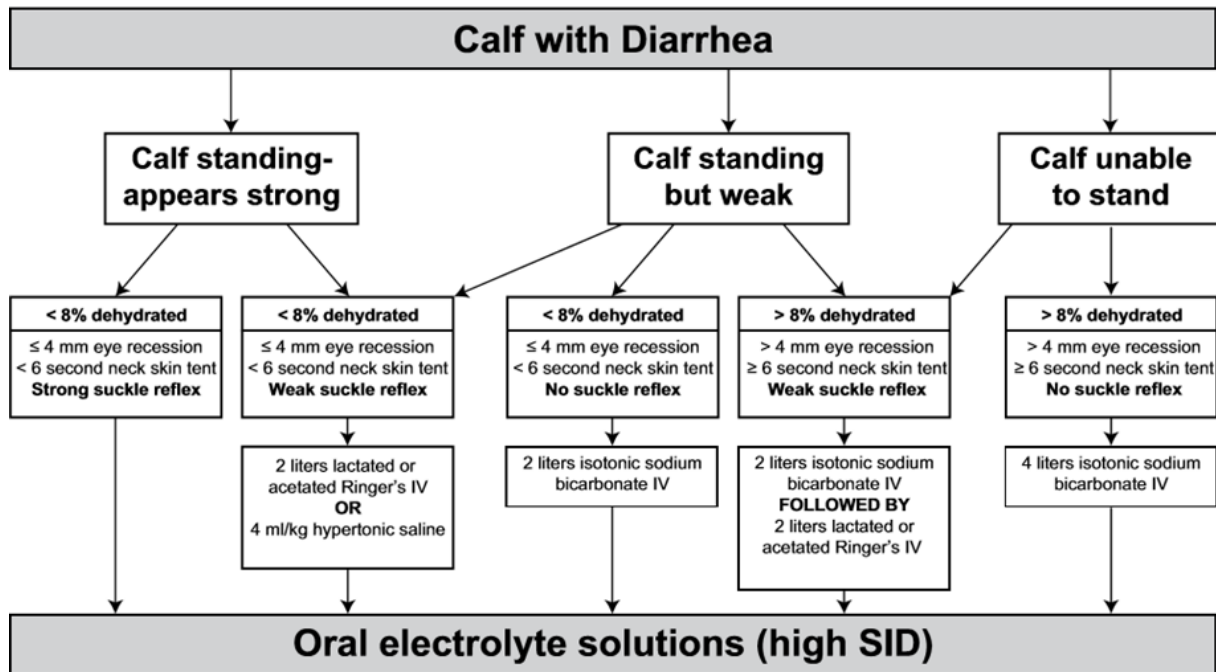


Figure 4. Calf's symptoms, proportion of dehydration and initial doses of fluid therapy (adapted from Berchtold 2009, p. 85).

In case of severe acidosis, rapid IV administration of an 8.4% bicarbonate solution at 5-10 ml/kg over 20 minutes, provides an effective and safe method to improve acid-base abnormalities (Koch and Kaske 2008; Coskun et al. 2010). Ideally, measurements of buffer needs are based on formulas for extracellular base excess (from blood gas analysis) or plasma total carbon dioxide concentration. Values calculated from blood gas analysis multiply base deficit with body weight and with a factor that considers the volume of distribution for bicarbonate ions in the body (0.5–0.6) according to the following equation (Berchtold 2009):

Equation 2. Acidosis correction.

$$\text{Bicarbonate requirement [mEq]} = \text{body weight [Kg]} \times \text{base deficit [mEq/L]} \times 0.5 - 0.6 \text{ [L/Kg]}$$

Legend: milliequivalent (mEq); Liters (L); Kilograms (Kg).

The fluids should be warmed so the calf does not need to spend extra energy to bring the given fluid to body temperature. When a calf's suckle reflex is re-established, further maintenance therapy can be given orally and should be continued as long the calf has diarrhea.

Although some consider the use of antimicrobials (AB) to be controversial and not indicated to treat diarrheic calves, they are systematically used (Sanders 1985; Kumaseran

et al. 2012; Meganck et al. 2014). Parenteral Gram-negative spectrum AB are advised to treat enteropathogenic bacteria, predominantly certain serotypes of *E. coli* that are assumed to be either the initial cause or at least contribute to the disease appearance and bacteremia. Broad spectrum antibiotics include fluoroquinolones, oxytetracycline and neomycin that are widely used in NCD treatment. More options are amoxicillin combined with clavulanic acid, potentiated sulfonamides and the third and fourth generation of cephalosporins, such as ceftiofur and cefquinome (Constable 2004; Constable 2009). Antibiotics should only be used at the proper dosage and frequency for three to five days at least. Their systematic use should be discouraged due to the increased levels of resistant bacteria populations. Ideally, the AB of choice depends on the drug sensitivity of the causative bacteria isolated from the feces of the calf (Radostits 1975). Non-steroidal anti-inflammatory drugs (NSAID) like a single injection of meloxicam (0.5mg/kg, SC) at the onset of diarrhea improves appetite and growth rate in calves, by reducing the malaise and gastrointestinal discomfort (Roussel and Brumbaugh 1991; Todd et al. 2010). For now, antiviral drugs known to treat rota- and coronavirus are not commercially available.

Halofuginone lactate is administrated orally and is so far, the only registered product in Europe to treat and prevent diarrhea caused by *C. parvum*. It decreases the duration and severity of diarrhea, as well as decreases fecal oocyst concentration and environmental contamination (Constable 2009; Meganck et al. 2014). In the United States, azithromycin is used to treat *C. parvum* infections in NDC.

If the calf is showing improvement, cow's whole milk or milk replacer is reintroduced to the calf in small amounts frequently. If it has not improved within six hours of beginning intravenous fluid therapy, it may be necessary to re-evaluate the diagnosis and/or treatment.

6. Prevention and control of neonatal calf diarrhea

Dairy farms with confirmed NCD should consult a veterinarian for a proper approach to disease control and prevention. Appropriate advice on nutrition, colostrum feeding, vaccination, hygiene and the use of antibiotics should be given.

The bovine placenta does not allow the passive transfer of antibody to the fetus. As a result, newborn calves are deficient in immunoglobulins and must ingest colostrum and absorb lactoglobulins, to obtain passive protection against environmental pathogens and to prevent increase morbidity and mortality rates due to acute NCD. The colostrum contains concentrated levels of antibodies, immune cells (neutrophils, macrophages, T cells and B cells), complements, interferon and other soluble factors along with many important nutrients (sugars and fat-soluble vitamins) (Cortese 2009; Cho and Yoon 2014). Immunoglobulin G (IgG) is the primary antibody isotype in bovine colostrum. Resistance of the calf to enteric

disease is closely related to the timely consumption of high-quality colostrum in enough quantities (Radostits 1975). These quality and quantity of colostrum is associated with body condition and vaccination of the cow (Odde 1988). Calves born from underfed cows have poor growth performance and higher susceptibility to develop disease. To obtain appropriate passive immunity from the cows, calves should uptake 3 to 4L of adequate colostrum within the first 6 h after birth (Cortese 2009; Moore et al. 2005). Absorption of immunoglobulins from colostrum is maximal only during the first 12h after calf life (Radostits 1975). Calves that do not ingest colostrum voluntarily, should be tube fed. Pasteurizing colostrum (60°C, 60 min) can reduce microbial counts while the colostrum IgG concentration remain within acceptable limits for feeding (Donahue et al. 2012). Moreover, calves fed pasteurized colostrum had a significantly decreased risk of scours (Godden et al. 2012). Colostrum replacement should be obtained from the farm of origin or from a historically disease-free facility when the mother's cow milk is insufficient (Meganck et al. 2014). Alternatively, colostrum supplements (containing specific antibodies or general IgG concentrations) in milk replacer during the first 2 weeks of life have significant value in decreasing the mortality or the severity of disease in colostrum-deprived calves (Cortese 2009; Meganck et al. 2014; Meganck et al. 2015).

Currently, there are available commercial multivalent vaccines for pregnant cows, to increase the concentration of specific antibodies in the colostrum against etiological agents such as rota-, coronavirus and *E. coli* (Uetake 2013; Durel et al. 2017). Most vaccines contain either live modified or killed organisms or a combination of both (Cortese 2009; Durel et al. 2017). Some vaccines are specific for cows while others are designed for calves. In any case, consistent vaccination program is an effective tool to prevent NCD if the other management aspects are controlled. Exposure to contaminated environment and stress factors like extreme weather conditions and moisture increases the susceptibility of young calves to develop diarrhea. Key successful principles and corrections are required to reduce environmental risk factors associated with NCD outbreaks. A controlled breeding program can be applied to adjust the calving season to a more favorable environmental condition (Meganck et al. 2014). The pens should be sanitized and packed with dry bed due to immune impairment of the newborn calves. Grouping calves by their age to keep clean (pathogen-free) the calving area after occupation by the previous calving group. Even more, calves must be separated to reduce level of exposure and contamination from feces and urine of other calves. Strict hygiene practices should be maintained in all feeding facilities and equipment (Sanders 1985; Smith 2012). Insufficient maternal pelvic or calf size can also induce dystocia, which frequently causes calf diarrhea (Sanders 1985). To prevent dystocia, the dam's genetic inheritance should be taken into consideration during heifer selection. The literature is contradictory or inconclusive regarding ancillary preventive products such as

phytopharmaceuticals (e.g. clinoptilitezeolite) and probiotics (e.g. *E. coli* Nissle 1917) (Meganck et al. 2014).

Altogether, prevention is better than treatment not only in a productivity viewpoint but also in animal welfare. Nonetheless, it is not easy in practice as in theory for farmers and cattlemen to perceive hidden problems without help from outside specialists such as veterinarians (Uetake 2013).

IV – Etiology of Neonatal Calves Diarrhea in Lower Bavaria, Germany

1. Prevention and control of neonatal calf diarrhea

The aim of this study was:

- a) to investigate the etiology of neonatal calves diarrhea in dairy farms from Lower Bavaria region in Germany;
- b) to identify the presence of rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* in neonatal calves diarrhea using rapid tests for diagnosis;
- c) to evaluate the suitability of these rapid tests for practical use in the stable and to compare the negative results with the standard laboratory methods to mislead false negatives.

2. Material and methods

2.1. Case definition, sample size and collection of fecal samples

In the present study, 18 fecal samples from diarrhea calves were collected between the 1st of October 2018 and the 31st of January 2019 from 17 different dairy herds. The time for this internship was beforehand planned in order to coincide with the most frequent periods of births and thereby of new-born calves that would require medical care. Calves, Fleckvieh breed, aged under 3 weeks old at sampling collection and belonged to cattle herds located in the Lower Bavaria region. Calves were apathic at the first visit, showing an evident dehydration condition, poor appetite with a weak or absent suckling reflex as well as with diarrhea characterized by pasty-watery feces.

Case definition: diarrhea was considered if feces were semi-liquid to liquid, with or without other abnormal characteristics such as presence of blood or mucous.

Fecal samples were collected directly by rectal stimulation of the calf with a sterile plastic glove while avoiding environmental contamination (by soil, urine, or other feces) and immediately identified. When possible, on-site analyzes were performed to apply a better treatment and prevention managements according to the result obtained. When logistically impossible, the samples were conserved in a refrigerated place until the end of the day and the analysis performed in the laboratory.

The name of each calf's owner, animal identification number, age, and data of sample collection were recorded. The clinical data included consistency of feces, clinical condition and treatment protocol for each animal. It was also investigated whether the farms had any vaccination program for calves or for adult animals, for at least one of the pathogens under study (*E. coli*, rotavirus or coronavirus) (Table 1). Follow-up consultations were made to each calf with NCD at 12h and 36h after the first visit (D0) and then daily if necessary, until prognosis was favorable. The cases were considered closed in two ways: when animal did not respond to the treatment and died, or at the end of the treatment protocol with the animal showing signs of recovery. In some cases, it was not possible to perform follow-up visits.

All negative samples from the rapid test kits were sent to the Bavarian State Office for Health and Food Safety Laboratory for confirmation by microbiological culture and ELISA (Antigen) to mislead possible false-negative cases.

2.2. Laboratory analyses

As explained all fecal samples were tested for the presence of four diarrhea pathogens (rotavirus, coronavirus, *Cryptosporidium* and *E. coli* K99) by the commercial immunochromatographic rapid test Fassisi® BoDia (Figure 5), from Fassisi GmbH in Germany, allowing for the identification of mixed infections.



Figure 5. Fassisi® BoDia test.

Following manufacturer's instructions, the test provides in ten minutes an overview of the current pathogen situation at individual or herd level, enabling the early detection of infected animals and the initiation of an effective therapy. Table 1 gathers information concerning the sensitivity (Se) and specificity (Sp) of the Fassisi® BoDia test for each pathogen.

Table 1. Sensitivity and specificity for each pathogen of the Fassisi® BoDia test.

Pathogen	Sensitivity (%)	Specificity (%)
Rotavirus	96.43	95.45
Coronavirus	99.99	99.99
<i>E. coli</i> K99	88.89	99.99
<i>Cryptosporidium</i>	99.99	95.85

Additionally, the test also gives critical information concerning the need to vaccinate adult cows or born calves.

2.2.1. Rapid immunoassay kit protocol

The protocol used was the one provided by the manufacturer instructions (Figure 6). A liquid stool sample was collected with a pipette provided by the kit (1a) and diluted 3 to 6 drops in the kit dilution solute (2a). If the sample was solid or semi-solid, it was taken with a sterile swab (2a) and diluted in the same dilution solution (2b). The sample was further homogenized by circular movements of the container. After homogenization, the spike of the cover (3) was broken and 3 to 4 drops of the diluted solution were poured into each of the four points of the cassette (4), specific to each pathogen, waiting about 10 minutes to obtain the result.

The result interpretation was based on the key provided by the manufacturer (5). The test was completed when the lines of the different pathogens showed one or two pink lines: the presence of 2 lines indicates a positive sample, as for rotavirus in Figure 5; the sample is considered negative if only 1 line in control appear. The test was only considered valid after the control line was revealed.

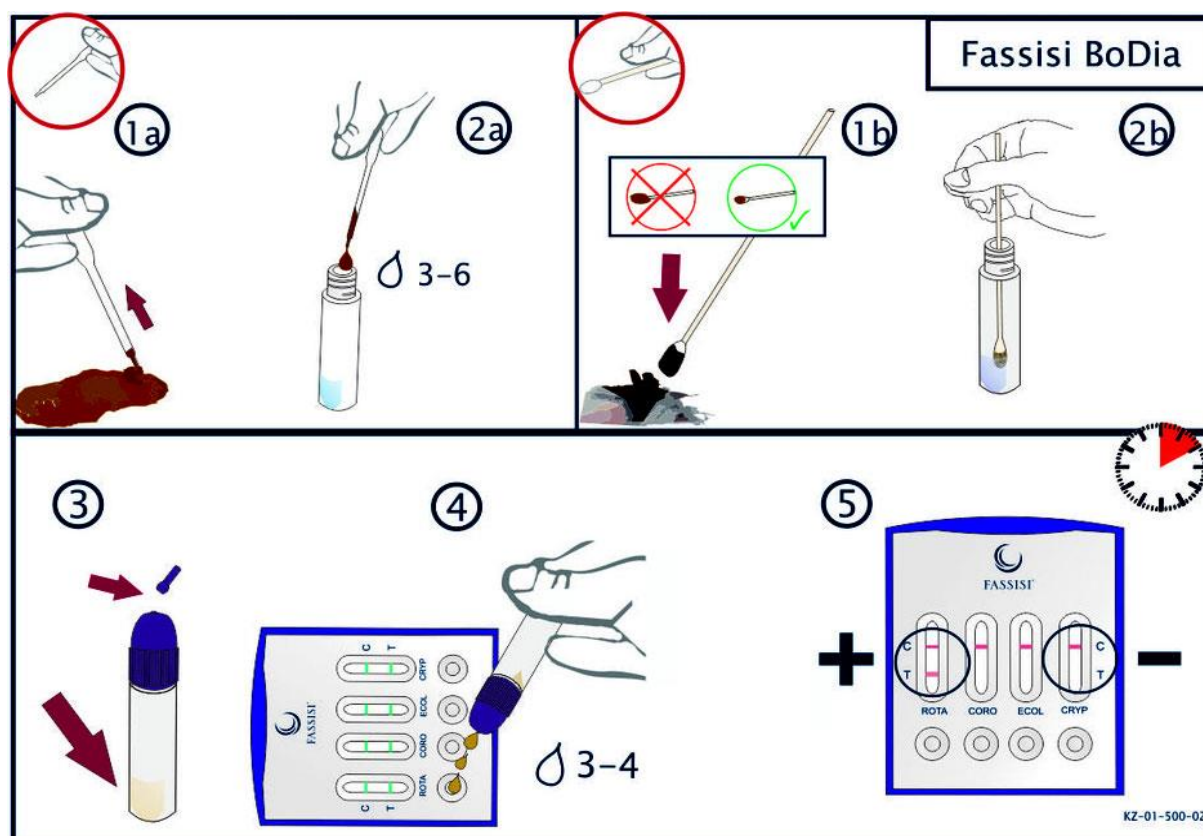


Figure 6. Fassisi® BoDia test protocol instruction (illustration adapted from en.fassisi.de website).

2.2.2. Statistical analyses

The statistical analysis was performed using Microsoft Excel for Office 365 MSO, version 1908 (Compilation 11929.20708) and the Statistical Package for the Social Sciences 23 (SPSS version 23).

The descriptive statistics analyses allowed to characterize the etiological agents and age at diagnosis based on the frequencies, medians, means and standard deviation (Mean \pm SD).

The inferential analysis tried to relate different variables. Statistical associations were tested using the Pearson Chi-square test when the results were higher than 5 or the Fischer Exact Test when the results were lower than 5. All results were considered statistically significant when p-value <0.05 .

3. Results

For the present study, fecal samples were collected from 18 calves, coded from A to R, from 17 different dairy herds. The analyses of the fecal samples in diarrheic calves with the rapid immunochromatographic assay were positive for at least one etiologic agent in 88.9% of the cases (n = 16), as is shown on Table 2.

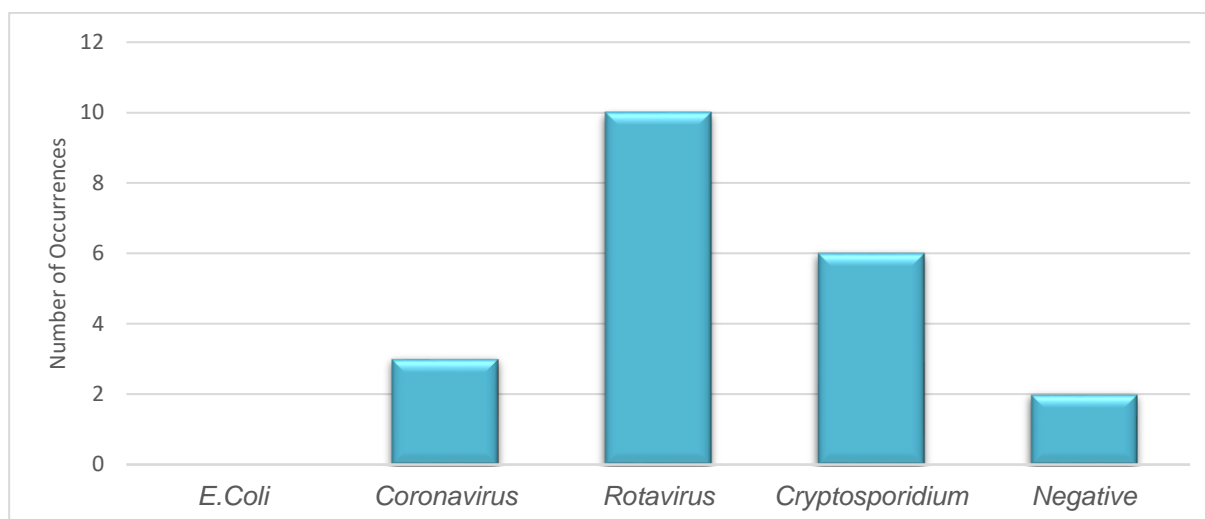
Table 2. Clinical cases description at the first veterinary visit.

Case	Age (days)	Feces description	Test result	Previous vaccination programme	Live status after treatment
A	3	Yellowish watery diarrhea, mucus present	<i>Cryptosporidium</i>	-	alive
B	5	Yellowish watery diarrhea	Rotavirus	Maternal vaccination	alive
C	3	Yellowish watery diarrhea	Rotavirus	-	alive
D	7	Yellowish diarrhea with with stripes of living blood	Negative	-	alive
E	10	Yellowish diarrhea	Rotavirus and <i>Cryptosporidium</i>	-	alive
F	3	Yellowish diarrhea	Rotavirus and <i>Cryptosporidium</i>	-	alive
G	4	Yellowish diarrhea	Rotavirus and <i>Cryptosporidium</i>	-	n.s.
H	7	Yellowish diarrhea	Rotavirus	-	n.s.
I	2	Yellowish diarrhea	Rotavirus	-	n.s.
J	4	Yellowish diarrhea	Rotavirus	<i>E. coli</i> specific vaccination	alive
K	7	Yellowish & very watery diarrhea	<i>Cryptosporidium</i>	-	alive
L	10	Yellowish diarrhea	Negative	-	died
M	4	Yellowish diarrhea	Coronavirus	-	n.s.
N	7	Yellowish watery diarrhea	Rotavirus	-	n.s.
O	2	Yellowish diarrhea	Rotavirus	-	alive
P	3	Yellowish diarrhea	<i>Cryptosporidium</i>	-	died
Q	8	Yellowish diarrhea	Coronavirus	-	n.s.
R	4	Yellowish diarrhea	Coronavirus	-	alive

n.s. – not specified

The most frequent pathogen was rotavirus (n=10) followed by *Cryptosporidium parvum* (n=6) and coronavirus (n=3). No *E. coli* K99 (n=0) was detected with the rapid test assay (Graph 1).

Graph 1. Frequency of cases per pathogen according to the rapid test.



Two samples that gave negative results in the rapid immunoassay were retested for the four investigated pathogens by microbiological culture to mislead false-negatives. Both cases revealed a significant infection by *E. coli* (Table 3).

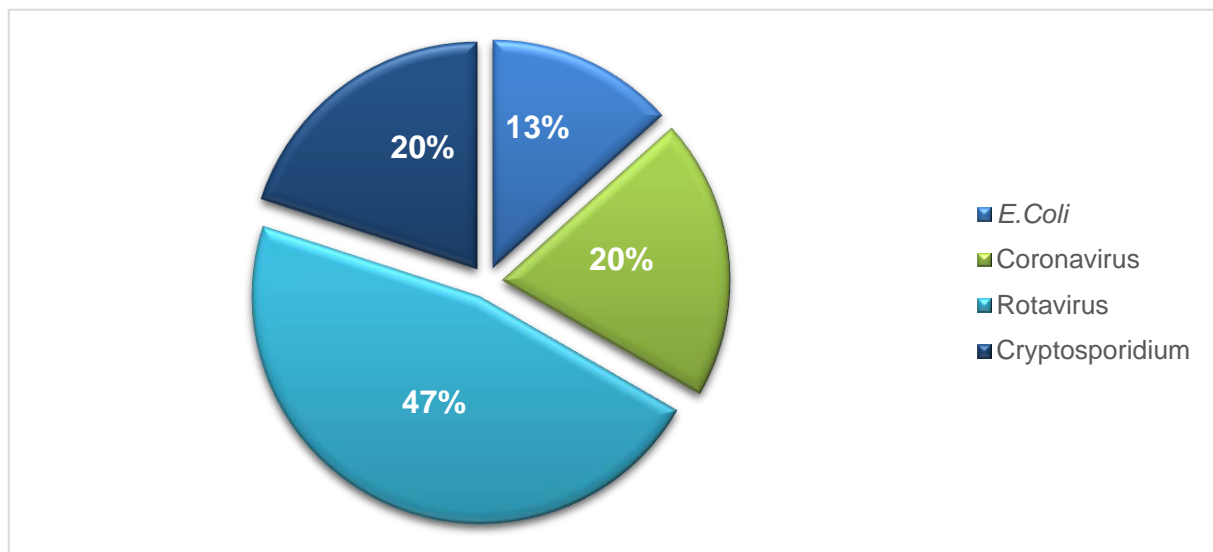
Table 3. Microbiological laboratory results for fecal samples of cases D and L that tested negative for all the four pathogens on the rapid immunoassay.

Case	Rapid Kit result	Laboratory result
D	Negative	<i>E. coli</i> (++++)
L	Negative	<i>E. coli</i> (+++)

Bacterial growth scale: (+) low; (++) moderate; (+++) strong; (++++) massive.

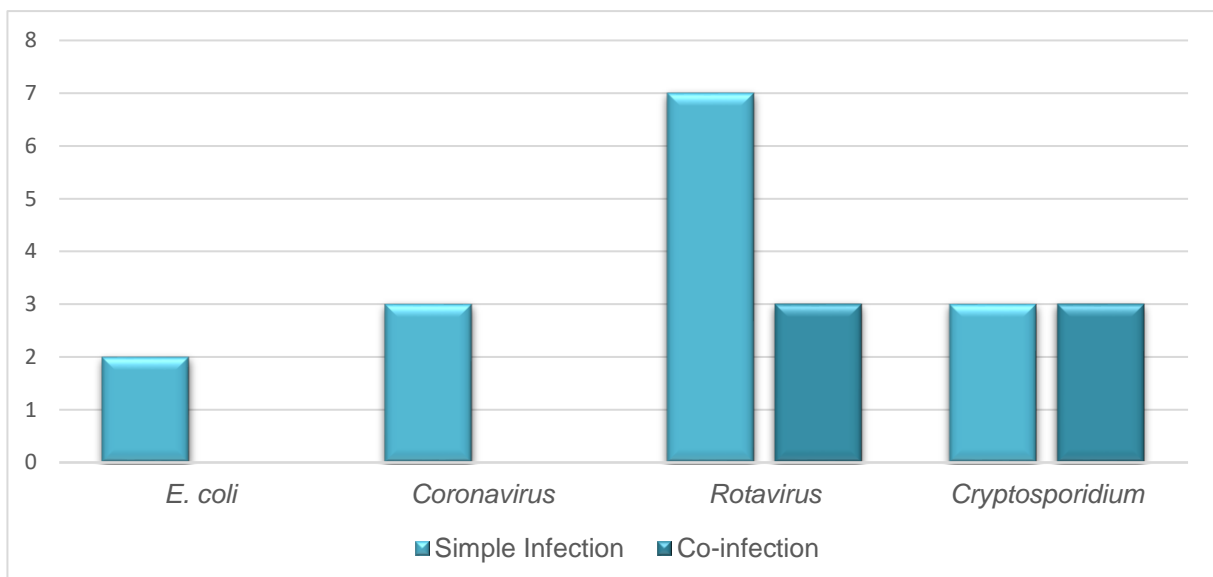
Simple infections were led by rotavirus (n=7), followed by coronavirus and *Cryptosporidium*, both at an equal detection frequency (n=3) and in the end by *E. coli* (n=2) (Graph 2).

Graph 2. Simple infections per pathogen.



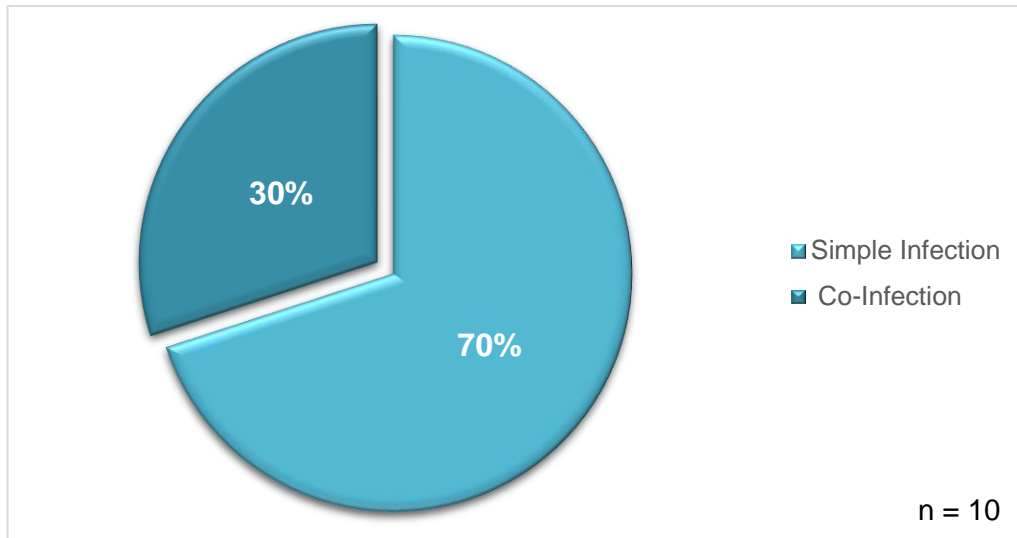
There was a significantly large number of single infections (83.3%) compared to co-infections (16.7%) (Graph 3). Rotavirus and *Cryptosporidium* were the only two pathogens associated in co-infections. The number of simple infections by rotavirus were more than double the co-infections with rotavirus. No differences in the frequencies of simple infections and co-infections were observed regarding *Cryptosporidium*.

Graph 3. Simple and co-infections per pathogen.



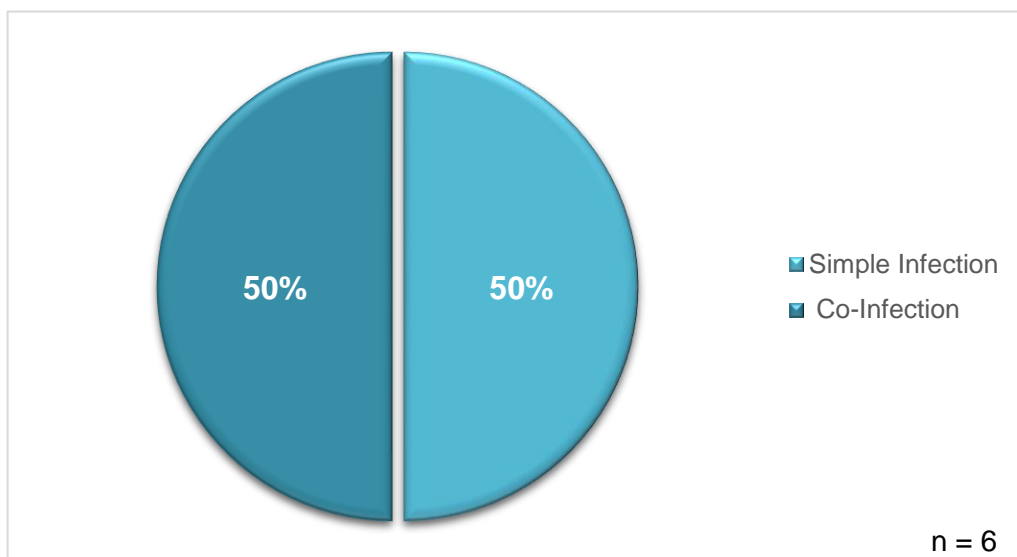
Co-infection by rotavirus and *Cryptosporidium* are represented in Graphs 4 and 5, respectively.

Graph 4. Infections and co-infections with rotavirus.



Comparing rotavirus frequency in both simple and co-infections *versus* the other isolated agents (coronavirus + *Cryptosporidium* + *E. coli*) either in simple or co-infection, we did not find any statistical association ($\chi^2=2.88$; $p = 0.09$). In this study there was no involvement of coronavirus in co-infections in episodes of neonatal calf diarrhea.

Graph 5. Infections and co-infections with *Cryptosporidium*.

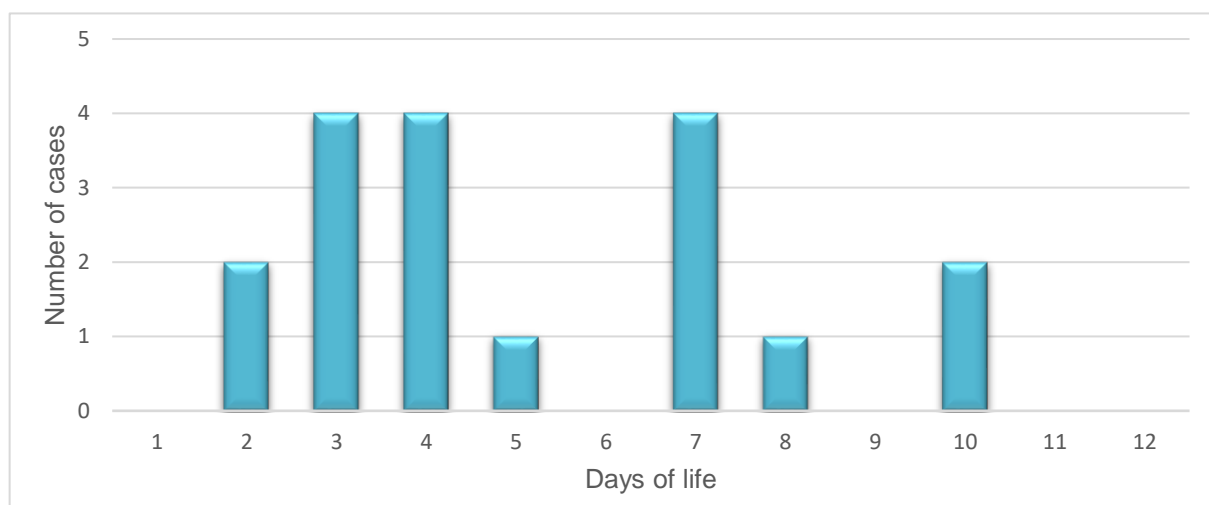


On the other hand, when comparing *Cryptosporidium* frequency in simple and co-infections *versus* the other isolated agents (coronavirus + rotavirus + *E. coli*) either in simple or co-infection infections, we found a statistical association ($\chi^2=7.2$; $p=0.025$). There is a tendency for *Cryptosporidium* to be associated with co-infection in episodes of neonatal calf diarrhea.

It was not possible to statistically assess any co-infection tendencies with *E. coli* and *coronavirus* because those pathogens were only isolated in simple infections and in a very low frequency.

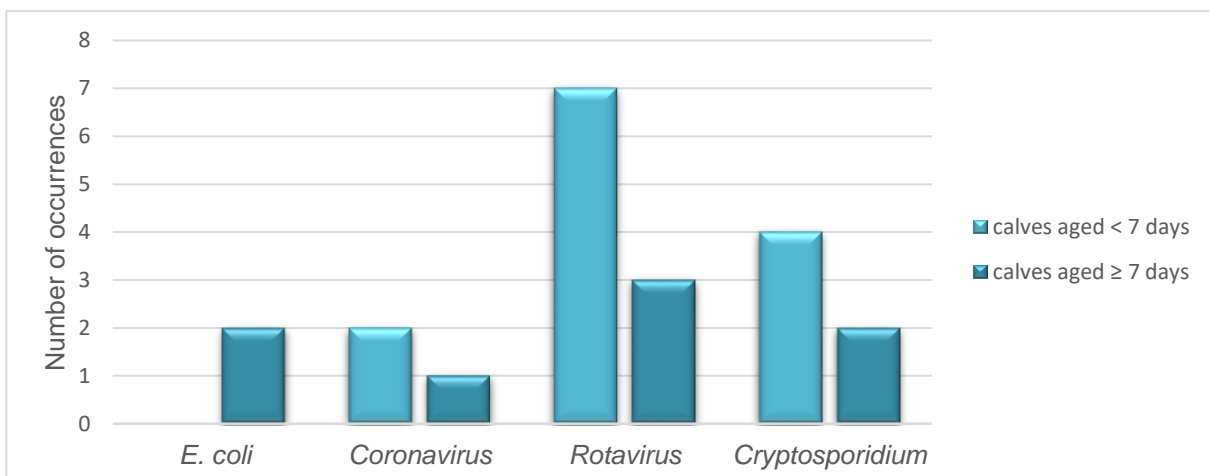
The calves diagnosed with neonatal diarrhoea aged between 2 and 10 days old, a mean age of 5.17 ± 2.57 days (Mean \pm S.D.) and a median age of 4 days. There were observed a greater number of cases at 3rd, 4th and 7th day of calf live (Graph 6).

Graph 6. Age (days) at the time that calves were diagnosed with neonatal diarrhea.



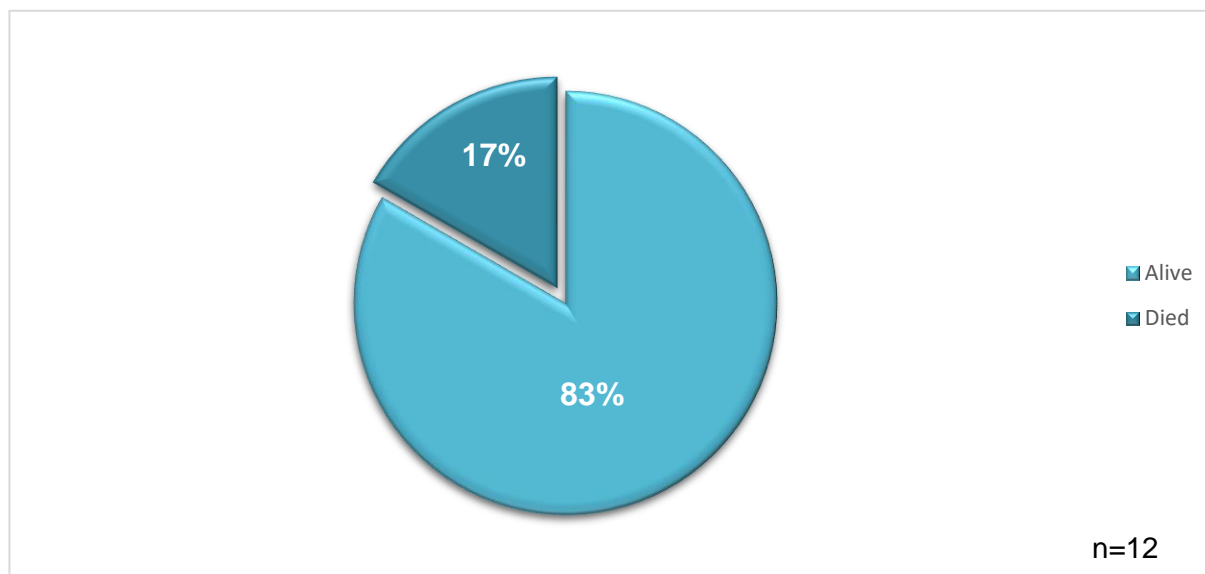
There was a higher frequency of calves less than 1 week of age to exhibit signs of neonatal diarrhea (61.1%). Calves less than 7 days old were more likely to develop diarrhea caused by rotavirus and by *Cryptosporidium* in comparison to calves more than 7 days old, although there was no statistically difference observed between age groups ($p=0.37$ and $p=0.74$) (Graph 7). There were no cases of *E. coli* recorded in younger age group.

Graph 7. Occurrences of each pathogen in calves less or over 1 week of life.



Two of the 12 cases (16.7%) that we were able to follow-up since D0, died during their treatment protocols. Case L with a strong *E. coli* infection and case P with a *Cryptosporidium* infection. The success rate was calculated at the end of the therapy with the animal being alive and in good health, which occurred in 83.0% of the cases (Graph 8).

Graph 8. Treatment succesful rate.



Intravenous (IV) fluid therapy was the most used method (66.67%) for hydration (n=12). Seventy five percent of the cases that were treated with IV fluid therapy, in jugular or auricular veins (n=8) survived. Half of the animals that received fluid therapy through the auricular vein died. In the other hand, jugular fluid therapy and oral fluid therapy had a 100% success rate. Due to the very low number os cases, inferential statistics was not performed. All fluid therapies cases are compiled in Table 4.

Table 4. Outcome of fluid therapy cases.

Fluid therapy method	Alive	Died	Not specified
IV (jugular) fluid therapy	4	-	3
IV (auricular vein) fluid therapy	2	2	-
Oral fluid therapy	4	-	3
Total cases	10	2	6

4. Discussion

Neonatal diarrhea in calves is the most reported disease and the leading cause of morbidity and mortality in calves. Calves with less than 30 days of life are the most likely to contract the disease representing an important source of economic loss to the farmer not only due to the loss of the present value of the calf and treatment costs but also the loss of genetic potential for herd improvement (Bendali et al. 1999). Furthermore, it is of great importance to detect and understand the etiology of NCD to rectify the causes and to apply suitable preventive measures in order to prevent or at least mitigate the incidence and severity of new outbreaks. For that reason, we focused on studying the etiology of NCD with main emphasis on *E. coli*, rota- and coronavirus and *Cryptosporidium parvum* and in evaluating the usefulness of a rapid immunoassay test to diagnose those pathogens.

Given the sampling procedure, we cannot exclude the results may be biased, since we tended to select only one calf out of the severe cases of diarrhea in calves for sampling and, thus, mild or transient cases may be under-represented in this study. Three cases were excluded due to the lack of enough data from the anamnesis or the follow-ups. There were dairy herds where there was more than one calf with NCD signs at the time of the visit, but we could only test one animal due to cost and time reasons. The inclusion criteria in those situations were to collect the coprological sample from the calf showing the most severe signs of the disease for analyses. Moreover, we considered the enteropathogen identification of the rapid test valid for a period of 15 days. Therewith, we considered any new case of NCD in the farm during the 2 weeks following the first diagnosis, as being the most likely the agent concerned, and thus not included in our data. Additionally, as it is possible to establish a therapeutic protocol considering the characterization (age), history (onset of symptoms) and clinical signs presented by the patient, there were some cases where the producers were not interested in screening for the four pathogens. We saw that the same drawback frequently exists for other studies involving NCD. In any case, it seems reasonable to compare our results with those reported in similar studies.

The prevalence of each pathogen and disease incidence varies by geographical location of the farms, farm management practices and herd size (Cho et al. 2014). In 18 calves with NCD, from 17 herds in Lower Bavaria, one or two agents were identified. Rotavirus (55.6%) and *Cryptosporidium* (33.3%) were the two most frequent enteropathogens, confirming data reported worldwide (Spence 2006; Luginbühl et al. 2005; Ok et al. 2009; Gulliksen et al. 2009; Bartels et al. 2010; Izzo et al. 2011). Even if there are concurrent infections with other agents as well as environmental, management and nutritional factors, that may influence the outcome of NCD in a calf with rotavirus and

Cryptosporidium, the results of this study show the importance of each of these microorganisms as primary pathogens causing acute diarrhea in neonatal calves.

Diarrhea caused by bovine coronavirus tends to be more severe than rotavirus because coronavirus also invades the large intestine (Clark 1993; Foster and Smith 2009; Izzo et al. 2011). However, the frequency of coronavirus observed in diarrheic calves in the current study was lower than for rotavirus. This trend was also found in other countries (Reynolds et al. 1986; de la Fuente et al. 1999; Uhde et al. 2008; Gulliksen et al. 2009; Bartels et al. 2010; Izzo et al. 2011). *E. coli* K99 (F5) was present in a very low frequency (11.1%, n=2). The results of other research groups corroborate this finding (Reynolds et al. 1986; Luginbühl et al. 2005; Gulliksen et al. 2009; Bartels et al. 2010).

Although a single primary pathogen can be the cause of NCD in calves, co-infection is common (Cho and Yoon 2014). Also, Reynolds et al. (1986) suggested that the presence of more than one enteropathogen may be one of the factors determining whether an infection results in a clinical or subclinical event. The proportion of calves with mixed infections was low in our study (16.7%), but it stood within the range reported in other European countries (5-50%) (de la Fuente et al. 1999; Uhde et al. 2008). All the co-infections detected were a combination of rotavirus and *Cryptosporidium*. While we have not found a statistical association of co-infections by rotavirus ($p>0.05$), there was a significant association of *Cryptosporidium* in co-infections ($p<0.05$). This result aligns with previous studies that reported a significant association between the detection of *C. parvum* and rotavirus (de la Fuente et al. 1999; Bartels et al. 2010; Izzo et al. 2011).

Most of the prevalence surveys of enteropathogens in calves fail to detect pathogens in 5-30% of fecal samples (de la Fuente et al. 1999; Uhde et al. 2008; Izzo et al. 2011) which is a significant source of frustration for clinicians that are trying to establish the causative agent in order to apply control methods. In our study, 11.1% of the samples gave a false-negative result in the immuno-chromatographic rapid test used. Ideally, to confirm the reliability of these rapid tests, we should confirm all the results obtained by golden standard exams in the laboratory. However, this was not possible to do due to cost-control limitations. Only samples with negative results in the immuno-chromatographic rapid test were sent to a Reference Laboratory to be investigated with standard methods (antigen-ELISA and microbiological culture). This was the cases of calves D & L that presented false-negative results for *E. coli* in the rapid test, but that later gave positive results in bacteriological culture. As *E. coli* is present in the intestinal tract of healthy animals, it is expected to be identified when cultured (Ok et al. 2009). Even if previous works indicate a role for *E. coli* in the occurrence of NCD (Bendali et al. 1999) with K99 being the most common strain isolated, little is known about its prevalence in dairy farms. Overall, *E. coli* is shed during a short

period of time, that helps to explain its low prevalence (Bartels et al. 2010), even if its incidence and thus impact on calf morbidity and/or mortality might be relatively high.

The isolation of causative agents is higher when animals are sampled early in the disease course and prior to the use of antibiotics that may lead to false-negatives. For that reason, veterinarian practitioners appreciate a less time-consuming, reproducible, sensitive and simple diagnostic tests for quick decisions on therapeutic and prophylactic strategies. In our experience, we considered the use of this rapid test of great practical value, thanks to the on-site diagnosis after 10 minutes in almost 90% of the cases, helping to implement a proper treatment protocol and adequate preventive measures in each farm. Like other rapid tests, the Fassisi® BoDia test depends essentially on correct handling, either ensuring a good mixing of the feces with the dilution buffer, or in case of watery feces, ensure a correct use of the sample pipette due to the risk of insufficient antigen amount that could also give false-negative results. As the Fassisi® BoDia test is not a quantitative but rather a qualitative to semi-quantitative test, the exact amount of pathogen excretion per calf could not be assessed. Nevertheless, as a large amount of antigen in the stool is expected during the acute phase of the disease, the use of this rapid test to enlighten the NCD etiology is worthwhile.

According to the manufacturer, the Fassisi® BoDia test has a great sensitivity and specificity for detection of rotavirus (Se=96.4%; Sp=95.5%), coronavirus (Se=99.9%; Sp=99.9%) and *C. parvum* (Se=99.9%; Sp=95.9%) in diarrheic calves (Table 1). We were unable to find external validation of the used kit in published literature, but following some authors reports, the use of similar antigens-ELISA tests specially for detecting *C. parvum* may not be appropriate for detection of mild infections where few oocysts are shed (Luginbühl et al. 2005; Izzo et al. 2011; Papini et al. 2018).

Therefore, calves shedding small numbers of oocysts may not have been detected and consequently a possible number of co-infections are missing in our samples. For *E. coli* K99 detection, the Fassisi® BoDia test has a very high specificity (99.9%) but low sensitivity (88.9%). The test low sensitivity explains why two calves shedding *E. coli* were not detected, and probably, another *E. coli* infections were not found in the current study.

Differences in prevalence rate among studies may be attributed to different diagnostic methods used, and to the age distribution of calves. The four NCD pathogens were detected in calves aged between 2- and 10 days old. The majority of NCD cases were diagnosed during the first week of life (61.1%) like Ok et al. study (2009), while in other studies, the prevalence of NCD was higher in the second week of age (Bartels et al., 2010; Izzo et al., 2011). Likewise, Bendali et al. (1999), reported more than 50% of all neonatal diarrheas in France appearing during the first week and only 15% after the second week of life, although, exceptionally, the highest prevalence of rotavirus was seen at 2-4 weeks of age. The

presence of *C. parvum* in calves aged less than 4 days was higher (66.7%), comparable with other studies (de la Fuente et al. 1999; Izzo et al. 2011). We justify this trend by the constant monitoring of dairy producers or animals' keepers for early detection of illness signs, together with the short time until the veterinarian arrived at the herd for a quick intervention on diseased calf/calves.

Another relevant point to discuss is that the etiology of NCD may differ according to geographic parameters. NCD is frequently diagnosed in Lower Bavaria state, with higher incidence in winter (peak seasonal period between October and February). During the 2018/19 winter season, in which the fecal samples for this study were collected, the state of Bayern was affected by heavy snowfall and low temperatures for a period longer than usual (WAZ 2019). In general, it is known that calf mortality rate increases in winter period (Bendali et al. 1999; Gulliksen et al. 2009; Millemann 2009; Uetake 2013) linked to cold, moisture and windy weather. These climatic risk factors are severe for calves due to their lower basal metabolic rate and heat production, especially for calves born of dystocia (Uetake 2013). Also, the major enteric pathogens can survive in the environment and water sources for weeks to months (sometimes over a year) in cool, damp conditions, increasing the infection pressure and the severity of the disease (Millemann, 2009). Although we did not find a statistical association between each pathogen infection and calf mortality rate, 2 out of 12 calves with NCD (16.7%) did not survive despite the treatment protocol. One calf (case L) was diagnosed with a *E. coli* infection at 10 days old. The other calf (case P) tested positive for *Cryptosporidium parvum* at 3 days of age.

The cause of mortality of newborn calves with severe diarrhea is due to acute dehydration and a metabolic development of acidosis (Coskun et al. 2010). The primary treatment indication is a well-executed oral and intravenous (IV) fluid therapy as a key of success (Koch and Kaske 2008; Meganck et al. 2014). In this study sick calves were treated with a fluid therapy protocol adjusted to individual needs, with a continuous infusion of an isotonic solution in the jugular or in the ear vein (0.9% saline solution), along with a non-steroidal anti-inflammatory drug (NAIDs) and antibiotics for a few days. To treat cryptosporidiosis infections halofuginone lactate, or in a few cases paromomycin, were added. According to several studies (Coskun et al. 2010; Trefz et al. 2015) calves with acidemia and concurrent hyperkalemia benefit from a rapid alkalization with sodium bicarbonate (8.4% bicarbonate solution) to correct strong ion acidosis, in order to restore renal function and enhance renal potassium ion (K^+) excretion. The IV fluid therapy is used predominantly based on the degree of dehydration and acidosis, but its use can be extended to calves that are recumbent or severely depressed, or do not have a sucking reflex (Berchtold 2009). Catheterization of the jugular vein is widely used for fluid therapy in calves, but ear vein catheterization is also possible, and a surgical cutdown of the jugular vein (which

is often performed in severely dehydrated calves) should be avoided (Berchtold 2009). Auricular vein catheterization in neonatal calves using a small flexible catheter, became popular among veterinary practitioners in Germany. Even if there is a lack of studies comparing the use of ear vein catheters with other approaches in calves, this technique is believed to result in fewer complications compared with jugular catheters. Given our small number of cases followed until their resolution (n=12), we could not assess statistically the recovery rate between the two methods of fluid therapy applied and life status. Despite that, more than half of the calves that were followed until NCD resolution received IV fluid therapy, with 75% of them being cured at the end of the treatment. A treatment failure was defined as finding no improvement in the health status of the calf or a deterioration of the general condition resulting in death, between two consecutive visits during the first 72-hour monitoring period. Greater the degree of dehydration, the slower was the administration of fluids for an effective perfusion of the tissues. Thanks to catheterization in the auricular vein (Figure 7) the infusion is usually slower compared to jugular IV fluid therapy, turning the rehydration more effective. In the present study, the two calves that died (cases P and L) during treatment, represented 2 out of 4 calves that got auricular IV fluids administration. This half rate of success may be explained by the small sample size, the advanced critical condition on the first visit, taking into account the interval between the appearance of first signs of NCD and the therapy implementation, the major loss of fluids and electrolytes (dehydration >8%), and the severe weather conditions (moister and very low temperatures) at the time.

The efficacy of antimicrobials in treating calf diarrhea is still controversial because it creates a potential health risk to humans in terms of drug residues and the development of resistant bacterial strains (Constable 2004; El-Seedy et al. 2016). Ideally, the antibiotics (AB) of choice should depend on the drug sensitivity of the bacteria isolated from the feces of the calf. However, in the current study, the single isolated causes of diarrhea from different farms turn impossible to assume that the *E. coli* isolated from the stool of a diarrheic calf was the primary cause of the NCD. Only when the same enteropathogenic serotypes are isolated consistently in the same herds do their presence assume significance (Radostits 1975; Constable 2009).

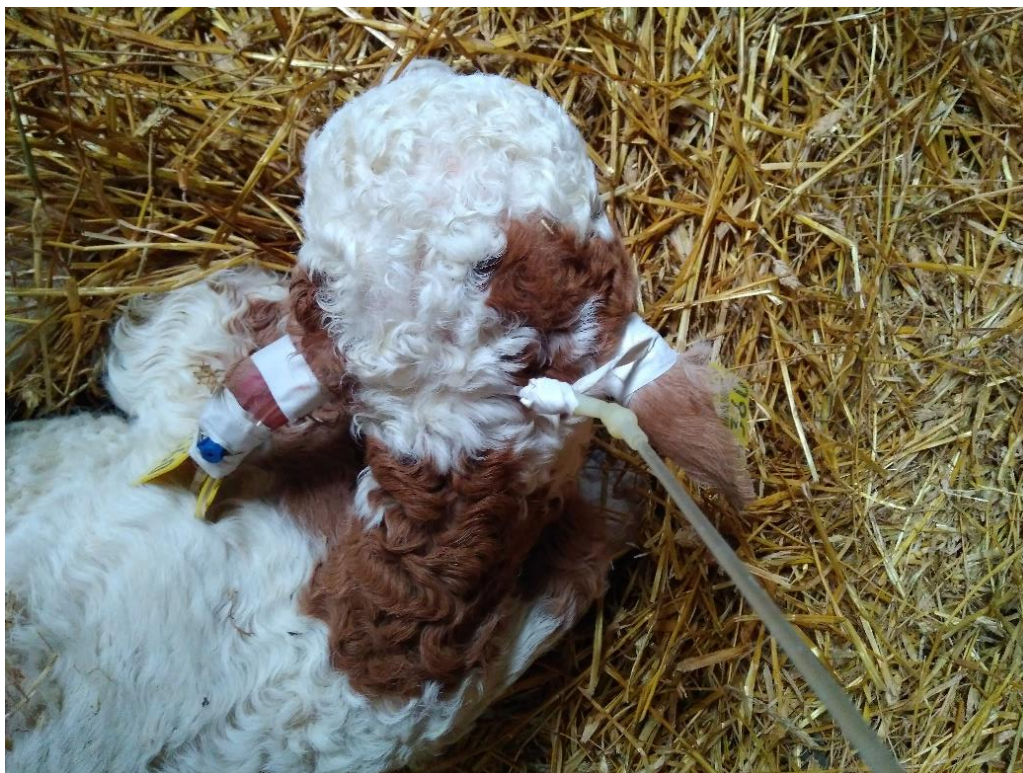


Figure 7. Diarrheic calf under auricular vein catheterization for IV fluid therapy (Case L).

The importance of colostrum in protecting neonatal calves against enteropathogens infection is also a very valuable point to address. Despite this, we were not able to determinate the IgG to evaluate if there was an adequate passive immunity or not, a good quality colostrum provides passive immunity to the calf, if ingested within the first 6 hours after birth (de Graaf et al. 1999; Cortese 2009; Staněk et al. 2019), increasing the resistance to enteric diseases. When seeking the causes for new NCD outbreaks, we found that there were sometimes failures in the milk replacer's supply, forcing the farmers to alternate between cow's milk and milk replacers. Several studies demonstrated that colostrum replacement products have a highly variable performance (Meganck et al. 2014), and reported that dairy calves fed with non-saleable pasteurized milk have a higher growth rate and lower morbidity and mortality rates than do calves fed with commercial milk replacer until they are weaned (Uetake 2013; Meganck et al. 2014). Additionally, the harsh winter already mentioned and most of the investigated herds being of small size, may have been the main trigger factors for a high frequency of NCD outbreaks. These two NCD environmental disease determinants were also described in the neighboring country, Czech Republic (Staněk et al. 2019).

To prevent or reduce the prevalence of NCD, farmers should implement a vaccination protocol against the NCD pathogens involved (de Graaf et al. 1999; Millemann 2009). Among

the 17 herds studied, only 2 farms (11.7%) followed a vaccination protocol against diarrhea for more than 2 years, including calf's protection against *E. coli* (herds of origin of the calves B and J). It is well documented that calves fed with colostrum from immunized mothers with high titers of specific antibodies are partially protected against some NCD enteropathogens infection (de Graaf et al. 1999). The specific *E. coli* strain vaccination protocol for newborns is equally of great value especially in farms where infection with *E. coli* is endemic. However, vaccines may not induce enough cross-protection and the pathogens, mainly *E. coli*, may evade the protection afforded by vaccination by evolving away from vaccine strains (Smith 2012), forcing the need to develop new vaccines for the new strains. The reason for that, is the virulence attributes that are frequently encoded on genetic elements of *E. coli* that can be mobilized into different strains to create novel combinations of virulence factors or on genetic elements that might once have been mobile, but have now evolved to become 'locked' into the genome (Kaper et al. 2004). Unfortunately, a vaccine against *C. parvum* is still not available (Panini et al. 2018). Yet it appears that calves born from dams vaccinated against *E. coli*, rotavirus and coronavirus shed less *C. parvum* oocysts (Meganck et al. 2014). This lower shedding of *C. parvum* oocyst may reflect a higher standard of herd management and biosecurity levels, rather than a direct protective effect (de Graaf et al. 1999; Meganck et al. 2014).

Lastly, the improvement of the quality of calf rearing conditions is other key to success in the prevention of NCD (Uetake 2013). The diagnosis of the NCD etiological agent, helped us to set up suitable preventive hygiene measures, vaccination programs of dams before calving or to the newborn calves, deworming programs and give proper advice on feeding management.

V - Conclusion

Neonatal diarrhea is frequently a neglected problem in dairy herds. The current study confirms the complexity of the etiology of NCD observed in dairy farms in southern Germany.

In 18 calves with NCD, four major pathogens were detected, rotavirus, coronavirus, *C. parvum* and *E. coli* K99, emphasizing the widespread dissemination of these microorganisms.

The frequencies of these four enteropathogens are in the same range of prevalences reported in neighbors' countries and overseas, being rotavirus and *C. parvum* the two most frequent agents diagnosed, both in simple and co-infections. Despite there were more simple infections than co-infections, *Cryptosporidium* was more associated to mixed infections.

Most calf births in Lower Bavaria are concentrated between October and February, which corresponds to winter season, with very low temperatures and moisture. These environmental factors are at the base of the increase of cases of NCD, usually observed in this region.

Other predisposing factors for NCD outbreaks found were small herd size with low investment on biosecurity ("old" dairy farms), either because the animals were closer to each other's, making easier the development of a chain of transmission, or failure on preventive and control measures, due to the lack of awareness of farmers.

When proceeding to the outbreak investigation (whether qualitative or quantitative), the veterinarian is responsible to identify disease risks factors, in order to increase the effectiveness of control measures and to mitigate the probability of future outbreaks. The veterinarian is also the most adequate professional to advice and educate farmers on how to manage and prevent NCD.

We confirmed the helpfulness of using a rapid immunochromatographic test to identify the enteropathogens causing NCD. This rapid test is highly appreciated by veterinarians in Lower Bavaria because it detects rotavirus, coronavirus, *Cryptosporidium parvum* and *E. coli* K99 in calves, just in one-step. In 16 out of 18 calves suffering from NCD, the detection for at least one pathogen was successful, although the missing detection of *E. coli* due to the low sensitivity of the test for this pathogen should be taken into account.

Enteropathogens diagnosis with the rapid test helped us to choose a specific therapy, especially in cases with *Cryptosporidium* infections, and to implement targeted prophylactic measures for calves or dams. A further advantage of these rapid tests is that they may be used to test on site large numbers of samples in a cost-effective manner. Samples are processed quickly in the stable with minimum effort, without the need for technological expertise or specialized laboratory equipment, allowing fast and accurate decision-making of intervention strategies.

The survival rate of calves was high probably due to the identification of the enteropathogen within a short time, allowing for the adjustment of a treatment protocol and measures to prevent the spread of infection to other animals, such as isolation of infected calves and disinfection of contaminated facilities.

In a nutshell, the results of this study provide reliable insight into the most frequent pathogens associated with outbreaks of NCD in dairy calves in Lower Bavaria state, although increasing the sample size would have allowed to generate more reliable information. Likewise, in future studies, it would be important to compare the rapid test results with standard microbiological methods in all samples, positive or negative, to better contribute for the rapid test validation on the field. Nevertheless, the technical advantages of rapid tests are indisputable. Identifying the enteropathogens involved in NCD outbreaks allows for a proper outbreak investigation and the timely implementation of therapeutic protocols and prophylactic measures, to promote calf health and welfare and to safeguard public health on a One Health perspective.

VI - Bibliography

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