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CALF NEONATAL PERIOD DIARRHEA PATHOGENS AND ASSOCIATION WITH ON-FARM MORTALITY

VASIKATE NEONATAALPERIOODI KÕHULAHTISUSE PATOGEENID JA SEOS SUREMUSEGA

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Important information is missing on the prevalence of neonatal diarrhea causing pathogens in Estonian dairy calves, and how they affect calf mortality. Aim in this study was to find out the prevalence of *Cryptosporidium parvum*, rotavirus, enterotoxigenic *Escherichia coli* and coronavirus in diarrheic calves reared in large-scale Estonian dairy farms and their impact on mortality during the first three months of calves life. Fecal samples were collected from 615 dairy calves living in 116 large-scale (including more than 100 cows) farms. Samples were analysed with ELISA kit, which detectes antigens of these four pathogens. Descriptive statistics were used to represent the prevalence of the pathogens. Survival analysis (Kaplan-Meier analysis and Cox proportional-hazards regression models) was used to identify if there is association between calf positive status for the pathogens and mortality probability.

In this study, *Cryptosporidium parvum* (33.3%) and rotavirus (19.7%) were the most prevalent pathogens among calves under three months of age. Single pathogen infections did not affect calf mortality probability significantly, but a trend of increasing the calf mortality risk in calves harbouring coinfection with *Cryptosporidium parvum* and rotavirus was identified ($p = 0.136$). Male calves had higher probability of death compared to female calves ($p = 0.006$). Breed did not affect the mortality risk. The study findings indicate that effective control programs are needed to prevent *Cryptosporidium parvum* and rotavirus infections and thus lower the mortality rate of calves under three months of age in large-scale Estonian dairy farms.

Keywords: *Cryptosporidium parvum*, rotavirus, coronavirus, enterotoxigenic *Escherichia coli*, mortality risk

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Tänaseni puudub info vasikate neoonataalperioodi kõhulahtisuse patogeenide levimusest Eesti piimakarja vasikatel ning nende mõjust suremusele. Käesoleva uuringu eesmärk oli välja selgitada *Cryptosporidium parvum´*i, rotaviiruse, enterotoksilise *Escherichia coli* ja koroonaviiruse levimus kõhulahtisust põdevatel vasikatel, keda peetakse suurtes Eesti piimafarmides, ning nende mõju suremusele vasikate esimese kolme elukuu jooksul. Roojaproovid koguti 615 piimavasikalt, keda peeti 116 suures (rohkem kui 100 lehmaga) farmis. Proove analüüsiti ELISA meetodil, mis tuvastab nende nelja patogeeni antigeene. Kirjeldavaid statistilisi meetodeid kasutati patogeenide esinemissageduse esitamiseks. Elumusanalüüsi (Kaplan-Meieri analüüsi ja Coxi proportsionaalsete ohtude regressioonmudeleid) kasutati selleks, et tuvastada seost vasikate patogeeni suhtes positiivse staatuse ning suremise tõenäosuse vahel.

Käesolevas uuringus olid *Cryptosporidium parvum* (33.3%) ja rotaviirus (19.7%) kõige levinumad patogeenid alla kolme kuu vanustel vasikatel. Üksikud patogeeniinfektsioonid ei mõjutanud vasika suremuse tõenäosust oluliselt, kuid täheldati, et vasikatel, kes põdesid samaaegselt *Cryptosporidium parvum´*i ja rotaviiruse infektsioone, oli mõnevõrra kõrgem suremusrisk ($p = 0.136$). Pullvasikatel oli surma tõenäosus kõrgem võrreldes lehmvasikatega ($p = 0.006$). Tõug ei mõjutanud suremusriski. Uuringu tulemused näitavad, et vajalik on tõhustada *Cryptosporidium parvum´*i ja rotaviiruse nakkuste ennetusmeetmeid farmides, mis võiks kaasa tuua vasikate surmajuhtude vähenemise esimesel kolmel elukuul suurtes Eesti piimafarmides.

Märksõnad: *Cryptosporidium parvum*, rotaviirus, koroonaviirus, enterotoksiline *Escherichia coli*, suremusrisk

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LIST OF ABBREVIATIONS

BCoV	bovine coronavirus
C. parvum	*Cryptosporidium parvum*
E. alabamensis	*Eimeria alabamensis*
EARIB	Estonian Animal Recording and Information Board
E. bovis	*Eimeria bovis*
E. coli	*Escherichia coli*
E. zuernii	*Eimeria zuernii*
ETEC	enterotoxigenic *Escherichia coli*
FPT	failure of passive immunoglobulin transfer
IgG	immunoglobulin G
S. Dublin	*Salmonella Dublin*
S. Typhimurium	*Salmonella Typhimurium*

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INTRODUCTION

Neonatal calf diarrhea causes economical losses, due to high morbidity and mortality, and treatment costs (Al-Alo et al., 2018), but also has impact on animal welfare and health. In the United States, 56.5% of preweaned heifer deaths were caused by diarrhea and digestive problems (Bristol et al., 2021). In Estonia, metabolic and digestive disorders and feet/claw diseases were the most common reasons for death in dairy cows (Reimus et al., 2017). According to Welk et al. (2023), 75% of mortality events occur during the first month of life. As young calves are the most vulnerable groups mortality-wise, it is important to find ways to decrease the mortality among young calves (Reimus et al., 2020). Mortality is a key indicator for calf-related health problems and welfare.

Escherichia coli, Cryptosporidium parvum, rotavirus and coronavirus are the four most important pathogens causing neonatal calf diarrhea (Al-Alo et al., 2018). There are many environmental factors influencing the onset of the disease. Factors increasing the risk are larger farm size, presence of other farm animals and housing calves outside, for example (Lorenz et al., 2021). There are not many studies comparing how these pathogens affect the mortality in diarrheic calves. The main risk factors for developing neonatal diarhhea include low level of immunoglobulin G (IgG) (Al-Alo et al., 2018) as well as herd management and environmental stress factors (Cho and Yoon, 2014).

Calf diarrhea is the most common disease and cause of death in neonatal calves (Lorenz et al., 2021). Estonian farms follow the same trend and according to farmers´ reports, the most common cause of death among youngstock is metabolic and digestive disorder, followed with respiratory diseases (Reimus et al., 2017). Rotavirus has been identified in 94% of calves with or without diarrhea, but the prevalence can be as low as 2% (Bristol et al., 2021; Gomez and Weese, 2017). Coronavirus prevalence varies from 5% to 17% (Gomez and Weese, 2017) and for enterotoxigenic *Escherichia coli* (ETEC) in diarrheic calves, the prevalence was 10% (Younis et al., 2009). *Cryptosporidium parvum* has been found to cause over 90% of diarrhea cases in calves (Jang et al., 2021).

Common calf neonatal period diarrhea pathogens are quite well studied already all over the world, but we are still lacking knowledge about the epidemiological situation in Estonia. As herd sizes are becoming larger in Estonia (ELPR, 2020), and thus increasing the risks for higher

mortality (Ismail and Muhaffel, 2022), it is important to know more about the factors causing calf mortality. In this study, we aimed to find the prevalence of different diarrhea-causing pathogens in diarrheic calves reared in large-scale Estonian dairy herds and reveal their impact to mortality during the first three months.

1. LITERATURE REVIEW

1.1. Dairy calf neonatal period

Calf neonatal period indicates time after birth to 28 days of age. This period overlaps with perinatal period which means time from 270 days of pregnancy to 24 hours after birth. In dairy heifers, neonatal period is very sensitive time, since 75% of mortality occurs during the first month of life. During neonatal period, calf goes through changes to adapt from intrauterine to extrauterine life. Gas exchange happens through umbilical cord up until the rupture of the umbilical cord. This causes hypoxia to the calf which stimulates the gasping reflex in calf and after that gas exchange occurs through lungs. Hypoxia leads initially to respiratory acidosis and after that to minor metabolic acidosis. Therefore, normal venous pH for healthy neonatal calves is 7.2-7.3. Metabolic acidosis is corrected within couple of hours, but respiratory acidosis can persist up to 48 hours. Usually, venous pH is normalized within 12 hours (Astiz et al., 2017). During the first 4 weeks of life calves rely on milk as their energy intake and nutrition. Calves are born pseudo-monogastric, and therefore it is important to introduce starter feeds to promote rumen development. Gradual weaning method four weeks before weaning can improve solid feed consumption, prevent weight loss during post-weaning period and reduce signs of hunger (Welk et al., 2023).

Immune system develops until six months of age. Calves are born agammaglobulinemic, but all essential immune components are present at birth, but not fully functional until at least two to four weeks of age and continues to develop until puberty. Complement activity in neonates is 50% compared to adult cattle. Neutrophil amount is approximately four times higher than in adults. Neutrophils and macrophages have reduced phagocytic ability, but the capacity increases after ingestion of colostrum. Neutrophils are functional at one week of age and it improves to the level of adult cattle by the age of five months. Also, high levels of cortisol during the first week of life suppress immune system. Calves rely on maternal antibodies from colostrum for protection against pathogens. Most of the maternal antibodies have a half-life of 16 to 28 days, which creates an opportunity to pathogens to infect calves, called “window of susceptibility”. Maternal antibodies protect from the pathogens, yet at the same time, it delays the development of active immunity. The timing of vaccination is important to estimate around a time, when the level of maternal antibodies is low enough so that it does not interfere with the development of active immunity (Chase et al., 2008).

B cell amount is around 4% of total lymphocytes in a neonate calf, compared to 20 to 30% in adults. 6 to 8 weeks old calves the amount of B cells increases to 20% of lymphocytes. Ratio of CD4 helper cells and CD8 cytotoxic T cells is the same as in adults. Gamma-delta T cell amount is approximately 25% of lymphocytes in one week old calf, but the number decreases to 16% by 19 to 21 weeks of age, total number remaining the same. Meanwhile B cell percentage and T cell number increase in the blood (Chase et al., 2008).

1.2. Dairy calf mortality

In Europe, calf mortality rates vary from 3,8% to 7%. Mortality rate differences between countries can be seen in Table 1. Dystocia is the most common cause for perinatal mortality, and pneumonia and diarrhea are the most common causes for neonatal mortality. Usually, the cause of death is however multifactorial (Probo and Veronesi, 2022).

Table 1. Epidemiological features of calf mortality

Country	Mortality	Age group	Number of animals	Number of herds	Reference
Great Britain	3.4%	24 hours to 28 days	494	19	Brickell et al., 2009
France	4%	3 days to 1 month	3 302 976	98 832	Raboisson et al., 2013
Denmark	8.6%	1-180 days	NA	NA	Nielsen et al., 2010
Sweden	0.7%	1-7 days	4839	131	Olsson et al., 1993
Sweden	0.6%	8-30 days	4839	131	Olsson et al., 1993
Sweden	3.1%	1-91 days	8962	122	Svensson et al., 2006
Finland	4,5%	Average 24 days followed for 180 days	28 228	87	Sandelin et al., 2021
Estonia	0.15 per 100 calf months	21-90 days	294 243	212	Reimus et al., 2020

NA - data was not available

Svensson et al. (2006) did a study to estimate mortality risk and its causes in medium-sized dairy herds in Sweden and found out that median age of dying from enteritis is 20 days and from pneumonia 67 days. The number of deceased calves due to those two conditions drop after 210 days of age. First week of life has the greatest risk of mortality. Overall mortality in this

study was 3.1% (Svensson et al., 2006). Neonatal mortality is associated with poorer growth rate and affects negatively the herd genetic improvement. Poor growth rate are directly related to farm management and calf-rearing practices which include calving and pre-calving management, biosecurity, sanitation, vaccination, housing and feeding. There are infectious causes such as viruses, bacteria, and protozoa, causing diarrhea and pneumonia. Non-infectious causes are related to dystocia, inadequate colostrum feeding, low birth weight and poor management practices. Animal-level risk factors for neonatal mortality include sex, twinning, dam parity, dystocia. Herd level risk factors include herd size and birth season (Ismail and Muhaffel, 2022).

1.2.2. Causes for calf mortality

Main causes for calf mortality are enteritis and pneumonia (Svensson et al., 2006). Bacteremia increases the risk of diarrhea and death (Uetake, 2013). Pathogens isolated from calves that died from pneumonia were *Pasteurella multocida, Escherichia coli* (*E. coli*), *Trueperella pyogenes, Mannheimia hemolytica, alpha- and beta*-hemolytic streptococcae and *Histophilus somni*. The main pathogens isolated from calves died from diarrhea were rotavirus, *E. coli* and *Cryptosporidium* (Svensson et al., 2006). According to National Animal Health System for US dairy cattle, 57% of calf deaths in pre-weaning period were caused by neonatal calf diarrhea, and similar findings were reported in Korea (53%) and Iran (58%) (Gomez and Weese, 2017). Neonatal calf diarrhea occurs during the first week of life in 50% of cases and in 15% after the second week of life (Uetake, 2013).

Similar findings were reported in 1996, study done by Sivula et al. (1996), which stated that 44% of deaths were from enteritis and 30% from pneumonia. Difference in this study compared to Svensson et al. (2006), was that the risk of death was highest at two weeks of life. Risk of enteritis was highest in the first 3 weeks of life and risk of pneumonia was highest at 10 weeks of age. They did not find difference in mortality or morbidity whether the calf had adequate passive transfer or not.

Other causes for calf mortality include abomasal ulcer, septicemic salmonellosis, meningitis, rumen drinkers, aspiration pneumonia, septic arthritis, omphalitis (Ismail and Muhaffel, 2022) and congenital defects (Mee, 2023).

1.2.3. Risk factors for calf mortality

Passive transfer of immunoglobulins is the major aspect to prevent calf mortality. Calves are born without immunoglobulins, and they do not start to produce them before five weeks of age. Maternal immunoglobulins are the only immunoglobulins protecting calves from infectious diseases. Calves suffering from failure of passive transfer (FPT) have higher rate of mortality. Calves with FPT also have lower productivity until the end of first lactation in dairy heifers, meaning higher morbidity and mortality, lower daily weight gain during pre- and postweaning period and lower milk yield during the first lactation. Timing of colostrum ingestion, method and volume of colostrum ingestion and immunoglobulin G (IgG) concentration affect absorption of immunoglobulins (Uetake, 2013). Quality of colostrum varies for several reasons. Heifers have lower number of antibodies than multiparous cows, due to that it is important to assure that a calf of a heifer gets the required volume of colostrum so it will receive adequate number of antibodies. Nutritional status and vaccinations of dam also affects colostrum quality (Cho and Yoon, 2014). FPT threshold value for serum total protein is ≤ 52 g/L which means that the serum IgG concentration is ≤ 10 g/L. Good quality colostrum gives the calf's serum total protein value of 58 to 60 g/L. Serum total protein value being ≥ 57 g/L has lowered the risk for calf to get respiratory disease (Lorenz, 2021). Calves with diarrhea have lower levels of IgG compared to healthy calves. IgG concentrations in dam and colostrum affect calves' IgG levels, therefore it is crucial that the calf will receive good quality colostrum (Al-Alo et al., 2018).

Lower mortality rates have been associated with the owners looking after their cows, instead of farm employees (Uetake, 2013).

Calf mortality has been connected to seasonal variability. It has been studied that mid-winter and mid-summer have higher rates of mortalities, but winter season having more deaths compared to summer. This is related to cold, wind and moisture during the season. Especially among dystocia calves are suffering from winter due to their lower metabolic rate and therefore weaker heat production. But also heat stress influences immunoglobulin absorption due to higher corticoid levels (Uetake, 2013).

Induction of premature parturition causes higher risks of stillbirths and mortality during the first four weeks of life. Increase in mortality rate is associated with severity of dystocia. Bull calves have also higher risk compared to heifer calves. Younger calves have higher risk of death since 50% of neonatal deaths happen in the first week of life (Uetake, 2013).

Lower mortality, morbidity and higher growth rate have been noticed in calves fed with pasteurized nonsaleable milk compared to calves fed with commercial milk replacement (Uetake, 2013).

Higher mortality is also reported in group-housing systems compared to individual housing. In stable groups the daily weight gain was higher compared to those in dynamic groups. Dynamic group means that new calves are introduced and exited the group continuously. Also, prevalence of diarrhea and pneumonia is reported to be higher in dynamic group-housing, compared to stable groups. Morbidity risk decreases significantly when calves of similar age are grouped together. Housing calves alone and outdoors with shelter reduces the risk of disease. There has been no difference in morbidity or average daily weight gain whether calves were housed alone indoors or outdoors within 2 weeks of life, but it has been noticed that outdoor housing gives long term benefits reducing the occurrence of diarrhea and pneumonia (Lorenz et al., 2011).

1.3. Calf neonatal period diarrhea causes

1.3.1. Rotavirus

Rotavirus is one of the primary agents in neonatal diarrhea. It has double-stranded RNA segments and is very stable in environment for pH and temperature (Cho and Yoon, 2014). In a study from California, they discovered that 94% of calves aged 0-14 days old, shed rotavirus. 96% of calves did not have any symptoms of illness (Bristol et al., 2021). In some studies, the prevalence of rotavirus in healthy calves was found to vary between 2% to 12% and in diarrheic calves between 7% to 30%. There were also studies that did not find rotavirus in healthy calves but only from diarrheic calves (Gomez and Weese, 2017). Some studies have found out, that 20-60% of diarrheic calves are positive for rotavirus infection (Geletu, et al., 2021). In Estonia, the prevalence of rotavirus is 70% of farms (Viidu and Mõtus, 2022), but this study only included 15 farms.

Route of rotavirus infection is fecal-oral (Bristol et al., 2021). Rotavirus has short incubation time, 24-48h, and it causes usually self-limiting mild to moderate diarrhea. In addition, infection causes poor growth rate, and increased mortality (Geletu et al., 2021). Infection can stay subclinical, and most calves that fall sick, have diarrhea already on the first day of rotavirus excretion. Rotavirus replicates in the small intestine, especially in the upper part. It causes

enterocytes to vacuolate and disarrange. Villi fuse and detache from the epithelial layer (Reynolds et al., 1985). Villus atrophy thereafter causes maldigestion and malabsorption leading to undigested feed in the colon, bacterial overgrowth, and higher osmotic pressure (Blanchard, 2012).

Rotavirus is one of the most common causes for diarrhea in calves less than one month old. It has many hosts, and it is also a major pathogen causing diarrhea in humans. To reduce economic losses caused by rotavirus, it is important to take care of adequate colostrum intake, vaccinations and farm management (Geletu et al., 2021). Pre-calving vaccination of dams against rotavirus, coronavirus and ETEC significantly decreases the calf mortality rate and positively impacts calves' health (Viidu and Mõtus, 2022).

1.3.2. Coronavirus

Bovine coronavirus (BCoV) causes enteric disease or respiratory disease. Enteric disease can be divided into calf diarrhea in calves and winter dysentery in adult cattle. These diseases provide cross-immunity between each other to some extent. The disease is spread worldwide. Morbidity is high but mortality is generally low. It is species-specific but other species can present as reservoirs (Hodnik et al., 2020). BCoV usually causes clinical enteritis illness in calves 5 to 30 days of age. Incubation time is two days and clinical signs continue three to six days. Diarrhea caused by coronavirus contains blood clots and has watery consistency. Due to reduced fluid and feed intake, calf becomes dehydrated quickly and suffers from metabolic acidosis and hypoglycemia (Gomez and Weese, 2017). Infection starts from small intestine, but spreads to the whole intestine. Infection leads to atrophy of the villi and colonic crypts, and eventually necrosis of the lamina propria (Cho and Yoon, 2014).

Infection with the virus itself is not enough to cause a disease, but stress and immune deficiency can cause disease in individuals infected with the virus (Hodnik et al., 2020). BCoV is isolated from healthy and diarrheic calves. In one study, the prevalence of BCoV in calves varies from 5% to 17%. In healthy calves, prevalence of BCoV was found to be 13% and in diarrheic calves 2,3%, therefore the importance of BCoV as primary pathogen is hard to evaluate (Gomez and Weese, 2017).

1.3.3. Enterotoxigenic *Eschericia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common cause of calf diarrhea and it usually causes disease in calves' less than four days old (Al-Alo et al., 2018). It is gram-negative bacterium and part of a commensal intestinal microbiota. Some strains are able to cause an illness in immunologically challenged hosts (Umpierrez et al., 2019). ETEC causes intestinal epithelial cells to adhere, and it remains in biofilms (Umpierrez et al., 2017). ETEC produces heat-stable and heat-labile enterotoxins which cause hypersecretion of fluids into the intestinal lumen, resulting in diarrhea, dehydration, and acidosis (Umpierrez et al., 2017). It can cause mild to peracute diarrhea, and sometimes causes also general signs. Heat-stable enterotoxin and F5 fimbriae are most common combination of virulence factors in cattle. ETEC is shown to be antimicrobial resistant in many calves in Spain. In one study, 100% of isolated ETEC were multidrug resistant and 44% were resistant to several antimicrobials simultaneously, including tetracyclines, trimethoprim/sulphonamides, fluoroquinolones, and some beta-lactam antibiotics. 100% of ETEC strains were resistant to ampicillin and 76% to amoxicillin/clavulanic acid. These findings emphasize that the treatment of choice in case of *E. coli* should not be antibiotics unless there is general illness present (Prieto et al., 2021).

1.3.4. Salmonella

Salmonella enterica serovars Typhimurium and Dublin are the most common *Salmonella* pathogens in cattle. *S. Typhimurium* is the most common amongst calves in the USA. Although salmonella can cause clinical signs in cattle of any age, it usually causes clinical signs in calves aged 10 days to 3 months. *S. typhimurium* is associated as diarrhea pathogen and *S. Dublin* is associated with general illness. It invades intestinal mucosa and colonizes lymphoid tissues, enterocytes and survives in macrophages. It spreads throughout the body by invading mononuclear cells and phagocytes. *Salmonella* causes watery diarrhea with mucus, blood and fibrin in feces (Cho and Yoon, 2014). Animals shed pathogens in their feces intermittently and variable periods of time, depending on the degree of infection. The most common source of infection is contaminated feed and water (El-Seedy et al., 2016). It can present as subclinical or clinical infection (Cho and Yoon, 2014). Up to 5% of infected animals become carriers and shed the pathogen in their feces. *S. typhimurium* is a non-host-adapted serotype and therefore not so likely to establish a carrier state in animals. *S. Dublin* in the other hand, is host-adapted

in cattle, and can produce a carrier state in animals. Determining the infected and carriers is important to prevent further human and other animals´ infections (El-Seedy et al., 2016). In Estonia, *S. Dublin* was the most common serotype at farm level in cattle, with the prevalence of 3,3%, found in research done in 5-year-period (Kuus et al., 2021). Other study found seroprevalence of 24.2% in large-scale Estonian dairy herds (Mõtus et al., 2021).

1.3.5. Cryptosporidium

Cryptosporidium is a protozoan, causing illness mainly in calves less than 4 weeks of age. It is a major pathogen causing diarrhea in both cattle and humans. It has a wide range of hosts, and it is found all over the world (Jang et al., 2021). Estimated prevalence of cryptosporidium infections in humans is 7.5% in the world (Dong et al., 2020). Intensity of diarrhea can vary between calves, some get more severe disease than others, but mortality is low (Duquesne and Harvey, 2020). Four different cryptosporidium species have been discovered to cause a disease in cattle, *Cryptosporidium parvum* being the most common and causing illness mainly in calves less than 20 days of age, highest rate of infections being in 11-20 days old calves. It causes over 90% of infections in neonatal calves. *Cryptosporidium ryanae* and *Cryptosporidium bovis* are commonly found in post-weaned calves up to one year old, but they usually do not cause an illness. *Cryptosporidium andersoni* infections are mainly reported in adult cattle (Jang et al., 2021).

Route of infection is fecal-oral or through contaminated water or feed. Calves start to excrete oocysts shortly after birth (Jang et al., 2021). Diarrhea starts three to four days after ingestion of oocysts (Adkins, 2022). Once *Cryptosporidium* can be found on a farm, it is difficult to get rid of *Cryptosporidium* oocysts are resistant to most disinfectants and can remain viable for a year and a half in the environment (Duquesne and Harvey, 2020).

Cryptosporidia replicate in the intestine, causing epithelial cells to become more permeable (Gerace et al., 2019). Destruction of epithelial cells of small intestine cause atrophy and impairment in nutrient digestion (Urie et al., 2018). Protozoan goes through its whole life cycle in one host, including asexual and sexual phases. Those produce thick and thin-walled oocysts, of which thick-walled oocysts are excreted in feces and thin-walled oocysts cause autoinfection of epithelial cells (Centers for Disease Control and Prevention, 2023).

Risk factors for infection are summer months, big dairy herds, usage of milk replacer, and feeding starter grain early on life. Concrete floors and good hygiene decrease the risk of

infection (Adkins, 2022). Higher prevalence of cryptosporidiosis cases has been associated with poor farm hygiene, feeding pasture and milk, and calves in contact with domestic animals. Also, calves with diarrhea were more susceptible to cryptosporidium infection (Gattan et al., 2023).

1.3.6. Eimeria

Eimeria is an intracellular protozoan parasite. There are 20 subspecies of eimeria in cattle feces but only four of them are considered to cause clinical disease (*E. alabamensis, E. auburnensis, E. bovis,* and *E. zuernii*). All subspecies are host-specific. Clinical disease occurs usually when calves are turn out to grass in Scandinavia. *Eimeria* thrives in moist and warm conditions, therefore summer season is a common time to have clinical signs of *Eimeria* in herd. It is causing less clinical disease in herds kept outside than indoors, which is related to the exposure rates. Exposure rates stay lighter in outdoors, and host is infected with smaller number of oocysts, allowing host to develop immunity against *Eimeria* before clinical illness. It infects most commonly calves of age one to two months, but sporadic cases in adults have been seen, usually related to some underlying condition. Infections are usually related to poor environmental conditions, chronic stress, and simultaneous infection with other pathogens, causing calf to be more susceptible to *Eimeria*. *Eimeria* are opportunistic pathogens, meaning that it usually does not cause clinical disease (Andrews, 2022).

Every subspecies affects different parts of intestines and different cells, causing destruction of affected cells. *E. bovis* and *E. zuernii* are affecting distal part of small intestine, cecum and colon. In *E. bovis* and *E. zuernii* infections, prepatent period lasts 15-21 days and patent period 11 days. In *E. alabamensis* infection, the prepatent period lasts 8 days and patent period 6 days. Clinical signs usually occur three weeks after infection with *E. bovis* and *E. zuernii* and after three to four days for *E. alabamensis* infection. Calf gets immunity to that specific *Eimeria* subspecies it has been infected with. Lifecycle is self-limiting unless reinfection occurs, it also suppresses the immunity making the host more susceptible to other diseases (Andrews, 2022).

Most infected calves do not have any clinical signs. Subclinical infection causes poor growth rate, loose or soft feces, and dull hair coat. It is common to calf to only have a decreased growth rate and no other clinical signs. In more severe cases calf has diarrhea, sometimes bloody, reduced growth rate, inappetence, lethargy and abdominal discomfort. Severe cases can cause anemia, recumbency, dehydration, bloody diarrhea and death. Central nervous system signs sometimes develop. Chronic infections cause poor hair quality and reduced growth rate. Sulfonamides as well as toltrazuril are treatment of choice. Treating calves with clinical signs

prevents reinfections, secondary infections, decreases mortality and decreases discharge of oocysts (Andrews, 2022).

1.3.7. Non-infectious causes

Non-infectious causes for diarrhea include abomasal hairballs and diet-related disturbances. Rearing management has a big part on inducing grooming behaviours, which are related to abomasal hairballs (Uetake, 2013). Calves can have a problem with cross-sucking if they do not get the opportunity to suckle. Incidence of cross-sucking is considerably higher in non-suckling calves compared to suckling calves. On the other hand, artificially reared calves consume more feed, compared to suckling calves. Abnormal sucking behaviour continues until the consumption of solid feed is high enough to stimulate rumination (Margerison et al., 2003). Teat feeding and feeding 20% of body weight or above prevents abnormal sucking behaviour such as pen fixtures. High amount of milk replacer has been associated with diarrhea. This might be due to the composition of milk replacer rather than the amount. Milk replacer has high amounts of total solids, which creates an osmotic gradient, causing fluid transfer to the intestines (Welk et al., 2023). Decreased prevalence of diarrhea is associated with feeding 3 litres or more of colostrum at the second feeding time after birth, feeding milk freely during the first week of life. Increased prevalence of diarrhea is associated with administration of iron soon after birth (Lorenz et al., 2021).

Dystocia has been reported to be one of the reasons associated with neonatal diarrhea. Dystocia is commonly related to too large calf size or too small pelvic size of the dam. With heifer selection, dystocia risks can be lowered when better genetic inheritance is used for breeding, favoring heifers with adequate pelvic size and family history of calving ease. Dystocia can cause swelling in the head area of the calf and therefore reduces the colostrum uptake from dam. Due to that, the amount of received immunoglobulins are lower than normally.

Herd management, colostrum quality and environmental stress factors are main aspects to predispose to neonatal diarrhea (Cho and Yoon, 2014). Environmental stress factors, for example low temperature, moisture, rain and wind increases the susceptibility for diarrhea. Calves are not able to regulate their body temperature as sufficiently as adult cows and are more prone to get hyper- or hypothermia. This impairs calves' immune system and makes them more vulnerable to pathogens. Dams are more capable to handle hard environmental conditions, but dystocia and metabolic diseases are associated with environmental stress factors, such as

weather. Cows close to calving should be placed somewhere dry and draft-free to reduce those risks (Cho and Yoon, 2014).

2. AIMS OF THE STUDY

This thesis aimed to describe the prevalence of rotavirus, ETEC, *Cryptosporidium parvum* and coronavirus in up to three-weeks old diarrheic dairy calves reared in large-scale Estonian dairy herds and analyze their association with mortality risk.

3. MATERIALS AND METHODS

3.1. Animals and sampling

3.1.2. Herd selection

This study is part of the wider research project aiming to identify the reasons and risk factors for dairy cattle culling and mortality in 120 large-scale Estonian dairy herds. The inclusion criteria for the study farms were farm size of at least 100 cows (based on the herd size data from beginning of 2019), loose-housed keeping system for milking cows and no intention to terminate production in the near future. A list of herds meeting the size criterion was obtained from Estonian Livestock Performance Recording Ltd and included 182 farms.

A random sample of 120 herds were taken from this list of farms and the farm managers or veterinarians were contacted individually to determine the compliance with the other two inclusion criteria and willingness to participate in the study. In total, 169 herds were contacted until the desired sample size of 120 herds was achieved. There were two farms later excluded from the present study due to not keeping calves on the farm for more than a few weeks.

3.1.2. Animal selection and sampling

All herds were visited once between August 2019 and July 2020 to gather data and samples. The aim was to sample at least five diarrheic calves aged up to 21 days from each farm. The samples were taken directly form the rectum or while the calf was defecating and collected into a rubber glove, marked, stored in a cooler and transported to the laboratory on the same or the following day where the samples were frozen until analysis. In farms where five fecal samples could not be obtained during the first visit (n = 65) due to not having enough diarrheic calves within suitable age range, another round of sample collection was done in December 2020 and January 2021. In the second round of sample collection, calves with visible signs of diarrhea were preferred, but in cases where no such calves were present, the samples were taken from apparently non-diarrheic calves within the determined age range. Also, before additional visit, farms were advised to collect fecal samples from diarrheic calves on a running basis and store these in the freezer until the sample collection visit. There was a total of five farms where five samples could not be obtained with two sampling rounds and the total number of analyzed fecal samples was 640.

3.2. Laboratory methods

To analyze calf fecal samples, we used ELISA kit BIO K 348 (Bio-X Diagnostics, Rochefort, Belgium) for rotavirus, coronavirus, *E. Coli* F5 attachment factor and *Cryptosporidium parvum* antigen testing. The kit uses sandwich method for diagnosing the pathogens from fecal samples. Kit has 96-well microtitration plates. Half of the rows have immobilized specific antibodies against those pathogens, and the other half of the rows have controls, containing non-specific antibodies. With controls we can differentiate between true and false positives. Every row has a specific antibody against one pathogen, to indicate which pathogen antigens can be found in calves' feces.

Fecal samples need to be diluted in dilution buffer and after that, samples are incubated in microplates for one hour at 21°C +/- 3°C. After incubation, plates need to be washed so conjugates, in this case, antibodies can be added. These monoclonal antibodies are peroxidase-labelled. After that, plates are incubated for the second time and chromogen is added. If there are these specific pathogens present, they will be attached to the immobilized antibodies and peroxidase catalyzes chromogen to change its color. After that, plates need to be incubated for a third time. Results can be read with a photometer, reading optical densities. Positive control antigen yield needs to have greater values than those given on the QC data sheet, or the test is not valid.

3.3. Data handling

Some calves were not ear-tagged at the time of sampling causing lack of relevant information (date of birth and possible death). Due to this, 25 samples had to be neglected from the final data analysis resulting in exclusion of two more herds. Consequently, this study included 615 calf-based records and sample results from 116 herds. Average number of calf-based samples/records included to the study was 5 (range 1-8) (Figure 1).

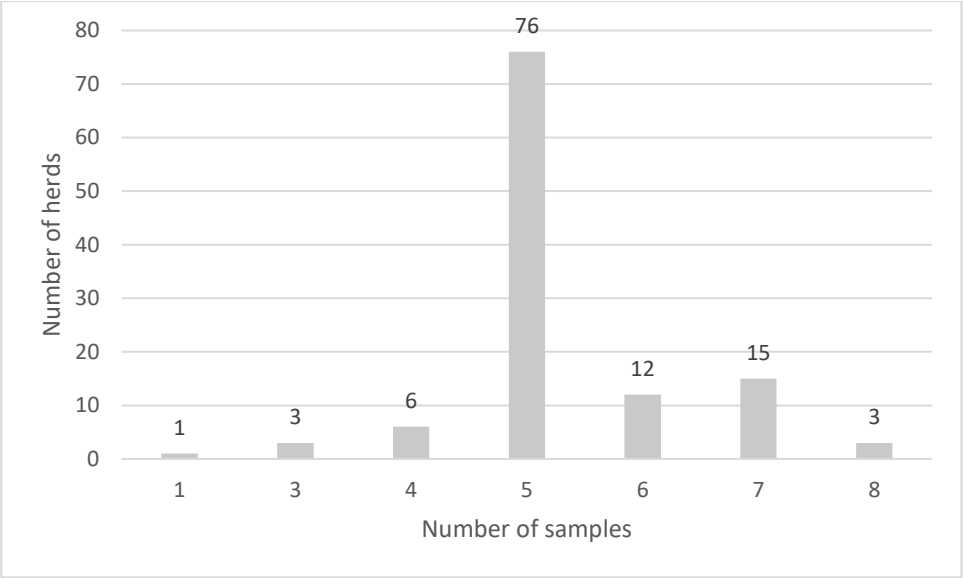

Figure 1. Distribution of herds based on number of calf-based observations/samples included to the analysis (n = 116 herds).

Calf-based information including the birth date, gender (male/female), breed, date of selling, export, slaughter or death was achieved from the Estonian Animal Recording and Information Board (EARIB) in Excel format. Two datasets (calf-based data and laboratory results) were merged by calf id-numbers. Calf age at sampling was missing for 125 observations due to not registering the exact date the sample was collected by farm personnel.

For each herd, data regarding the number of cows and average 305-day milk yield (on the date of the visit and exactly one year before) was collected from Estonian Livestock Performance Recording Ltd. The average number of cows in the herd and average milk yield were calculated as means from the number of cows in the herd/milk yield at the day of the farm visit and one year before the visit.

3.4. Statistical analysis

Descriptive statistics were used to represent the prevalence of ETEC, coronavirus, *Cryptosporidium parvum* and rotavirus. Proportion of calves positive for a certain pathogen out of all calves sampled was calculated as a percentage. Exact 95% Confidence Intervals (CI) were calculated for the pathogen prevalence using "*ci*" command in Stata® MP14.2 (College Station, TX: StataCorp LP).

Due to calves contributing different time to the analysis, survival analysis was used to identify if there is association between calf positive status for different diarrhea-causing pathogens and

mortality probability during the first three months of age. For this, the data was declared as survival data using the "*stset*" command. The start of the observation period was set as the birth date and calf-based observation period lasted until death (declared as the event of interest in the analyses), slaughter or selling (the observation period was ended at the date of these events resulting with right-censoring of the observation). Half a day was added to the date which ended the analysis in order to retain calf (n = 1) that was sold at its birth date in the analysis.

Kaplan-Meier graph was used to graphically display the survival probability across calves with different pathogens tested. To calculate the mortality rate, "*strate*" command was used and mortality rate estimates were calculated for the unit of 100 calf-months. Cox proportional-hazards regression model was chosen for the data analysis due to allowing to investigate animals' survival probability related to multiple predictors and including the information about time-to-event (George et al., 2014). Tested predictors in the model were calf fecal sample status for ETEC, *Cryptosporidium parvum*, rotavirus, coronavirus antigens, breed and gender. In comparison of breeds there were three different groups. Three calves were put into one group (Estonian native, Blonde d'Aquitaine and Simmental) for the statistical analysis. Outcome variable was calf mortality (no / yes) during the first three months. Due to herd clustering effect, farm was included as random effect to the model. At first, the effect of pathogens to calf mortality hazard were tested individually (called univariable analysis). After this, a multivariable model was composed including all the pathogens but also the calf gender and breed were included due to their possible confounding effects. Due to large number of missing data, calf age was not included to the model. Herd size was also tested for the confounding action. Interaction between the tested pathogens was included to test for the combined effect of pathogens. Insignificant predictors were removed from the model. The associations were considered statistically significant if p-value was below 0.05 and showing a trend if $p < 0.20$.

3.5. Ethics

The study was approved by Animal Experiment Project License Commission of the Ministry of Rural Affairs – License nr. 147, date 03.07.2019. Farm data was used in anonymous way in the data analysis with the permission of the farm manager. This work was supported by an Estonian Research Council Grant (PSG268). There is no conflict of interest

4. RESULTS

4.1. Sampled calves and farm data

In total, 615 calf-based observations were included from 116 farms. Calf average age at sampling was 9.9 days, minimum being 0 days and maximum 43 days (for 125 calves the age data was missing).

The mean number of cows per herd was 517. Smallest herd had on average 92 cows and the biggest herd had on average 2275 cows. Average milk yield per herd was 10 330.8 litres, minimum being 5983 litres and maximum being 13 155 litres.

4.2. Pathogen prevalence

From the sampled calves, number of rotavirus-positives were 162 calves, sample prevalence was 19.7% (95% CI 16.6; 23.0), coronavirus-positives were 12 calves, sample prevalence 2.0% (95% CI 1.0; 3.4), ETEC-positives were 27 calves, sample prevalence 4.4% (95% CI 2.9; 6.3), *Cryptosporidium parvum*-positives were 205 calves, sample prevalence 33.3% (95% CI 29.6; 37.2). Prevalence and number of pathogens can be seen in Table 2. *Cryptosporidium parvum* and rotavirus were the most common pathogens found in our study and the combination of these two were the most common combination of pathogens (Figure 1).

Table 2. Pathogen prevalences of 615 diarrheic calves tested in 116 Estonian dairy herds

Variable	Rotavirus	Coronavirus	ETEC	*Cryptosporidium parvum*
Negative	494	603	588	410
Positive	121	12	27	205
Mean prevalence (%)	19.7	2.0	4.4	33.3

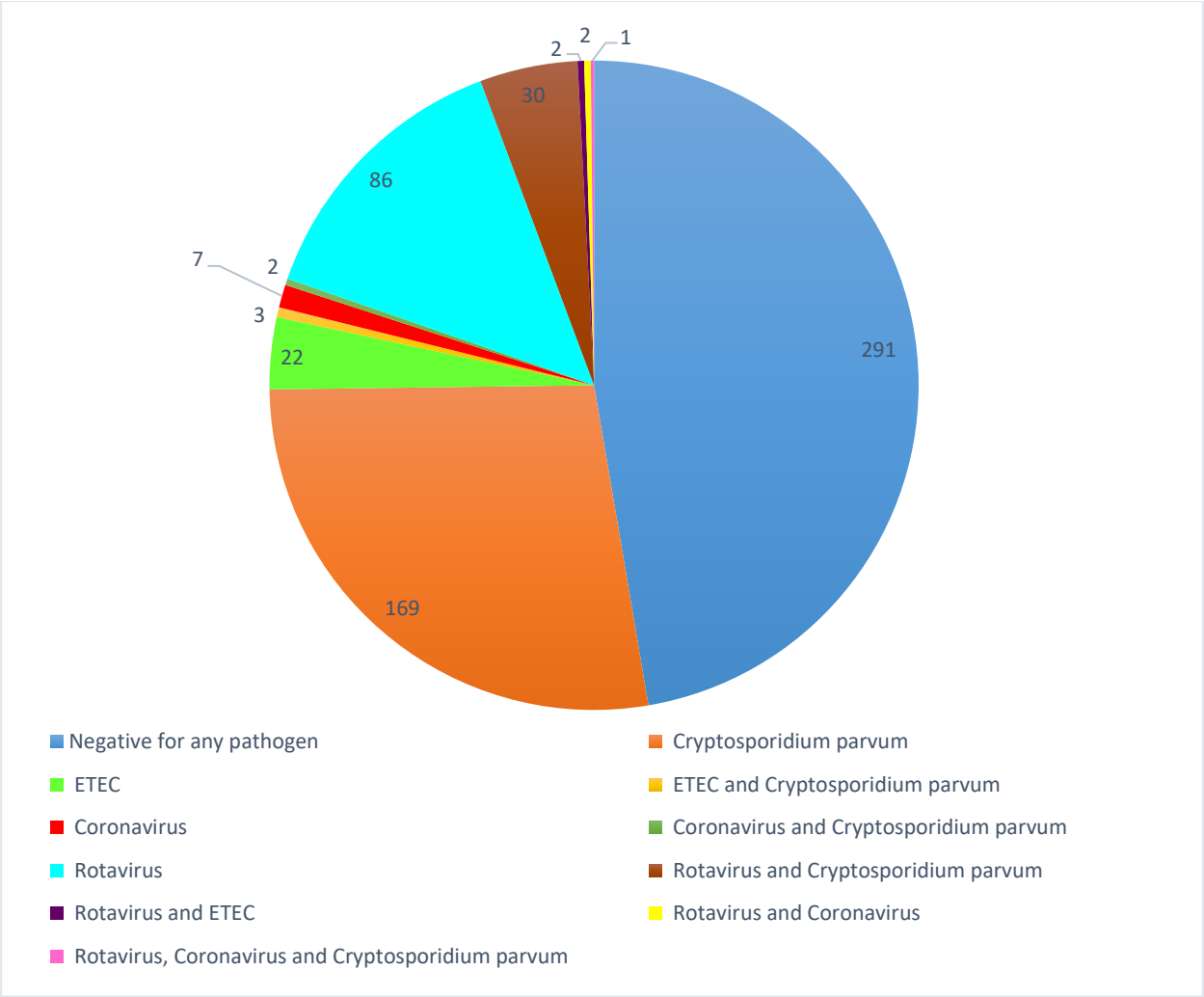

Figure 1. Distribution of calves based on the tested faecal pathogens (n = 615).

Age distribution in pathogen-positive calves can be seen in Table 3. Mean age of rotavirus positives was 8.9 days, coronavirus 9.7 days, ETEC 4 days and *Cryptosporidium parvum* 10.8 days.

Table 3. Average age of infected calves, minimum, maximum, and standard deviation included (n = 490, the data was missing in for 125 calves).

Variable	Mean age (days)	Minimum days of age	Maximum days of age	Standard deviation (days of age)
Rotavirus	8.9	1	39	4.9
Coronavirus	9.7	2	19	5.7
ETEC	4	1	15	3.3
Cryptosporidium parvum	10.8	0	39	4.4

4.3. Mortality of diarrheic calves

Among 615 calves, 330 stayed in the herds and were alive up to the age of three months (53.7%), 41 calves died (6.7%), 4 were slaughtered (0.7%), 23 were exported (3.7%) and 217 were sold (35.3%) before reaching three months old (Figure 2). The death prevalence of the calves enrolled in this study was 6.7% (95% CI 4.8; 8.9) and the overall mortality rate was 3.3 (95% CI 2.5; 4.5) per 100 calf-months.

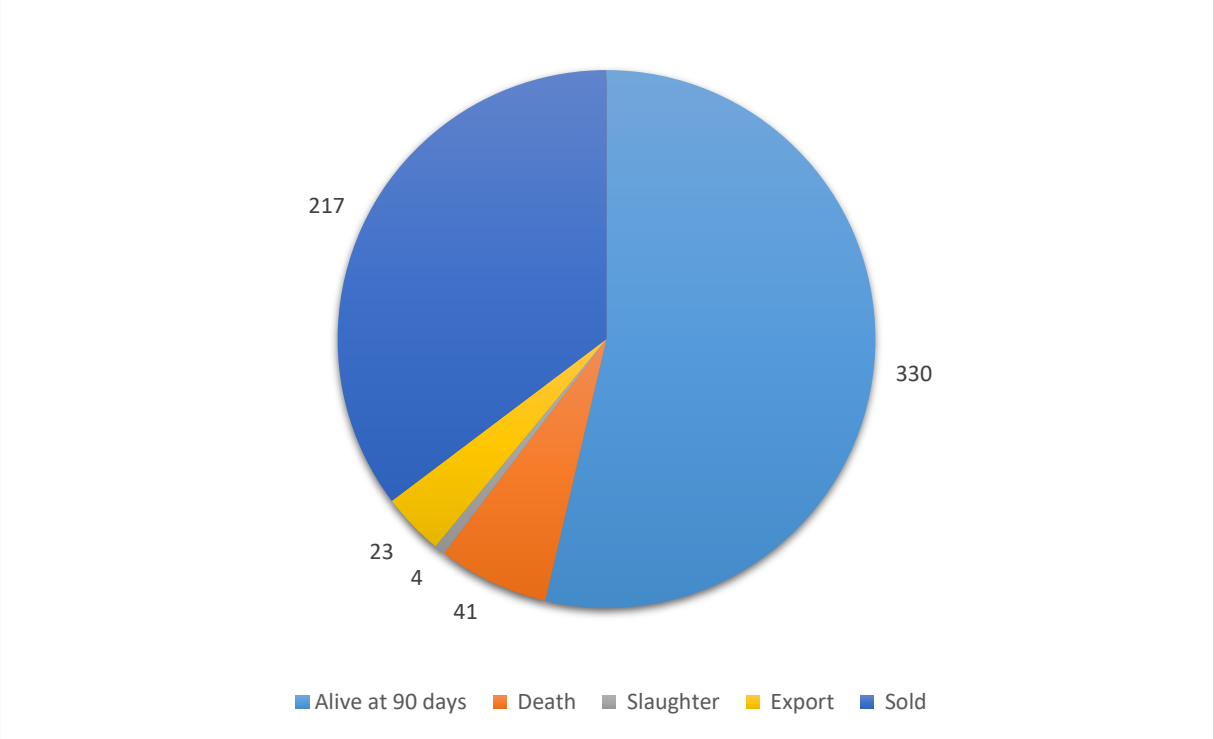

Figure 2. The events occurring for sampled calves during the 90 days observation period (n = 615 calves from n = 116 herds).

Calves contributed to the analysis on average 60.6 days (standard deviation (SD) = 33.6), minimum being 0.5 days and maximum 90.5 days. Survival time for the 41 deceased calves was on average 31.7 days, range being 2.5 to 85.5 days. The median age at death of the deceased calves was 18.5 days (Figure 3).

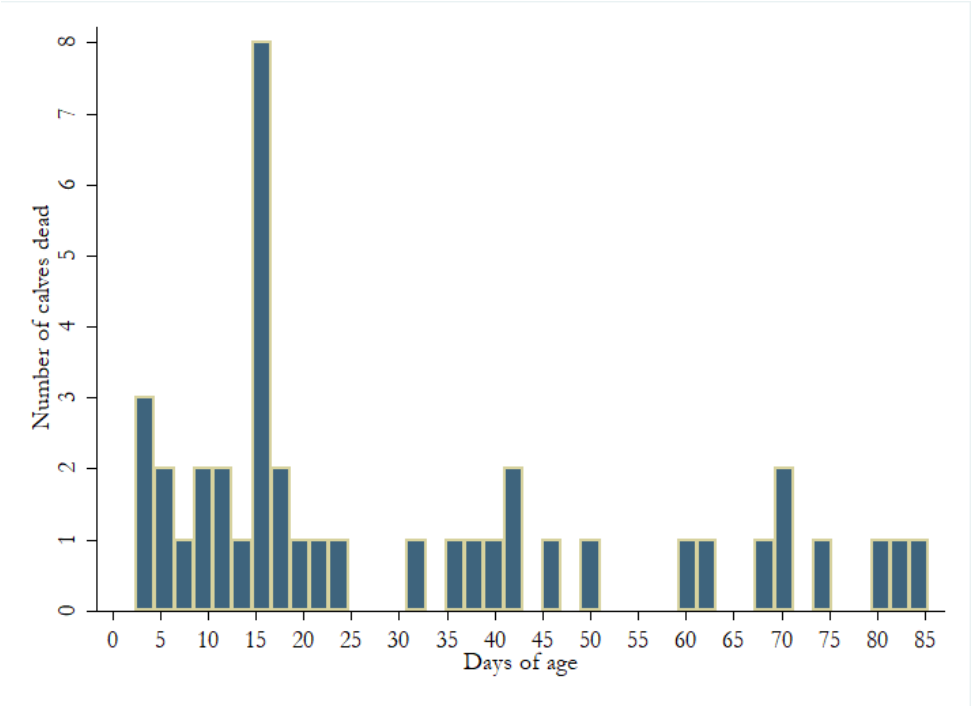

Figure 3. Distribution of survival times for calves that died (n = 41 calves)

There were 296 (47.6%) bull calves and 322 (52.4%) heifer calves in the analysis. Bull calves' mortality rate was 6.6 (95% CI 4.3; 10.2) per 100 calf-months and 2.2 (95% CI 1.4; 3.4) per 100 calf-months for heifer calves. According to the univariable random-effects Cox regression model, the mortality hazard of bull calves was HRR = 2.7 (95% CI 1.3; 5.3, p = 0.005) compared to heifer calves.

There were 549 Estonian Holsteins (89.3%), 63 Estonian Reds (10.2%), one Estonian native (0,2%), one Blonde d'Aquitaine (0,2%) and one Simmental (0.2%) breed calf. The average mortality rate for Holstein calves was 3.3 (95% CI 2.4; 4.5) per 100 calf-months, and for Estonian Reds' 4.2 (95% CI 1.7; 10.0) per 100 calf-months. According to the univariable random-effects Cox regression model, the mortality hazard of Estonian Red breed calves was not statistically higher compared to Holstein breed calves (HRR = 1.2, 95% CI 0.4; 3.2, p = 0.725).

4.4. Association between diarrhea pathogens and calf mortality

The mortality rate of rotavirus-positive calves was 3.0 (95% CI 1.4; 6.4) and 3.4 (95% CI 2.4; 4.8) per 100 calf-months for the rotavirus-negative calves. According to the univariable Cox

regression model, the survival probability did not differ between groups of calves with different rotavirus status (p = 0.812) (Figure 4).

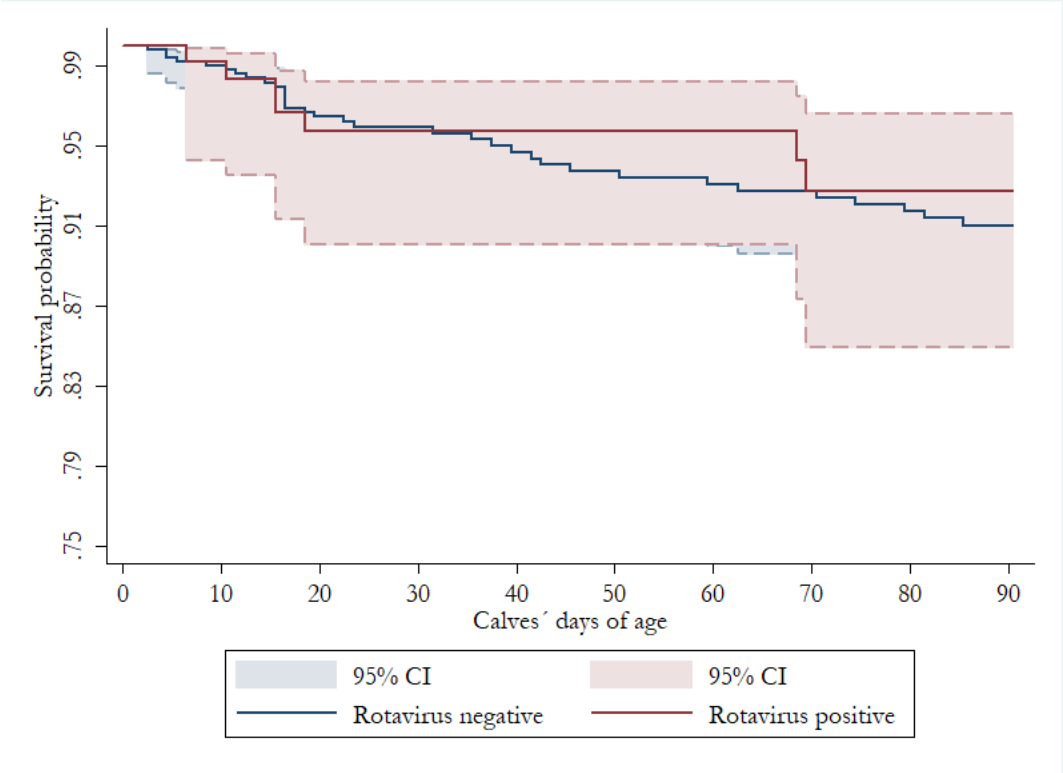

Figure 4. Kaplan-Meier graph showing the survival probability of rotavirus negative and positive diarrheic calves.

In coronavirus-positive calves, the average mortality rate was 8.4 (95% CI 2.1; 33.7) and for the negative group 3.2 (95% CI 2.0; 18.9) per 100 calf-months. According to the univariable Cox regression model, the survival probability did not differ between groups of calves with different coronavirus status (p = 0.280) (Figure 5).

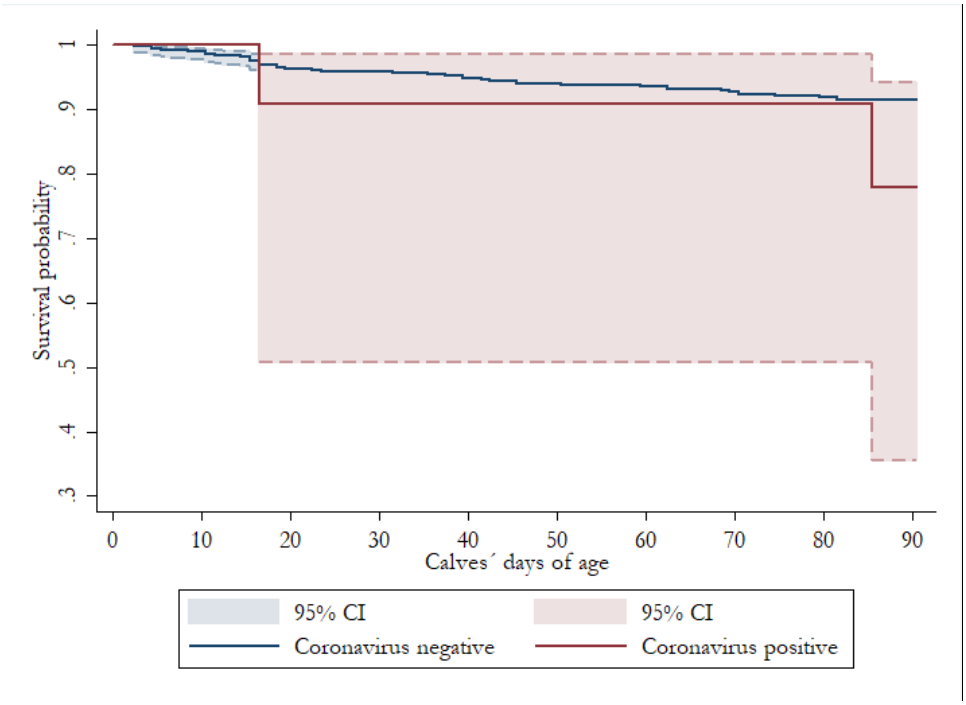

Figure 5. Kaplan-Meier graph showing the survival probability of coronavirus negative and positive diarrheic calves.

In ETEC-positive group of calves, the average mortality rate was 6.1 (95% CI 2.0; 18.9) and in ETEC-negative group 3.2 (95% CI 2.4; 4.4) per 100 calf-months. According to the Kaplan-Meier graph, the survival probability was somewhat higher for the ETEC-negative group of calves. According to the univariable Cox regression model, the survival probability during the first 90 days of age did not differ between groups of calves with different ETEC status ($p = 0.240$) (Figure 6).

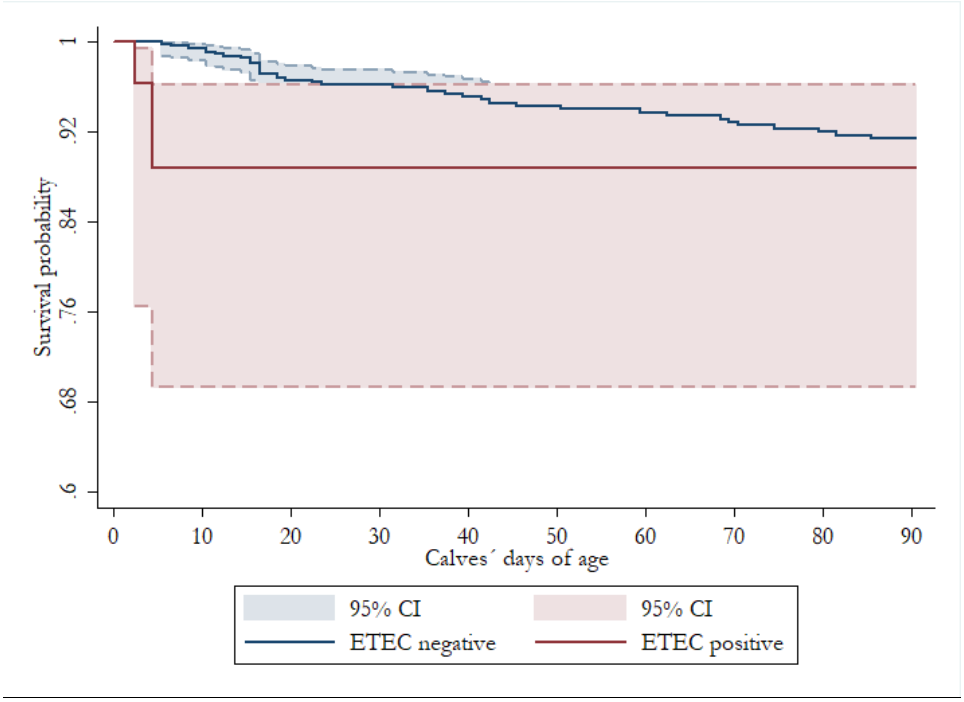

Figure 6. Kaplan-Meier graph showing the survival probability of ETEC negative and positive diarrheic calves.

In *Cryptosporidium parvum*-positive group of calves, the average mortality rate was 3.8 (95% CI 2.3; 6.3) and for negative-group 3.1 (95% CI 2.1; 4.6) per 100 calf-months. According to Kaplan-Meier graph, *Cryptosporidium parvum*-positive calves had somewhat higher survival probability compared to pathogen-negative calves. According to the univariable Cox regression model, the survival probability did not differ between groups of calves with different *Cryptosporidium parvum* status (p = 0.634) (Figure 7).

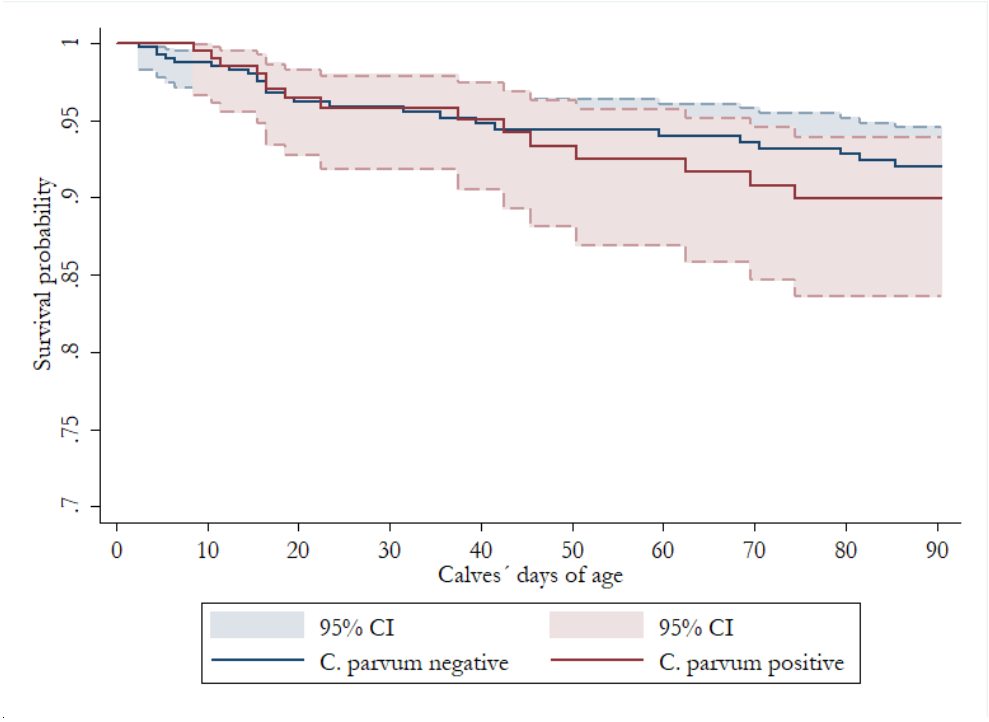

Figure 7. Kaplan-Meier graph showing the survival probability of *Cryptosporidium parvum* negative and positive diarrheic calves.

Mortality risks of different pathogens' positive and negative groups are shown in Figure 8.

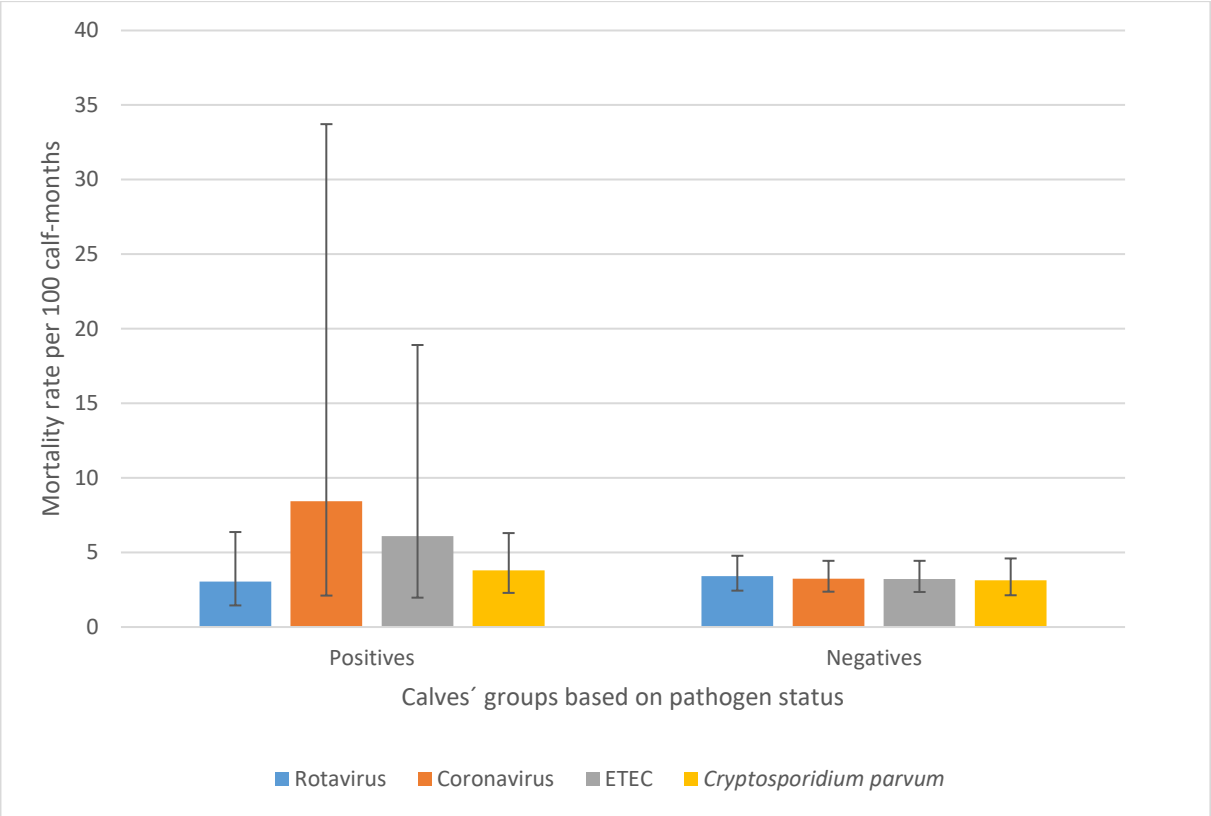

Figure 8. Calf mortality rates across different pathogen status (n = 615 calves from n = 116 herds).

Multivariable Cox regression analysis revealed that rotavirus and *Cryptosporidium parvum* coinfection had higher hazard ratio, although it is not significant according to p-value but is considered showing a trend. Single pathogen infections did not have statistically significant difference in mortality probabilities. Males had significantly higher mortality probability compared to females (p = 0.006) (Table 4).

Table 4. Multivariable mixed-effect Cox regression model including herd as random factor analysing the association between calf faecal pathogen status and mortality probability during the first 90 days of age in 615 diarrheic calves from 116 Estonian dairy herds

Variable	Category	N of animals	HRR[a]	95% CI	p-value
Rotavirus status	Negative	494	1		
	Positive	121	0.50	0.15; 1.72	0.273
Cryptosporidium parvum status	Negative	410	1		
	Positive	205	0.95	0.45; 2.00	0.884

Table 4 continued

Rotavirus and *Cryptosporidium parvum* coinfection	Negative	585	1		
	Positive	30	3.75	0.68; 20.83	0.130
Gender	Female	322	1		
	Male	293	2.62	1.32; 5.21	0.006

[a]Hazard Rate Ratio

5. DISCUSSION

The aims for this study were to describe the prevalence of four main diarrhea pathogens in up to three-weeks old diarrheic dairy calves in large-scale Estonian dairy herds and find the association with mortality probability. The main findings of the study were that *Cryptosporidium parvum* and rotavirus were the most prevalent pathogens in up to three-weeks old Estonian dairy calves and combined infection of these two pathogens resulted in somewhat increased mortality hazard during the first 90 days of age.

Cryptosporidium parvum is the most common diarrhea pathogen identified in our study, and similar findings have been found in previous studies (Blanchard, 2012). However, there seems to be a geographical factor since the prevalence differs from study to study (Falkenberg et al., 2022; Gattan et al., 2023). In a study done in Korea (Chae et al., 2021), *Eimeria* spp. (31.4%) was the most prevalent pathogen found in calves, followed by rotavirus (15.2%), coronavirus (10.3%), *Cryptosporidium parvum* (8.3%), and *E. coli* K99 (0.5%). On the other hand, *Cryptosporidium parvum* and rotavirus were the only pathogens that showed significantly higher prevalence in diarrheic calves compared to non-diarrheic calves. Prevalence of *Eimeria* spp. did not show significant difference when these two groups were compared. This study is not directly comparable with our study, since they had a healthy control group, versus our study which included mostly diarrheic calves.
Rotavirus was second, ETEC was third and coronavirus was fourth most common pathogen found in our study. This gives quite a good review on pathogen prevalence in Estonia in diarrheic calves. Result is similar as in Swedish study (Björkman et al., 2003) where *Giardia intestinalis*, *Cryptosporidium parvum* and rotavirus were the most common pathogens found from dairy calves. Coronavirus and *E. coli* K99+ were not that prevalent and only found from few calves.

Mean age for rotavirus infection was 8.9 days, for coronavirus 9.7 days, for ETEC 4 days and for *Cryptosporidium parvum* 10.8 days. This follows common age pattern as described in literature (Geletu et al., 2021; Gomez and Weese, 2017; Al-Alo et al., 2018; Jang et al., 2021; Naylor, 2009).

The univariable Cox regression model did not confirm increased mortality risk if the calf had rotavirus or *Cryptosporidium*, but calves that had these two pathogens combined, had increased

mortality risk compared to calves tested negative to both of these pathogens. P-value was over 0.05, meaning the result is not significant, but lower than 0.20, and this is considered as a trend. Snodgrass et al., (1986) studied the prevalence of coinfection of diarrhea causing pathogens and found that 15% of diarrheic calves had a coinfection with two or more diarrhea causing pathogens. It is proved by many studies that coinfections are common among diarrheic calves (Brunauer et al., 2021). As described in the literature review, many calves shedding rotavirus, do not have any symptoms of illness (Bristol et al., 2021). As our results describe, calves become infected with rotavirus earlier (8.9 days) than with *Cryptosporidium parvum* (10.8 days) and this could suggest that infection with rotavirus lowers the immune system increasing the susceptibility to *Cryptosporidium parvum* infections that compromises the immune system even further leading to severe disease and increased mortality risk (Chen et al., 2017).

ETEC infections entailed possibly time-dependent effect to calf mortality as ETEC-positive group of calves had somewhat lower survival probability during the first 20 days compared to ETEC-negative calves. As described in literature review, ETEC infects mostly very young calves, under four days old calves (Al-Alo et al., 2018). Our average age for ETEC infection was four days. Older calves are more ready to fight against the infection, as the cortisol levels in blood decreases after the first week of life and neutrophils become functional. All essential immune components are fully functional until two to four weeks and continues to develop (Chase et al., 2008), increasing the survival probability. Also, K99 antigen of ETEC is able to bind to small intestine in young calves, but older calves are naturally resistant (Foster and Smith, 2009). Mortality rate in ETEC positive group was 6.1 and in negative group 3.2. ETEC was the second smallest positive group we had, and sample size was only 27 animals.

Bull calves had much higher mortality rate compared to heifer calves. We need to consider also, that vast majority of sold, exported, or slaughtered calves were also bull calves, reducing the amount of animal-time at risk for bull calves in our study. As discussed in the literature review, bull calves suffer more from dystocia, and possibly making them weaker from the early life (Murray et al., 2015). The increased on-farm mortality rate identified in bull calves can also be explained by their shorted time period staying in the herds. Mortality probability is not equal during the three months of calves' life being the highest immediately after birth and during the first few weeks and declining thereafter (Astiz et al., 2017). In Estonian dairy herds, bull calves are sold roughly at the age of two to three weeks which means that the time-at-risk accounted in the analysis included the most hazardous time period for them. While the data analysis

adjusted the mentioned aspects by calculating the mortality rate per animal days-at-risk rather than presenting mortality risk as a percentage and by employing survival analysis considering the duration of each calf's contribution to the analysis, it still does not fully address the inherent variability in risk periods for calves based on genders.

Mortality rate for coronavirus positive group was 8.4 and negative group 3.2 per 100 calf-months. The survival probability did not differ between these groups (p-value = 0.280). Coronavirus positive group had only 12 calves, and there were two deaths, which makes the mortality rate seem quite high. Mortality in coronavirus diarrhea patients is generally low, only 5-10% in uncomplicated diarrhea cases, but it can increase rapidly in case of secondary bacterial or viral pathogens (Torres-Medina et al., 1985).

Previously, Reimus et al. (2017) found that Holstein calves had higher mortality probability compared to Estonian Red calves. In our research, we did not find a difference. In either study, there can be confounding effect from the herd management causing distortion of the result.

Calves came from 116 different farms, and therefore farm was included as a random factor. Herd sizes varied a lot, which creates a great difference in management. Most possible, a specialized calf care workers are responsible for dairy management and feeding of calves in such herds. As discussed in the literature, mortality risk is higher in calves handled by workers compared to the owners (Uetake, 2013). Also, increased herd size increases mortality rate (Alvåsen et al., 2012). We included herd size to the multivariable model as a fixed effect, but it did not affect mortality neither the tested associations. We did not include small farms (less than 100 cows) in our study, which might have had an effect to mortality. Nevertheless, the mortality rates identified in this study should be interpreted with caution as we preferably included diarrheic calves only without the healthy control group. It is known and shown in different studies all over the world, that calves suffering from diarrhea have higher mortality rates (Gomez and Weese, 2017; Sivula et al., 1996; Svensson et al., 2006).

It should also be acknowledged that testing all the possible co-infections was not technically feasible due to relatively low number of positive calves for combined infections. Therefore, comparing calves based on single pathogen status, both the pathogen-negative and -positive calves might entail calves positive or negative for other pathogens. Also, the observation period for calves was chosen 90 days which roughly corresponds to the pre-weaning period. Still, the

direct effect of a pathogen to mortality might be time-dependent. This was evidenced for ETEC but also for *Cryptosporidium parvum* in Kaplan-Meier analysis. In case of ETEC, the mortality usually occurs during the first four days so it is expected to see the increased mortality probability during the first few weeks. For *Cryptosporidium parvum,* the effect was opposite as the pathogen-positive group of calves had slightly increased survival probability in early life. It is difficult to provide explanation for this but we could speculate the opportunistic effects between pathogens might occur meaning that testing negative for one pathogen might increase the risk for suffering other infections or non-infectious diarrhea problems bearing in mind that majority of the included calves were diarrheic. Still, future studies should clarify these aspects.

5.1. Study limitations

Weaknesses in our study were the small study groups in some of the positive groups, as ETEC and coronavirus. Even though our sample size was rather high (615 calves), the mortality events were rather rare. Coronavirus positive group had only 12 calves and ETEC positive group had 27 calves, making it hard, if not impossible, to identify the difference in mortality rates. Also, we have got only one pathogen combination big enough to calculate mortality probabilities. In our study, mortality rates did not differ in single pathogen infections, but negative groups were only negative for those particular pathogens, meaning that mortality in negative groups could have been affected by other pathogens. It remains unknown, if there are secondary pathogens involved in our study calves causing neonatal diarrhea and increasing mortality risk as these pathogens, e.g. salmonella, eimeria or clostridia, were not detected in our study. Also, we do not know when the calves were infected and there could have been false negatives, due to timing of the testing.

We were comparing diarrheic calves to diarrheic calves, meaning our comparison group animals were also clinically compromised, possibly suffering from dehydration, metabolic acidosis, and hypoglycemia due to malabsorption or hypersecretion (Grünberg, 2021). This also causes higher mortality rates in our negative status groups. Probably there would be more drastic difference in mortality rates between the study groups, if we were comparing diarrheic calves to clinically healthy calves. Calves suffering from diarrhea have overall increased mortality probabilities (Gulliksen et al., 2009; Abebe et al., 2023). As we know, calf mortality is multifactorial (Ismail and Muhaffel, 2022), and we did not take into account every

confounding affect, for example vaccination status of the dam, twinning, dam parity, birth season (Ismail and Muhaffel, 2022).

CONCLUSIONS

In the present Thesis, the prevalence of rotavirus, ETEC, *Cryptosporidium parvum* and coronavirus in up to three-weeks old dairy calves reared in large-scale Estonian dairy herds are described. This study revealed that *Cryptosporidium parvum* was the most prevalent pathogen among diarrheic calves (33.3%), followed with rotavirus (19.7%). ETEC and coronavirus had rather low prevalence. We were not able to find a difference between single pathogen infections and the negative group across these four tested pathogens, but *Cryptosporidium parvum* and rotavirus positive calves had somewhat increased mortality probability. ETEC infection also entailed somewhat increased mortality probability during the first weeks of life.

Our study describes well the pathogen prevalence in Estonia, and we can state that cryptosporidium and rotavirus infections are prevailing diarrhea pathogens in Estonian dairy calves. By using this information, taking preventative measures against *Cryptosporidium parvum* and rotavirus infections to substantially lower the death occurrence among neonatal calves, is recommended.

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APPENDICES

Appendix 1. Non-exclusive licence for depositing the final thesis (indefinite restriction) and the supervisor's (supervisors') confirmation for allowing the thesis for the defence

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