Antimicrobial resistance, serologic and molecular characterization of *Escherichia coli* isolated from calves with severe or fatal enteritis in Bavaria, Germany

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Meiner Familie

Cuando tienes una familia que te deja que te expreses como eres, es lo mejor que te puede dar la vida.

– Rosario Flores

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ABBREVIATIONS

A/E lesions	attaching and effacing lesions
BCoV	bovine coronavirus
BRV	bovine rotavirus
BVD	bovine viral diarrhea
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAEC	diffusely adherent *Escherichia coli*
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	enteroaggregative *Escherichia coli*
ECD agar	*E. coli* direct agar
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *Escherichia coli*
ESBL	extended-spectrum β-lactamase
ETEC	enterotoxigenic *Escherichia coli*
EU	European Union
Gb3 receptor	globotriaosylceramide receptor
HC	hemorrhagic colitis
HUS	hemolytic uremic syndrome

IM	intramuscular
IV	intravenous
kg	kilogram
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
LT	heat-labile toxin
mg	milligram
PCR	polymerase chain reaction
q12hr	every 12 hours
q24hr	every 24 hours
RTX-toxin	repeats-in-toxin-toxin
SC	subcutaneous
ST	heat-stable toxin
STEC	Shiga toxin producing *Escherichia coli*
Tir	translocated intimin receptor
XLD	xylose-lysin-desoxycholate

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I. INTRODUCTION

Worldwide, the major causes regarding mortality in pre-weaned calves are dysentery and subsequent complications (SMITH, 2015; NGELEKA et al., 2019). The interactions of multiple factors lead to the severe diarrhea syndrome in animals of young age. These factors include environmental conditions such as low ambient temperature, or the farm management practice such as insufficient nutrition, hygiene, and calf housing (QUINN et al., 2011; CHO & YOON, 2014; SMITH, 2015). However, enteritis in young calves is primarily triggered by pathogenic microorganisms and parasites (CHO & YOON, 2014). *Escherichia coli* (*E. coli*) account to the major enteric and systemic pathogens within the family Enterobacteriaceae. Most of the *E. coli* colonizing the intestinal tract of animals and humans are commensal, but facultative pathogenic strains may cause intestinal disease or even severe and life-threatening extraintestinal disorders (KAPER et al., 2004; QUINN et al., 2011). In calves, especially enterotoxigenic *E. coli* (ETEC) are one of the most frequently detected pathogens that cause enteritis within the first four days of life (FOSTER & SMITH, 2009).

Neonatal diarrhea is a major economic problem on cattle farms and the therapy with antimicrobials is crucial in routine practice (FEDERAL OFFICE OF CONSUMER PROTECTION AND FOOD SAFETY & PAUL-EHRLICH-GESELLSCHAFT FÜR CHEMOTHERAPIE E.V., 2016). However, the therapy with bactericide antibiotics is highly indicated exclusively in the case of life-threatening septicemia (CONSTABLE, 2009; VETSUISSE-FAKULTÄT et al., 2019). Nevertheless, the emergence of multidrug- and pandrug-resistant *E. coli* isolates in fecal samples of diarrheic calves has been repeatedly reported (CAO et al., 2019; FEDERAL OFFICE OF CONSUMER PROTECTION AND FOOD SAFETY, 2020). Particularly drug-resistant *E. coli* isolates express extended-spectrum β-lactamases (ESBL) (MURPHY et al., 2017). These enzymes destroy broad-spectrum β-lactam antimicrobials by hydrolysis and render them ineffective (BUSH & JACOBY, 2010). Animals hosting ESBL-producing *E. coli* constitute a reservoir of resistance genes that may negatively affect the health of man and animals (MURPHY et al., 2017; ASTORGA et al., 2019)

This underlines the importance of establishing monitoring systems for antimicrobial resistance trends. These shall aim for the preservation of the efficiency regarding antimicrobial drugs (FEDERAL OFFICE OF CONSUMER PROTECTION AND FOOD SAFETY & PAUL-EHRLICH-GESELLSCHAFT FÜR CHEMOTHERAPIE E.V., 2016).

The present work intends to investigate and discuss recent trends in antimicrobial resistance and occurring pathotypes of *E. coli* isolates obtained from fecal samples of diarrheic calves in Bavaria, Germany. The gut microbiome including *E. coli* depicts an excellent mirror for the dynamics of resistance in farm animals and should constitute a main area of resistance research for the future (FEDERAL OFFICE OF CONSUMER PROTECTION AND FOOD SAFETY & PAUL-EHRLICH-GESELLSCHAFT FÜR CHEMOTHERAPIE E.V., 2016).

II. LITERATURE REVIEW

1. Infectious causes of diarrhea in calves

1.1. *Escherichia coli*

Escherichia coli (*E. coli*) accounts to the major enteric and systemic pathogens of the gram-negative rods within the family Enterobacteriaceae. Most of the *E. coli* colonizing the intestinal tract of animals and humans are commensal, but pathogenic strains are not part of the physiological microflora. They are oxidase-negative, facultative anaerobes. Culturing on selective media, such as MacConkey agar, enables easy detection due to their ability to ferment lactose (QUINN et al., 2011). In our study, *E. coli* isolates were confirmed by using fluorescence on *E. coli* direct (ECD) agar (Merck Millipore, Burlington, MA, USA) and a positive Kovacs-Indole reaction (Merck Millipore, Burlington, MA, USA). Some isolates showed a mucoid or hemolytic phenotype, that are potential signs for pathogenicity (QUINN et al., 2011)(Figure 1). Furthermore, some serotypes are more pathogenic than other ones. Therefore, serotyping may help to identify potentially pathogenic isolates that are responsible for disease (LINTON & HINTON, 1988). However this costs more time and money (FRATAMICO et al., 2016). Serotyping is accomplished by slide agglutination testing with antisera to detect O (somatic) and K (capsular) antigens (Figure 2) (ORSKOV & ORSKOV, 1992; QUINN et al., 2011). The bacteria may be enclosed by capsular antigens that protect them from phagocytic ingestion and improve their intestinal attachment accomplished by fimbrial adhesins. Lipopolysaccharide (LPS) structures on their surface, also known as endotoxins, are released after cell death and lysis. Once LPS enter the bloodstream and are transported to the brain, endotoxins may cause fever, damage of endothelial cells and in a worst case disseminated intravascular coagulation (DIC) that may lead to acute shock and sudden death(QUINN et al., 2011). The LPS also determines the O-antigen by its side chains (ORSKOV & ORSKOV, 1992). Further, proteinaceous fimbrial (F) antigens accomplish the attachment to the enteric mucosa and prevent mechanical shedding from the gut by peristalsis (QUINN et al., 2011). The initial infection occurs from direct interaction with infected animals, after fecal-oral uptake or via contaminated food or water (FOSTER & SMITH, 2009).

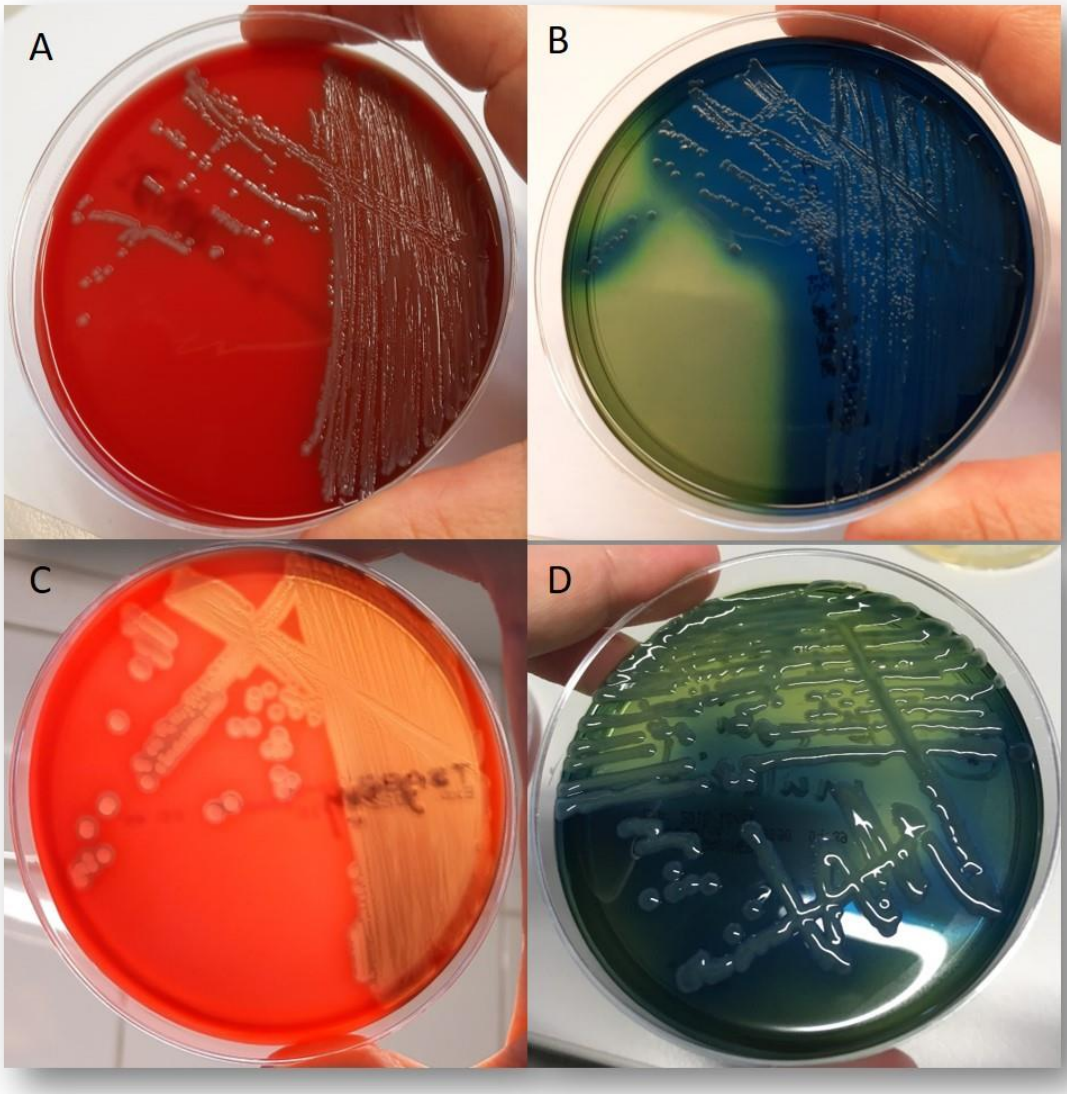

Figure 1: Growth of *E. coli* after a 24 h incubation at 37°C on Columbia Sheep Blood Agar (A); the lactose-positive metabolism revealed by blue coloring on Gassner Agar (B); the potentially pathogenic phenotypes of a hemolytic *E. coli* (C) and of a mucoid isolate (D).

In neonatal calf diarrhea, enterotoxigenic *E. coli* (ETEC) play a major role. Enteropathogenic *E. coli* (EPEC) and Shiga toxin producing *E. coli* (STEC) including enterohemorrhagic *E. coli* (EHEC) may sometimes be isolated from diseased and healthy calves, but their role in calf diarrhea is still not clear. Nevertheless, they are an important source for human disease. Enteroaggregative *E. coli* (EAEC),

diffusely adherent *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC) have not been clinically emerged in cattle yet (KOLENDA et al., 2015).

1.1.1 ETEC

As reported, pathogenic serogroups that lead to ETEC diarrhea in calves and lambs mostly include O8, O9, O20 and O101, whereat the serotypes O9:K30 and O101:K30 were the most common ones. In the case of systemic disease, the serotype O78:K80 plays a major role in the development of septicemia that leads to endotoxic shock, especially in calves suffering from failure of transfer of passive immunity (LINTON & HINTON, 1988; QUINN et al., 2011). Further, virulent *E. coli* may express various proteinaceous fimbrial adhesins to attach to the enteric mucosa and prevent mechanical shedding from the gut by peristalsis (QUINN et al., 2011). In recent studies, F17-fimbria encoding genes were the most frequently detected virulence factors in *E. coli* from diseased calves (STING & STERMANN, 2008). This finding was as well reviewed in a meta-analysis driven by Kolenda *et al.* 2015 collecting data from 106 studies originating from 27 countries between 1951 and 2013 (KOLENDA et al., 2015). Furthermore, ETEC may at least express two different types of enterotoxins, heat-stable toxin (ST) and heat-labile toxin (LT). LT is an AB_5-toxin, in which the subunit B is determined for receptor binding and the subunit A catalyzes enzymatical activity (DUBREUIL et al., 2016). By indirectly activating the adenylate cyclase enzyme, cyclic adenosine monophosphate (cAMP) levels rise within the enterocytes. ST consists of short polypeptides (GYLES, 1992; QUINN et al., 2011). It causes the activation of the enzyme guanylate cyclase, which in turn induces a rise of the cyclic guanosine monophosphate (cGMP) level (FIELD et al., 1978; GYLES, 1992). The increase of the second messengers cAMP or cGMP effect an active secretion of fluid in the small intestine, subsequently leading to extreme loss of fluid. At the same time, the chloride and bicarbonate secretion increases whereas the sodium absorption decreases. However, these toxins do not cause pathologic mucosal damage (GYLES, 1992; DUBREUIL et al., 2016).

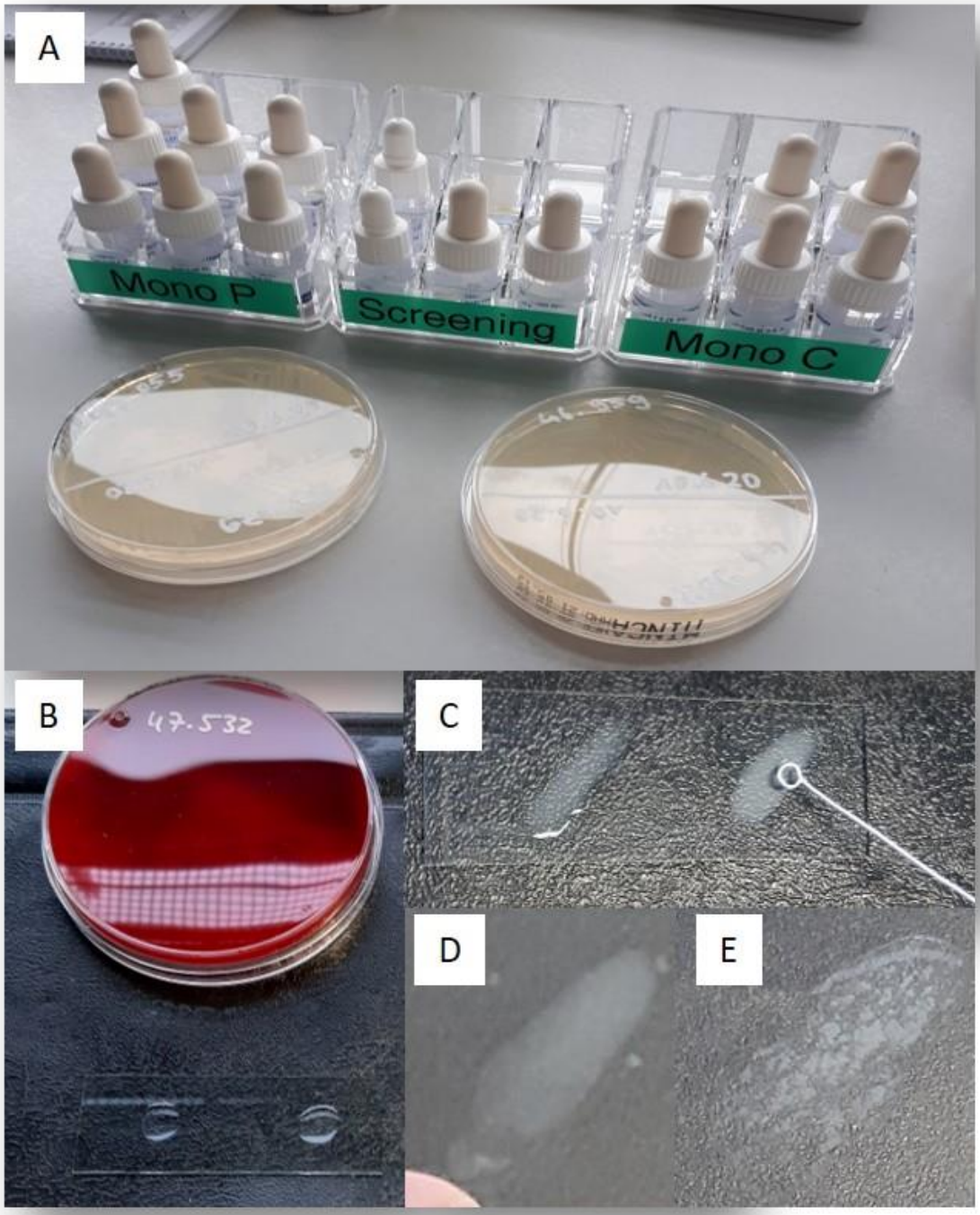

Figure 2: *E. coli* isolates were characterized by performing agglutination with three sets of antisera (A). A glass slide was prepared with the respective serum (B). For testing the potential agglutination reaction, colony material was suspended in the serum (C). The turbid color indicates a negative reaction (D). In case of agglutination, the positive result appears in flaking clouds (E).

1.1.2 STEC

STEC may constitute to the physiological intestinal flora of healthy cattle (MONTENEGRO et al., 1990; WELLS et al., 1991). Indeed, ruminants such as cattle are known to be a major reservoir of human pathogenic STEC (MONTENEGRO et al., 1990; BEUTIN et al., 1993; BLANCO et al., 1994; GYLES, 2007).

Shiga toxins, formerly called Verotoxins, here the two subtypes stx1 and stx2, account to the AB_5-toxins that are encoded via prophages. During bacterial stress, e.g. DNA damage or antibiotic treatment of the patient (ZHANG et al., 2000), these phages may become lytic, coding DNA sequences may be expressed and Shiga toxins may be formed and are released (NEELY & FRIEDMAN, 1998; TOSHIMA et al., 2007; CROXEN & FINLAY, 2010). The B subunits bind to specific glycolipid receptors on cell surfaces that lead to the internalization of the toxin. The subunit A removes an adenine base from the 28S rRNA, which ceases protein biosynthesis followed by apoptosis of susceptible cells (GYLES, 2007).

Shiga toxins may not clinically manifest in healthy cattle but may lead to enterocolitis with intimin associated attaching and effacing (A/E) lesions in neonatal and weaned calves. As well, symptoms as watery or bloody diarrhea were described in neonatal calves within the scope of previous infection studies (DEAN-NYSTROM et al., 1997; WIELER & BAUERNFEIND, 2003). The so-called enterohemorrhagic *E. coli* (EHEC) represent a subset of STEC strains. These additionally possess a locus of enterocyte effacement (LEE) coded on a genetic pathogenicity island (NATARO & KAPER, 1998). LEE encodes various virulence factors such as intimin. Intimin promotes the attachment to epithelial gut cells and binds the translocated intimin receptor (Tir) that causes pedestal formation and effacement of microvilli. These interactions again induce typical A/E lesions such as premature enterocyte desquamation and villous malformation (KAPER et al., 2004; QUINN et al., 2011). In calves, intimin encoding *E. coli* O157:H7 cause A/E lesions in the ileum and the large intestine as well as primarily watery, then bloody diarrhea, fibrinous exudates at the luminal site, and microvillus atrophy (DEAN-NYSTROM et al., 1997; DEAN-NYSTROM et al., 1998). In a previous study from 1998, two infected calves did not survive experimental infection, whereas calves exposed to intimin-deleted mutants and control strains remained healthy with

only minor clinical signs (DEAN-NYSTROM et al., 1998).

Another study from 2002 revealed that stx1 from *E. coli* O157 may bind to the globotriaosylceramide (Gb3) receptor. The latter is located on crypt epithelial cells within the small and large intestine as well as on kidney epithelium cells in tubules and finally on cells of collecting ducts in cattle at the age of one to ten months. Furthermore, stx1 binds to submucosal lymphoid cells, but not to vascular endothelial cells within the intestinal tract or kidney. It was shown that their vasculature lacks the Gb3 receptor (HOEY et al., 2002). According to recent studies, STEC is not associated with diarrhea in calves. The bacteria are even more frequently detected in healthy calves, as well (WELLS et al., 1991; KOLENDA et al., 2015; NGELEKA et al., 2019).

Human illness is often associated with exposure to food and water contaminated with cattle feces (GYLES, 2007). Beside the virulence factor intimin, Shiga toxins, especially stx2, are regarded the most important virulence factors for the development of clinical symptoms in humans such as abdominal pain, furthermore an initial watery, subsequently bloody diarrhea (hemorrhagic colitis, HC) and finally the hemolytic uremic syndrome (HUS) (BOERLIN et al., 1999; CROXEN et al., 2013). HUS is a life-threatening condition defined by the occurrence of thrombocytopenia, hemolytic anemia and thrombotic microangiopathy. This may cause acute renal failure and end potentially fatal (NATARO & KAPER, 1998; MAYER et al., 2012). Herein, HUS results from a direct cytotoxic effect of Shiga toxins on renal vascular endothelial cells that may be enhanced by the proinflammatory cytokine reaction (NATARO & KAPER, 1998).

The majority of human EHEC disease outbreaks is caused by the serotype O157:H7. Further outbreaks are associated with various non-O157:H7 serogroups, namely O26, O103, O111, O117, O128, O145, O146, and O157 (BEUTIN et al., 1994; BOERLIN et al., 1999; GYLES, 2007; BIELASZEWSKA et al., 2013). Especially the serotype O157:H7 is considered human pathogenic, which is shown in several case reports where infection resulted from the ingestion of undercooked hamburgers. In this case, the patties may be produced from EHEC-contaminated ground beef (RILEY et al., 1983).

In *E. coli* caused disease, there may be an association between the clinical picture of bloody diarrhea and the occurrence of the repeats-in-toxin (RTX)-toxin enterohemolysin, which forms pores in erythrocytes. During this process, released heme and hemoglobin represent an iron source for bacterial growth (TANEIKE et al., 2002). Enterohemolysin detection rates are frequently higher in stx1 carrying *E. coli* strains from diarrheic than from healthy calves (WIELER et al., 1992). The role in the development of HUS and HC is still not clear, but the prevalence of the ehxA gene in STEC especially in human pathogenic serotypes is high (BOERLIN et al., 1999; NIELSEN & ANDERSEN, 2003; BLANCO et al., 2004), thus it may constitute a diagnostic marker for STEC causing severe human disease (BOERLIN et al., 1999).

STEC plays a major role in the edema disease of piglets often detected in combination with F18 fimbria. The Stx2e causes endothelial damage in the vasculature of target organs which leads to edema, e. g. in the central nervous system, in the larynx and in the eyelids, soon after weaning. This results in clinical signs such as hind limb paresis, muscular tremors, swollen eyelids, aphonia and sudden deaths (QUINN et al., 2011).

1.2. *Salmonella* spp.

Regarding the genus *Salmonella,* clinically relevant strains belong to *Salmonella* (S.) *enterica* subsp. *enterica,* the host-specific serotype *S. Dublin* and the zoonotic serotypes *S.* Typhimurium and *S.* Enteritidis as described by Kaufmann and White (QUINN et al., 2011; CHO & YOON, 2014). These are capable to survive within macrophages, which is important for the development of systemic disease (HOLSCHBACH & PEEK, 2018). Further, they express fimbrial adhesins to attach to the enteric mucosa and replicate in vesicles within the enterocytes. Their virulence factors accomplish fluid secretion, alteration of ions and cause inflammation resulting in diarrhea (QUINN et al., 2011). While accounting the gram-negative Enterobacteriaceae, their LPS may cause septicemia, fever, depression and recumbency, especially in young animals. This may lead either to death or the development of arthritis, meningitis and/or pneumonia (MOHLER et al., 2009). Herd infections may occur latent whereas acute disease is triggered by pregnancy, overcrowding and antimicrobial treatment. *S.* Dublin causes abortion, subclinical fecal excretion, latent carriers, acute or chronic intestinal disease and septicemia

at all ages, as well as terminal dry gangrene, osteomyelitis and joint ill, especially in calves (QUINN et al., 2011; HOLSCHBACH & PEEK, 2018). The clinical signs of the enteric salmonellosis are rotten smelling mucus, fibrin and/or blood comprising profuse diarrhea with fever, deranged general condition and anorexia resulting in dehydration (MOHLER et al., 2009; QUINN et al., 2011).

For diagnostics regarding salmonellosis in cattle, feces samples or rectal swabs are investigated according to the ISO 6579 standard. Further, identified isolates are agglutinated to determine one out of more than 2500 known *Salmonella* serotypes. Finally, further polyvalent antisera may be used to screen for pathogenic surface structures regarding all members of the Enterobacteriaceae (QUINN et al., 2011). Isolates may as well be characterized by further PCR screening for virulence genes. In pathogenic bacteria such as *Salmonella*, antimicrobial susceptibility testing is crucial prior to intravenous antibiotic therapy, which is obliged in a septicemic disease (HOLSCHBACH & PEEK, 2018). Supportive treatment with intravenous fluids and electrolytes may be beneficial (MOHLER et al., 2009; QUINN et al., 2011).

1.3. *Clostridium* spp.

Clostridium spp. are anerobic gram-positive rods with *Clostridium (C.) perfringens* types A to E accounting to the group of histotoxic Clostridia. *C. perfringen*s type A may cause gas gangrene and malignant edema when introduced via wounds as well as necrotic enteritis (QUINN et al., 2011). Furthermore, α-toxin, a phospholipase that is produced by *C. perfringen*s type A - E, causes hemolysis, necrosis, and digests lecithin. Type A also produces θ-toxin, which is a cytolysin (MACLENNAN, 1962; SONGER, 1996). The types A - D are present in the physiological microflora within the gut and ubiquitous in the environment but may proliferate, produce toxins, and finally may cause enterotoxemia determined by multifactorial causes such as changes in the nutrition or in the environment. *C. perfringens* types B - D occasionally may cause hemorrhagic enteritis in calves with a rapid course of disease which may end in sudden death (QUINN et al., 2011). The anaerobic environment in the gut or in wounds enables the replication of the clostridia that express exotoxins and may lyse tissue. These factors lead to an inflammation of the gut and the clinical picture of enterotoxemia (SONGER, 1996).

For diagnostic purposes, *C. perfringens* is cultured anaerobically on blood agar for 48 hours at 37 °C. Colonies show a double-zone hemolysis on blood agar. There are PCR techniques established for the detection of specific toxins. As well, the Nagler reaction identifies the α-toxin. In early stages of the disease, penicillins may be effective, but the best strategy is the vaccination with bacterin or toxoid components and the avoidance of predisposing conditions such as sudden changes in the nutrition (QUINN et al., 2011).

1.4. Bovine rotavirus (BRV)

BRV is a major pathogen responsible for diarrhea in calves between one and two weeks of age. It belongs to the family Reoviridae, genus *Rotavirus (CHO & YOON, 2014)*. Reoviridae are non-enveloped, possess a segmented double-stranded RNA and have an icosahedral structure with a double or triple-layered capsid (QUINN et al., 2011). It is very stable in the environment and may survive the gastric acid. It infects and then destroys enterocytes at the upper third of the villi within the small intestine. The villi become malformed because of the slow replacement rate of the enterocytes and malabsorption and fluid accumulation within the gut thereof results (QUINN et al., 2011; CHO & YOON, 2014). Diseased calves develop anorexia, depression and bright semi-liquid diarrhea (QUINN et al., 2011). They may recover quickly in uncomplicated cases but may develop severe or fatal disease, as there are coinfections with other diarrheic pathogens. For the diagnosis, feces and intestinal contents can be investigated for viral antigens with an ELISA. For treatment, oral rehydration solution and in severe cases intravenous fluid therapy and the administration of antibiotics is recommended (QUINN et al., 2011).

1.5. Bovine coronavirus (BCoV)

BCoV belongs to the order Nidovirales and the subfamily Coronavirinae with the genera Alpha-, Beta- and Gammacoronavirus. The BCoV is classified as Betacoronavirus 1. It is an enveloped positive-sense single-stranded RNA virus with a helical nucleocapsid. It causes diarrhea in calves at an age of one to two weeks and winter dysentery in adult cattle. The virus replicates in enterocytes and causes cell lysis within the small intestine and colon (CHO & YOON, 2014). This

results in malabsorptive profuse diarrhea that leads to exsiccosis and acidosis. The virus is also a respiratory tract pathogen leading to mild disease, which may complicate with secondary infections. The agent may be diagnosed using an antigen-ELISA or real-time PCR assays from fecal samples or intestinal contents. The treatment includes the administration of oral rehydration solutions and in case of severe disease an intravenous fluid therapy and antibiotics (QUINN et al., 2011).

1.6. Bovine viral diarrhea virus (BVDV)

The bovine viral diarrhea viruses BVDV 1 and 2 account to the family Flaviviridae, genus *Pestivirus*. These possess a positive-sense single-stranded RNA genome (QUINN et al., 2011). Regarding an infection within the first 30 days of gestation, the dam returns to estrus after early embryonic death. Between 30 and 150 days of gestation, abortion, mummification or fetal abnormalities may occur. It comes to persistently infected animals when non-immunocompetent bovine fetuses get infected with a non-cytopathic strain before day 120 of gestation (BAKER, 1995). At the age of six months to two years, the virus may recombine with a cytopathic strain or mutate. The affected animals tend to develop mucosal disease (QUINN et al., 2011). This includes profuse watery diarrhea, fever and ulcerative mucosal lesions in the mouth and interdigital clefts (CHO & YOON, 2014). Serum, spleen, ear punch tissue, lymph node and gastrointestinal lesions can be examined via ELISA or PCR. As the majority of losses results out of prenatal infections, persistently infected animals have to be detected and eliminated in combination with vaccinations (QUINN et al., 2011).

1.7. *Cryptosporidium parvum*

The parasite *Cryptosporidium (C.) parvum* causes gastrointestinal disease in neonatal calves as a sole agent. After fecal-oral uptake, its sporozoites invade enterocytes in the ileum and cecum. The asexual and sexual reproduction results in sporozoites released from oocysts which re-infect the host or either are released via feces and may infect other animals. The calves show clinical signs such as profuse malabsorptive diarrhea, anorexia, dehydration and recumbency that may lead to death (THOMSON et al., 2017). The highest shedding rates of

Cryptosporidium occur at an age of two weeks (FOSTER & SMITH, 2009). Thick-walled oocysts are long-term infectious in the environment; thus, the reduction of environmental contamination is a key measure for the eradication. Steam cleaning, subsequent drying and disinfection of calf houses are recommended (FOSTER & SMITH, 2009; THOMSON et al., 2017). As a drug therapy, only halofuginon is licensed for the prevention and reduction of diagnosed *C. parvum* caused diarrhea, which is administered orally for seven consecutive days (FOSTER & SMITH, 2009; INSITUTE FOR PHARMACOLOGY et al., 2021). It reduces the number of shed oocyst , but must be accompanied by hygiene measures (FOSTER & SMITH, 2009). *Cryptosporidium* spp. can be detected via fecal flotation and microscopy, antigen-capturing ELISA or PCR (CHO & YOON, 2014). (QUINN et al., 2011).

1.8. *Eimeria* spp.

The protozoa *Eimeria (E.)* are host-specific. In calves, the pathogenic *E. bovis* and *E. zuernii* are likely to cause hemorrhagic diarrhea that contains fibrin and intestinal tissue. Calves are susceptible primarily at an age of three weeks to six months. Frequently, bovine eimeriosis ends fatal (DAUGSCHIES & NAJDROWSKI, 2005). After the ingestion of sporulated oocysts, the released sporozoites infect enterocytes in the small intestine. These reproduce asexually and sexually while invading new host cells. Finally, non-sporulated oocysts are excreted via feces and sporulate in the environment leading to a high contamination of the surrounding soil (KEETON & NAVARRE, 2018). The pathogen may cause subclinical diarrhea that results in low feed efficiency or severe clinical disease with bloody or mucous diarrhea, abdominal cramps, dehydration, weight loss and can end fatal. As soon as clinical signs occur, the lifecycle of *Eimeria* is almost completed, so that a curative treatment is not possible anymore. Thus, the pro- or metaphylactic single dose application of anticoccidial drugs such as toltrazuril is recommended (INSITUTE FOR PHARMACOLOGY et al., 2021). Sulfonamides are often applied and may be beneficial in very early stages of coccidiosis, but in advanced stages of the disease, they only control secondary bacterial infections within the gut. Supportive treatment of diseased calves with intravenous fluids and glucose may be beneficial as well (DAUGSCHIES & NAJDROWSKI, 2005; KEETON & NAVARRE, 2018). *Coccidia*

spp. can be detected via fecal flotation and consecutive oocyst microscopy (DAUGSCHIES & NAJDROWSKI, 2005; KEETON & NAVARRE, 2018).

2. Non-infectious factors causing diarrhea in calves

Not solely infectious factors may cause diarrhea in young calves, but also factors like their immunity, the environment, and the farm management including insufficient hygiene and food supply may predispose young animals (QUINN et al., 2011; CHO & YOON, 2014; SMITH, 2015).

2.1. Immunity

As published, is the colostrum management the outstanding key factor that affects calf health and mortality (GODDEN et al., 2019). Especially an insufficient colostrum supply paves the way for diarrhea causing pathogens (QUINN et al., 2011). As there is no transfer of immunoglobulins via the placenta in cattle (MOON & BUNN, 1993), the key point for a good immunity against ubiquitous pathogens is a good and early colostrum supply for the calves immediately after birth to build up the adaptive immunity (QUINN et al., 2011). Then, only for limited time of approximately 24 hours, the alimentary uptake of antibodies may be accomplished in the small intestine by pinocytosis (BROUGHTON & LECCE, 1970). After these few hours, the absorption of macromolecules in the gut is terminated, the so-called “closure”. As published, this process can be prolonged up to 36 hours after birth, if feeding has occurred belated (STOTT et al., 1979). Despite this, neonatal animals bear innate immune mechanisms for the protection against pathogens. Calves without colostrum supply are likely to develop disease even though. Specific antibodies originating from the mother neutralize bacterial toxins and viruses and opsonize microorganisms so that they can be phagocytized by macrophages and neutrophils (QUINN et al., 2011). A recently published study from Bavaria found a positive correlation between the feeding of calves providing 2 or more liters of colostrum at their second meal and a less frequent development of diarrhea of calves (HUBER, 2021). Moreover, a so-called “animal individual factor” regarding the calves’ immunity is the age, because ETEC receptors are only distinct in the first week of life. Hence, only young calves are susceptible for an

ETEC infection (QUINN et al., 2011).

2.2. Environment and animal management

For the prevention of diseases and the reduction regarding the use of antimicrobials, adequate management and husbandry strategies are required (MURPHY et al., 2017).

2.2.1. Hygiene

The reduction of the risk regarding introduction, emergence and spread of diseases in an animal stock caused by anthropogenic and material measures is defined as 'biosecurity' (THE EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION, 2016). The entry of a pathogen may either occur through the introduction of animals as a major origin of infection, contaminated feed or water, or visitors, utensils for animal production and enrichment materials. The sum of policies to prohibit these feasibilities are called external biosecurity. The risk of a pathogen introduction can be reduced through the following measures: minimization of the introduction of animals or only introducing animals from the same source, cleaning, and disinfection of the transport vehicles, reduced mixing of different animal groups, and finally isolation of sick animals (MURPHY et al., 2017).

In the case that pathogenic organisms are already introduced, the transmission within a herd must be limited through internal biosecurity. This includes the prevention of overcrowding which enables efficient pathogen transmission (QUINN et al., 2011). Furthermore, the diagnosis of the pathogenic organisms is crucial to reduce the spread and initiate adequate treatment of diseased animals (MURPHY et al., 2017). The all-in-all-out occupancy benefits a proper hygiene regime, e. g. cleaning and disinfection of calf rearing areas before a new group of animals is introduced (MURPHY et al., 2017). Furthermore, cleaning the calving pen after every calving decreases the risk of diarrhea in newborn calves compared to sporadic cleaning (KLEIN-JÖBSTL et al., 2014).

2.2.2. Feed and housing

The feed should be nutritionally balanced, properly formulated and of constant stability following good manufacturing practice, as gut dysbiosis leads to disease liability (MURPHY et al., 2017). Moreover, a recently published study showed that calves, which are fed ab libitum from their first week of life, have a lower risk of developing neonatal diarrhea (HUBER, 2021). Feeding unpasteurized nonsaleable whole milk, which is not cooled, increases the risk for enteric disease (MCGUIRK, 2008).

Farm animal housings should be constructed so that a proper ambient temperature and an effectual ventilation is maintained (QUINN et al., 2011; MURPHY et al., 2017). Placing the individual calf housing into a barn significantly increases the positive impact on the development of diarrhea (KLEIN-JÖBSTL et al., 2014). Calf pens in open barns expose the calves to any weather, which is a risk factor for disease development (AL MAWLY et al., 2015). Dusts and gaseous pollutants should not accumulate, because they enable the pathogen transmission by inhibiting the mucociliary clearance mechanisms (MUNKHOLM & MORTENSEN, 2014). Feces and wastewater should be drained to ensure an evacuation of pollutants (MURPHY et al., 2017).

As newborn calves spend the most time lying, the most susceptible source for enteric pathogens is the bedding. It should be clean and dry. Especially rice hulls, wheat straw and wood shavings are recommendable. Furthermore, residual feed should be removed to protect calves from potential enteric pathogens. Continuous occupancy of calf housings increases the risk of pathogen transmission between the calves. Obtaining 10 % more calf housings than calves on the farm saves time for cleaning and following disinfection (MCGUIRK, 2008). Renewing the calf bedding every two to three days is associated with a lower prevalence of calf diarrhea within the calf housing (MEDRANO-GALARZA et al., 2018).

Calves born out of calving pens, e.g. in a loose-housing system, are at a higher risk to develop diarrhea in their first two weeks of life (FRANK & KANEENE, 1993). The misuse of calving pens for other purposes than calving increases the prevalence of calf diarrhea therein (MEDRANO-GALARZA et al., 2018).

2.2.3. Animal management

Stress factors that promote an immunosuppression should be minimized. Handling animals calmly with a minimum of stress is beneficial at standard husbandry procedures such as weaning, dehorning, castration, and transport. In the case of weaning, the separation from the cow into a new environment depicts a stress factor for the young animals (MURPHY et al., 2017).

Biosecurity is the collectivity of management factors established to reduce the risk of the carryover, initiation and spread of diseases within a livestock. Carryover of pathogens may occur through introduction of animals by purchase or contact of animals and external wildlife. Thus, the introduction of animals should be minimized. Transport vehicles should regularly be cleaned and disinfected to reduce the risk of pathogen transmission (MURPHY et al., 2017). Stocking density also has a great impact on disease outbreaks (MAES et al., 2000). All-in-all-out occupancy means that a barn gets cleaned and disinfected after each occupancy with animals and is beneficial for interrupting chain of infection (ANDRES & DAVIES, 2015). Further, minimization of contact between different age groups on the farm and avoiding mixing animal groups is feasible to reduce the spread of pathogens (MURPHY et al., 2017).

III. PUBLICATION

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Antimicrobial Resistance, Serologic and Molecular Characterization of *E. coli* Isolated from Calves with Severe or Fatal Enteritis in Bavaria, Germany

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Abstract: Worldwide, enterotoxigenic *Escherichia coli* (ETEC) cause neonatal diarrhea and high mortality rates in newborn calves, leading to great economic losses. In Bavaria, Germany, no recent facts are available regarding the prevalence of virulence factors or antimicrobial resistance of ETEC in calves. Antimicrobial susceptibility of 8713 *E. coli* isolates obtained from 7358 samples of diseased or deceased diarrheic calves were investigated between 2015 to 2019. Considerably high rates of 84.2% multidrug-resistant and 15.8% extensively drug-resistant isolates were detected. The resistance situation of the first, second and third line antimicrobials for the treatment, here amoxicillin-clavulanate, enrofloxacin and trimethoprim-sulfamethoxazole, is currently acceptable with mean non-susceptibility rates of 28.1%, 37.9% and 50.0% over the investigated 5-year period. Furthermore, the ETEC serotypes O101:K28, O9:K35, O101:K30, O101:K32, O78:K80, O139:K82, O8:K87, O141:K85 and O147:K89, as well as the virulence factors F17, F41, F5, ST-I and stx1 were identified in a subset of samples collected in 2019 and 2020. The substantially high rates of multi- and extensively drug-resistant isolates underline the necessity of continuous monitoring regarding antimicrobial resistance to provide reliable prognoses and adjust recommendations for the treatment of bacterial infections in animals.

Keywords: *E. coli*; calves; enteritis; antimicrobial resistance; serotypes; virulence; multidrug-resistant; extensively drug-resistant

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1. Introduction

Escherichia coli account to the major enteric and systemic pathogens of the Gram-negative rods within the family Enterobacteriaceae. Most of the *E. coli* colonizing the intestinal tract of animals and humans are commensal, but facultative pathogenic strains may cause intestinal disorder or even severe and life-threatening extraintestinal disease [1,2]. In calves, enterotoxigenic *E. coli* (ETEC) pose a leading cause of intestinal disease, especially within the first four days of life [3–5]. ETEC encode lipopolysaccharide structures (LPS) that may act as endotoxins, fimbrial adhesins and finally enterotoxins. The endotoxins within the blood stream cause fever, damage of endothelial cells and disseminated intravascular coagulation (DIC), that leads to acute shock and sudden death [1]. The serological LPS characterization in calves comprise the *E. coli* serogroups O8, O9 and O101, and respective serotypes O9:K35 and O101:K30, as these are known for endotoxin effect [6]. Further, the

serotype O78:K80 plays a major role in systemic disease, septicemia and endotoxic shock of newborn calves [1,6,7]. In piglets, the serotype O141:K85 in combination with F4 fimbria is specific for the postweaning diarrhea syndrome [6]. As well, three further serotypes O139:K82, O8:K87 and O147:K89 play an important role as pathogens for swine [6,8]. Proteinaceous fimbrial adhesins precipitate the bacterial attachment to the enteric mucosa that avert the mechanical shedding of virulent strains from the gut by peristalsis [1,4,9]. Former studies showed that the fimbrial adhesins F5, F17 and F41 are associated with calf diarrhea [4]. For ETEC, two different types of enterotoxins contribute to diarrhea in calves, the heat-stable toxin (ST) and heat-labile toxin (LT), respectively [1,10,11]. On a molecular level, the toxins increase the second messengers cyclic adenosine/ guanosine monophosphate (cAMP/cGMP), that effect an active secretion of fluid and electrolytes in the small intestine leading to extreme loss of fluid within the organism [11,12]. Further, ruminants are known to be a major reservoir of human pathogenic Shiga toxin-producing *E. coli* (STEC) [13–16]. Shiga toxins (stx1, stx2) may lead to enterocyte damage, subsequent bloody diarrhea and endothelial damage leading to internal hemorrhages and septicemia in susceptible neonatal calves [1,17,18]. Enterohemorrhagic *E. coli* (EHEC), a subset of STEC, further include intimin, an adhesin coded from the enterocyte effacement pathogenicity island (eaeA) [19,20] and enterohemolysin, a toxin encoded by the ehxA gene [21]. As published in several case reports, a majority of human EHEC disease outbreaks are caused by the serotype O157:H7 originating from contaminated ground beef [13,22,23]. This serotype is responsible for the hemorrhagic colitis and the life-threatening hemolytic uremic syndrome with the occurrence of thrombocytopenia, hemolytic anemia and thrombotic microangiopathy that may lead to acute renal failure and death [23–26].

Worldwide, neonatal diarrhea is still a major economic problem on cattle farms and the therapy with antimicrobials is crucial in routine practice [27]. However, the medication with bactericide antibiotics is solely, but highly indicated exclusively in the case of life-threatening sepsis [28,29]. The Swiss antibiotic therapy guidelines for veterinarians recommend amoxicillin-clavulanate as a first line, sulfonamide-trimethoprim as a second line and fluoroquinolones as a third line choice, here enrofloxacin [29]. A study from 2014 revealed that veterinarians in Europe mainly used polymyxins (44%), (fluoro)quinolones (18%), penicillins (13%), aminoglycosides (9%) and third and fourth generation cephalosporins (8%) in calves with diarrhea emphasizing the problem of an inappropriate use of antibiotics [30]. This contributes to a higher level of antimicrobial resistant bacteria in young animals compared to adults [31–33]. In addition, the emergence of multidrug- and pandrug-resistant *E. coli* in fecal samples of diarrheic calves has been recently and repeatedly reported [33,34]. According to the expert proposal for standard definitions for acquired resistance from the European Centre for Disease Prevention and Control (ECDC), strains are classified as "multidrug-resistant" if these are non-susceptible (resistant or intermediate) to at least one antimicrobial agent in more than three categories. Isolates meet the definition "extensively drug-resistant" if these are non-susceptible in all agents but two or fewer categories. Finally, isolates non-susceptible to all agents in all antimicrobial categories are ranked as "pandrug-resistant" [35].

Previous data show that the prevalence of extended-spectrum β-lactamase (ESBL)-producing *E. coli* in calves increased from 7% to 29% between 2006 and 2013 in Germany [27]. ESBL-producing strains do encode for numerous resistance genes and may transduce these to other, even commensal, bacteria [36]. Animals hosting these *E. coli* bacteria constitute a resistance gene reservoir that may affect the health of man and animals [36,37].

Only few data are available on the identification of ETEC from calves in Bavaria. However, the discrimination between the physiological intestinal flora and pathogenic *E. coli* is crucial [1,6,38]. The aim of the present study was to provide recent information about the most prevalent pathotypes of *E. coli*. These include the investigation of the current virulence factors, serotypes and trends in antimicrobial resistance [9,39–42].

2. Results

2.1. Antimicrobial Susceptibility

Within the study period 8713 *E. coli* were isolated from 7358 diarrheic calves at the federal state veterinary laboratory in Bavaria, Germany (Table S1). This number matches an average count of 1740 isolates per year that is in accordance with previous years (data not shown). The results on antimicrobial susceptibility testing revealed mean non-susceptibility values of 28.1% for amoxicillin-clavulanate, 37.9% for enrofloxacin and 50% for trimethoprim-sulfamethoxazole (Figures 1 and 2, Table S1). The highest non-susceptibility value of a substance within each antimicrobial class revealed 11.9% for tulathromycin (macrolides), 18.3% for colistin (polymixins), 61.9% for tetracycline (tetracyclines), 62.2% for spectinomycin (aminoglycosides), 69.7% for ampicillin (penicillins), 80.5% for cephalothin (cephalosporins) and 96.8 % for florfenicol (phenicols) (Figure 1). A 5-year tendency from 2015 to 2019, evaluated for amoxicillin-clavulanate, enrofloxacin and trimethoprim-sulfamethoxazole, revealed a statistically significant decrease of the non-susceptibility rates for amoxicillin-clavulanate and enrofloxacin ($p < 0.05$) (Figure 2, Table 1). Regarding trimethoprim-sulfamethoxazole a significant decrease was assessed from 51.9% to 47.8% between 2015 and 2017 regarding the non-susceptible *E. coli* isolates ($p < 0.05$). A subsequent increase was further revealed from 47.8% to 52.5% in the years 2017 to 2019 ($p < 0.05$) (Figure 2, Table 1). Categorizing the 8713 isolates according to the ECDC expert proposal, 84.2% of the isolates (7336/8713) were multidrug-resistant, 15.7% (1368/8713) were extensively drug-resistant, eight isolates (0.1%) were pandrug-resistant and one isolate was susceptible to all antimicrobials tested. As we only tested antimicrobials licensed for the veterinary use, and none of the latest antimicrobials available on the market, we rededicated the eight presumably pandrug-resistant as extensively drug-resistant summing up to 1376 isolates in this specification (Figure 3).

Table 1. Statistic parameters regarding the increase or decrease of resistance values within the five-year period for the three clinically relevant antimicrobials (Figure 2).

Antimicrobial	Years	OR	CI (95%)
amoxicillin-clavulanate	2015–2019	0.95	0.92–0.98 [1]
enrofloxacin	2015–2019	0.91	0.88–0.94 [1]
	2015–2017	0.92	0.85–1.0 [1]
trimethoprim-sulfamethoxazole	2015–2019	1.0	0.97–1.03
	2017–2019	1.11	1.03–1.19 [1]

OR: odds ratio, CI: confidence interval, [1] p-value (Wald test) < 0.05.

Treatment choice	Antimicrobial agent	Antimicrobial class	MIC values [µg/ml]														Susceptible	Intermediate	Resistant	Non-susceptible[4]	No result[5]	MIC_{50}	MIC_{90}
			0,02	0,03	0,05	0,06	0,13	0,25	0,5	1	2	4	8	16	32	64	128	%	%	%	%	%	[µg/ml]	[µg/ml]
1st line	Amoxicillin-clavulanic acid[1]	Betalactam combination agents									1614	2182	2468	1701	746			71,9	19,5	8,6	28,1	0,01	8	16
2nd line	Enrofloxacin	Fluoroquinolones	909	2937	-	546	228	790	272	54	2971							62,1	3,8	34,1	37,9	0,07	0,06	4
3rd line	Trimethoprim-sulfamethoxazole[2]	Folate pathway inhibitors			2			4119	141	47	47	4353						50,0		50,0	50,0	0,05	2	8
	Gentamicin	Aminoglycosides					16	2186	3119	875	660	138	449	1260				80,4	5,1	14,5	19,6	0,10	0,5	16
	Spectinomycin	Aminoglycosides										5	4	219	3064	1159	4262	37,8	13,3	48,9	62,2	0,00	64	128
	Cephalothin	Cephalosporins I & II								7	32	159	1505	2997	4013			19,5	34,4	46,1	80,5	0,00	16	32
	Ceftiofur	Cephalosporins III & IV					81	2519	2588	1152	350	241	1778					76,8	2,8	20,4	23,2	0,05	0,5	8
	Ampicillin	Penicillins					2	2	5	150	1375	1063	45	21	6050			30,3	0,2	69,5	69,7	0,00	32	64
	Florfenicol	Phenicols								15	263	3154	5281					3,2	36,2	60,6	96,8	0,00	8	16
	Colistin	Polymyxins							7116	1352	82	159						81,7	16,5	1,8	18,3	0,05	0,5	1
	Tetracycline	Tetracyclines					6	5	188	1943	1061	112	44	5352				38,1	0,5	61,4	61,9	0,02	16	16
	Tulathromycin[3]	Macrolides								15	778	2428	2942	882	373	579		88,1	4,7	7,2	11,9	8,22	8	32

Figure 1. Minimum inhibitory concentration (MIC) distribution of 8713 *E. coli* isolates on 12 antimicrobial agents from 11 antimicrobial classes. The three first lines represent the clinically relevant substances, first to third treatment choices in buiatrics. The red line demarcates the breakpoint towards resistance, the green line a breakpoint towards intermediate. Regarding the two combination compounds, only the concentration of the former substance is presented; the ratio of amoxicillin:clavulanic acid is 2:1 (1), concentration ratio of trimethoprim:sulfamethoxazole is 1:19 (2). Tulathromycin has not been tested in the first quarter of 2015 (3). The summation of intermediate and resistant isolates was named non-susceptible (4). Some results were not evaluable (5).

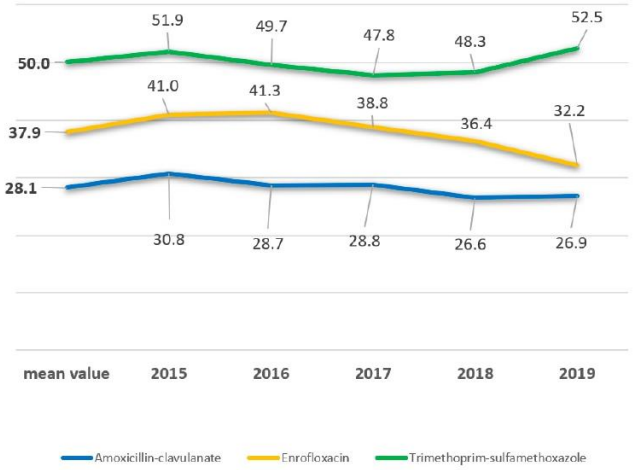

Figure 2. The mean value (bold) and the five-year trend on non-susceptible *E. coli* isolated from calves revealed the highest proportion of isolates against trimethoprim-sulfamethoxazole, followed by enrofloxacin and amoxicillin-clavulanate. The trends regarding enrofloxacin and amoxicillin-clavulanate remain at a stable level and rather tend towards a decrease regarding the number of non-susceptible isolates. The graph of non-susceptible isolates regarding trimethoprim-sulfamethoxazole reveals a decrease, 2016–2017, followed by a steep increase of non-susceptible isolates in 2019. The corresponding statistic parameters are presented in Table 1.

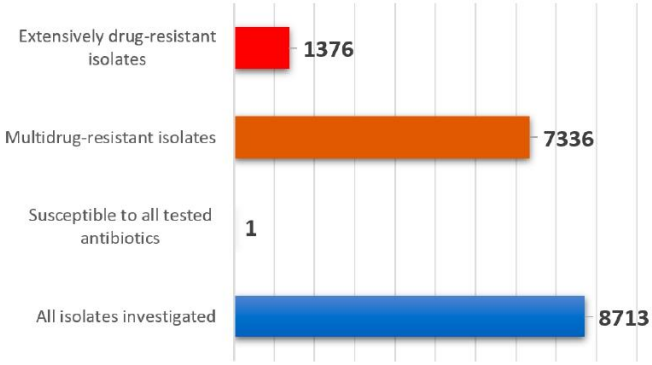

Figure 3. The classification of 8713 *E. coli* into extensively drug-resistant and multi drug-resistant isolates was carried out according to the expert proposal for standard definitions for acquired resistance. We categorized eight potential pandrug-resistant isolates in the category extensively drug resistant, as we only tested antimicrobials licensed for the veterinary use and did not include the latest antimicrobials available on the market.

2.2. Serologic Characterization

Serotyping of a randomly chosen subset of 108 *E. coli* isolated in 2019 and 2020 revealed 38 unequivocally typeable (35.2%), 29 untypeable (26.8%) and 41 seronegative (38%) strains

(Tables 2 and S2). The most frequently detected serotypes were O101:K28 (8.3%; *n* = 9), O9:K35 and O139:K82 (6.5%; *n* = 7), O101:K30 (3.7%; *n* = 4), O101:K32, O78:K80 and O8:K87 (2.8%; *n* = 3). The serotypes O141:K85 and O147:K89 were detected once each (Tables 2 and S2). Finally, the serotypes O138:K81, O149:K91 and O157:H7 were not detected at all.

The fimbrial antigen F5 agglutinated in 6.5% of the isolates (*n* = 7) in combination with the serotypes O101:K30, O101:K28 and O9:K35. The fimbrial antigen F4 agglutinated in 4.6% of the isolates (*n* = 5), and exclusively combined with the serotype O139:K82 (Tables 2 and S2).

Table 2. The serologic and molecular characterization revealed 13 different serotypes known to be pathogenic for cattle and other species. Furthermore, four different genotypes were detected with five different coding sequences for fimbria and/or toxins in one or more isolates. Some of the isolates were untypeable/ seronegative and did not reveal any of the investigated virulence factors (green box).

Serotype	Additionally Known for Pathogenicity in	Number of Isolates	Non-Virulent	Molecular Results			
				F17	F5ST-I	F5F41ST-I	stx1
O9:K35		6	5	1			
O9:K35/F5		1				1	
O101:K28		6	6				
O101:K28/F5		3			3		
O101:K30		1		1			
O101:K30/F5		3				3	
O101:K32		3	3				
O78:K80	Human/sheep	3	3				
O8:K87	Swine	3	3				
O139:K82	Swine	2	2				
O139:K82/F4	Swine	5	4	1			
O141:K85	Swine	1	1				
O147:K89	Swine	1		1			
untypeable		29	20	7			2
seronegative		41	37	4			
Total		108	84	15	3	4	2

2.3. Molecular Characterization

Within the molecular characterization, 14 PCR assays targeted genes for the expression of fimbria, adhesin, hemolysin and toxins. A positive result was obtained for 24 isolates and 35 single assays, respectively (Tables 2 and S2). The most frequently detected genes coded for the fimbria F17 (13.9%; 15/108), F41 (3.7%; 4/108) and F5. The latter was always detected in combination with the toxin gene coding for ST-I (6.5%; 7/108). Finally, the gene coding for stx1 was detected in two of 108 isolates (1.9%). Seven of 108 isolates (6.5%) carried more than one type of virulence-associated genes (Tables 2 and S2). The fimbrial antigens F4, F6, F18, O157, adhesin eaeA, hemolysin ehxA and the toxins LT, ST-II and stx2 were not detected in any isolate. The occurrence of F4 fimbria in the serotyping assays could not be confirmed in the PCR investigation (Tables 2 and S2). In all, 84 of 108 isolates were negative in all PCR assays (Tables 2 and S2).

3. Discussion

Antibiotic treatment is the fundamental therapy regarding serious or life-threatening bacterial infections in man and animals [28,29]. Records regarding antimicrobial susceptibility on single substances are collected in many countries all over the world [43]. Worldwide this is a critical topic in line with the One Health issue [44]. Monitoring on the application and more important efficacy of antimicrobials regarding bacterial infections of farm animals is possible on principle in industrial countries. However, it is costly and difficult to standardize [36]. Published data from Canada in 2018 revealed a 51.6% susceptibility rate of 489 *E. coli* against trimethoprim-sulfamethoxazole, which is in consensus with our

data (50%) (Figures 1 and 2) [45]. Tetracycline was accounted to be effective in 36.8% and resembles our findings at 38.1% (Figure 1) [45]. Further, authors from the United States and Germany determined similar high resistance rates for tetracycline, with 71.1% and 70.9%. These data rather resemble the rate of 61.4% revealed in the present study (Figure 1) [46,47].

The antimicrobial class of fluoroquinolones includes enrofloxacin which is one of the substances of choice for the treatment of diarrhea in young cattle [29,48]. In Germany, the usage of fluoroquinolones has risen from 2011 to 2013 in human and veterinary medicine. This trend needs close monitoring to preserve the efficacy of the agent [27]. Fluoroquinolones are assessed as highest priority clinically important antimicrobials and as one of the few options for the treatment of serious *Salmonella* and *E. coli* infections in children recommended by the World Health Organization (WHO) [49]. The legislation reacted and passed a law in 2017 including obligatory antimicrobial susceptibility testing in case of the application of fluoroquinolones or third or fourth generation cephalosporines in Germany [50]. In the present study, the investigated *E. coli* isolated revealed a resistance rate of 34.1% regarding enrofloxacin (Figure 1). This finding correlates with published results from South America in 2017, with 36.4% [51].

Antimicrobial substances or closely related compounds may likewise be licensed for the use in man and animals. The application in an organism does trigger the development of antimicrobial resistance in present bacteria [49]. Legal restrictions regarding the use of cephalosporines, especially from the third and fourth generation, aim at a high prioritization of critically important antimicrobials in human medicine [49]. This is again in accordance with the terms of One Health [27,44]. The use of cephalosporines for the therapy of *E. coli* diarrhea in calves is a malpractice, as the effective therapeutic concentration is not reached within the gut [29]. Nonetheless, cephalosporin is the fifth-most commonly prescribed antimicrobial in the case of diarrhea with 8% according to a recent survey in Europe [30]. Regarding the third generation cephalosporine ceftiofur, a susceptibility rate of 86.4% could be determined in a study from Canada between 1994 and 2013 [45]. Significantly, our findings revealed 76.8% (Figure 1). Compared to data from the USA collected within the years 1960 until 2002 and in 2007, the resistance rate was at 7.4% and 11%, whereas in the present study the resistance rate of ceftiofur revealed 20.4% (Figure 1) [46,52]. This result is concerning, and the use of ceftiofur must be scrutinized critically, if not avoided completely. The resistance rates of the first generation cephalosporine, cephalothin, were lower in a comparable study regarding data within the period of 1960 to 2002, with 20.1%, in contrast to our results with an average rate at 46.1% from 2015 to 2019 (Figure 1) [46]. Currently, the standard antimicrobial therapy of mastitis in cows includes penicillins as well as first and second generation cephalosporines in the EU. Traces of antibiotics may reach the calves through the feeding of antibiotic contaminated waste milk [36]. To predict a reliable trend regarding the prevalence of ESBL-producing *E. coli*, PCR and sequencing methods should be applied to investigate the existence of ESBL- encoding genes as these are probably more accurate than the phenotypic characterization [53]. A study from 2013 revealed high rates (32.8%, 196 of 598 samples) of ESBL-encoding *E. coli* on dairy and beef cattle farms in Bavaria [54].

Completely inconsistent data are publicly available regarding the resistant rates for *E. coli* isolates and the substance florfenicol within the phenicol group. A 78% share of resistant isolates was determined in a study from the USA in 2006, only a 28% share from Canada in 2018, and a share of 35% from Bavaria, Germany, in 2002 [45,52,55]. In the present study, a rather higher resistance rate of 60.6% was determined for florfenicol (Figure 1). There was no information about ages of animals within the American and Canadian studies [45,52]. Since lower resistance rates were previously published in older animals for the substances ampicillin, tetracycline, streptomycin, sulfamethoxazole and chloramphenicol, this might accordingly apply for florfenicol [32]. This argument, however, still does not explain the diverse results of the Bavarian study from 2002 and the present study (Figure 1) [55].

With a 9% share of the most frequently listed antimicrobials, aminoglycosides remain at the fourth top position for the treatment of diarrhea in calves [30]. As these are almost solely used in the therapy of enterococcal endocarditis and multidrug-resistant tuberculosis in humans, they account to the high priority, clinically important antimicrobials in human medicine [49]. An application in veterinary medicine should therefore be prudent and well considered. Gentamicin belongs to the aminoglycoside antimicrobial class and has a withdrawal time for meat of more than 200 days in Germany for cattle and the indication of gastrointestinal disease. As this is economically hardly acceptable, the application of gentamicin is quite limited [48]. However, resistance to gentamicin among *E. coli* isolated from animals has been increasing from 0% to 40% between 1970 and 2002 within the United States [46]. Another long-term investigation from Germany revealed a further decrease of resistance rates including data from 2010 until 2013, and 2016 until 2017, respectively [47]. In the present study, the resistance rate of *E. coli* against Gentamicin was at 14.1% (Figure 1). Likewise, spectinomycin is an aminoglycoside antibiotic as well, and frequently used in combination with lincomycin for oral application in the treatment of simultaneous infection of the respiratory and the gastrointestinal tract in calves. The meat withdrawal time of 21 days is acceptable for farmers and practitioners and may be an explanation for the frequent prescription [48]. Within the present study and correspondingly a resistance rate of 48.9% was revealed in calves (Figure 1).

As stated by the WHO, the antimicrobial class of polymyxins accounts for the highest priority in critically important antimicrobials regarding the treatment of serious infections with Enterobacteriaceae and *Pseudomonas aeruginosa* in human medicine [49]. Despite rather frequent prescription of polymyxins in the treatment of diarrhea in animals, investigated *E. coli* isolates are still highly susceptible [30]. In the present study, the resistance rate against colistin revealed to be only 1.8% (Figure 1). Corresponding to this suggestion, another study revealed that only 3.8% of the isolates were resistant to colistin [47].

The aminopenicillin family, as well as the preparation amoxicillin-clavulanate, belong to the high priority critically important antimicrobials for the therapy of *Listeria* and *Enterococcus* spp. infections in humans according to the WHO [49]. For the aminopenicillin, ampicillin, an alarming resistance rate of 76.3% was determined in *E. coli* published in a most recent study from Germany [47]. Regrettably, a rate of 69.5% was determined in the present work as a similar result (Figure 1). Consequently, the recommendation on the usage of ampicillin for the treatment of calf diarrhea cannot further be continued. The amoxicillin-clavulanate susceptibility rate averaged at 57% in Germany in 2013 [27]. In the present study, the average susceptibility rate was 71.9%, and the resistance rate was 8.6% (Figure 1). Accordingly, a recently published study reported 7% of resistant *E. coli* isolates in Germany in 2018 [34]. Analogical to the report on the resistance monitoring study 2018 of the Federal Office of Consumer Protection and Food Safety, Germany, we determined decreasing non-susceptibility rates regarding the clinically important antimicrobial amoxicillin-clavulanate [34]. In conclusion, the resistance rates of *E. coli* against amoxicillin-clavulanate have decreased since 2013 and remained on a constant level within the years 2015 and 2019. This is a positive trend is beneficial for the One Health point of view [27].

Comparing data originating from other continents and collected over the last 60 years clearly reveals an increase of resistance regarding *E. coli* in nine out of the 12 tested drugs, namely gentamicin, cephalothin, ceftiofur, enrofloxacin, trimethoprim-sulfamethoxazole, ampicillin, amoxicillin-clavulanate, florfenicol and tetracycline [27,34,45–47,51,52,55]. Out of the 12 tested drugs in the present study, eight substances are similarly suitable for the treatment of human patients, namely gentamicin, spectinomycin, cephalothin, ampicillin, tetracycline, amoxicillin-clavulanate, colistin and trimethoprim-sulfamethoxazole (Figure 1) [49]. The application of these in veterinary medicine should be prudent due to the One Health aspect.

In a published study from Canada in 2018, 48.7% of multidrug-resistant *E. coli* were isolated from ruminants [45]. Within another study from the USA covering the years 1950 until 2002, a significantly increasing trend in resistance was observed for ampicillin,

sulfonamide and tetracycline antibiotics regarding more than 1700 *E. coli* isolates. Two of these strains were identified as pandrug-resistant and originated from cattle in 2001 [46]. Further, multidrug resistance in *E. coli* increased from 7.2% to 63% between 1950 and 2002. Finally, 59.1% of the strains recovered form cattle were classified as multidrug resistant in the USA [46]. In the present study, we detected an even higher rate of 84.2% regarding multidrug resistance, 15.7% extensively drug-resistance and 0.1% pandrug-resistance (Figure 3). Furthermore, there were no exclusively susceptible isolates found amongst 108 isolates recovered in 2019 and 2020 from diarrheic calves in Bavaria (Table S2). Comparably high levels of antimicrobial resistance were published regarding the countries Brazil and Uruguay. Calves aged up to 60 days revealed a multidrug-resistance rate in *E. coli* at 78.7%, and at 61.6%, respectively [51]. As published, these bacteria occurred frequently in herds with high levels of diarrhea symptoms and subsequent antimicrobial therapy, as equally described in the present study [31].

Besides antimicrobial resistance, the determination of virulence regarding infectious agents is crucial in diagnostics. The discrimination from commensal *E. coli* was determined investigating virulence factors and evaluating the pathogenicity of isolates. As published, the *E. coli* serotypes O139:K82, O8:K87 and O147:K89 are pathogenic in swine [6]. However, in the present study, a fair amount of such isolates, six out of 108, were isolated from cattle, respectively (Tables 2 and S2). In laboratory diagnostics, implication of these serotypes should therefore be considered. Three isolates were identified as the serotype O78:K80, which frequently causes septicemia in calves (Table 2) [5,7,56]. However, more than one third, 38%, of the *E. coli* in this study revealed to be entirely seronegative (Tables 3 and S2), as it was as well published previously [57]. Preferably and in accordance with the One Health approach, the screening of *E. coli* isolated from diseased animals should always be of interest to identify zoonotic and human pathogenic serotypes [25]. As a matter of fact, formula associated with severe human syndromes included the serotypes O26, O103, O111, O117, O128, O145 and O146 respectively [13,22,23,58].

Table 3. In all, 16 different polyvalent and monovalent (mono) antisera were used for the agglutination and the characterization of *E. coli*. The listed serotypes are known for their pathogenicity in humans and farm animals.

Antiserum for Initial Screening	Respective Follow Up Agglutination	Specific Serotypes Occur in Cattle, but Are Found as Well/Especially in
Polyvalent anti-*E. coli* C	O9:K35, mono O101:K28, mono O101:K30, mono O101:K32, mono F5, mono	
O78:K80, mono		Human, sheep
Polyvalent anti-*E. coli* P	O8:K87, mono O138:K81, mono O139:K82, mono O141:K85, mono O147:K89, mono O149:K91, mono F4, mono	Swine
O157:H7, mono		Association with food-poisoning

In recent studies, the fimbrial adhesins F17, F41 and F5 were frequently and significantly correlated with diseased calves compared to healthy animals [4,9]. These findings clearly correspond to the results of the present study (Tables 2 and S2). Other selective fimbrial antigens, F4, F6 and F18, occur frequently in isolates from diarrheic piglets [1,10,59]. As to be expected, we did not detect these amongst our strains isolated from calves (Table S2). Even five serologically F4 positive isolates were not confirmed within our molecular in-

vestigation (Tables 2 and S2). We assume that none of these isolates carry the specific primer sites, or agglutination was non-specific [9]. However, working at a federal state laboratory, we do research cross species infections especially among farm animals [60]. Furthermore, we consider the One Health approach, here especially the idea from farm to fork, and therefore continuously consider possible correlations between food-borne human pathogens and isolates from farm animals [27,44].

As published, hemolysis in *E. coli* isolates from piglets is a reliable diagnostic marker for virulence and pathogenicity [61–63]. Within the present study, only few (3/108) isolates revealed a hemolytic phenotype that was not even confirmed within the molecular analysis (Table S2). We conclude that hemolysis is not a relevant marker for virulence of *E. coli* isolated from calves in the present study. This statement is in accordance with prior publications [64,65].

Regarding the present study, ST-I was found in similar prevalence at a rate of 6.5% (7/108) compared to published data (Tables 2 and S2) [4,66]. The enterotoxins LT and ST-II were not detected in the present study (Table S2) and this again resembles data of relevant previous studies [4,56]. Concluding published data, ETEC isolated from calves only produced ST-I, whereas ETEC isolated from pigs may encode varying combinations of the enterotoxins LT, ST-I and ST-II [11,67]. In the present study, the detection rate of stx1 was very low and stx2 as well as intimin were not detected at all among the diarrheic calves' isolates (Tables 2 and S2). This finding matches the results of previously published data to a high degree [9,51,68]. Obviously, the detection rate of Shiga toxins rose with the number of colonies isolated from each clinical sample, suggesting the selection of up to 35 colonies [69,70]. In the present investigation however, only up to three colonies were analyzed per clinical sample (Table S2). Other published results suggested a positive correlation between animal age and the amount of Shiga toxin, supporting our findings including animals of young age [69–71]. Targeted infection studies with STEC led to severe disease and bloody diarrhea in neonatal calves, but more recent studies disproved this observation revealing a still controversial discussion [4,72–74].

Limits of the Study

The antimicrobial susceptibility testing was carried out with a standard panel of antibiotics currently used in veterinary diagnostics in Germany. The results are therefore limited to substances only partially prescribed in human diagnostics and sometimes even in veterinary medicine regarding other countries of the world.

A thorough molecular investigation of single isolates is fairly time consuming and costly compared to the benefit that might be drawn from the results. In routine diagnostics, the molecular methods therefore can hardly be kept up.

4. Materials and Methods

4.1. Study Design and Bacterial Isolates

At the Bavarian Health and Food Safety Authority in Germany 7358 fecal samples of diseased or deceased calves with enteritis younger than six weeks of age were analyzed and included in the present study. Samples were collected between January 2015 and December 2019. Clinical symptoms ranged from low general condition, diarrhea, fever, sepsis and sudden death, respectively. A total of 8713 *E. coli* strains were isolated and confirmed through positive fluorescence on ECD agar (Merck Millipore, Burlington, MA, USA) and a positive Kovacs-Indole reaction (Merck Millipore, Burlington, MA, USA). All isolates were subject to antimicrobial resistance testing, further analysis and cryopreservation at the internal vaccine laboratory.

4.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was carried out according to the protocols published in CLSI VET01, 5th edition (Clinical and Laboratory Standards Institute, Wayne, PA, USA) [41]. Breakpoints were adopted from CLSI Vet01S, 5th edition, and national break-

points for farm animals [41,42,75]. We used the microbroth dilution method on the following twelve different antimicrobial agents (antimicrobial class): Amoxicillin-clavulanic acid (betalactam combination agent), enrofloxacin (fluoroquinolone), Trimethoprim-sulfamethoxazole (folate pathway inhibitor), gentamicin and spectinomycin (aminoglycosides), cephalothin (cephalosporin I and II), ceftiofur (cephalosporin III and IV), ampicillin (penicillin), florfenicol (phenicol), colistin (polymyxin), tetracycline (tetracycline) and tulathromycin (macrolide). A commercially available set was used according to the manufacturer's instructions (Micronaut-S, Grosstiere 4, Merlin, Bruker, Bornheim, Germany). The minimum inhibitory concentration (MIC) of each isolate and antibiotic substance was metered using a photometric plate reader system (Micronaut Scan and MCN6 software, Merlin/ Sifin, Bruker, Bornheim, Germany). Subsequently, the MIC value was reconciled with supplemented CLSI breakpoints, to categorize the respective *E. coli* isolate into "susceptible", "intermediate" and "non-susceptible" for each antimicrobial substance tested [41,42,75,76]. *E. coli* ATCC 25922 was used as quality control strain [41].

4.3. Phenotypic Analysis and Serotyping

We deeper investigated a subset of 108 *E. coli* isolated in 2019 and 2020 originating from 66 diarrheic calves. The isolates were subcultured on Gassner agar (Oxoid Deutschland GmbH, Wesel, Germany) to differentiate specific colony morphology. The expression of potential virulent F5 fimbria was investigated by subculturing the isolates on pH 7.5 stabile, "minimum of casein" (Minca) agar (Sifin Diagnostics GmbH, Berlin, Germany) as previously published [76]. Finally, potential hemolytic properties of isolates were interpreted as described with subcultures on Columbia Sheep Blood Agar (Sifin Diagnostics GmbH, Berlin, Germany) [77]. Growth incubation was carried out for 18 to 24 h at 37 °C at all times. Serotyping for specific O-antigens was carried out using two polyvalent and 14 monovalent agglutination sera in a hierarchical approach according to the manufacturer's instructions (Sifin Diagnostics GmbH Berlin, Germany) (Table 3). If an isolate showed a positive agglutination reaction with a polyvalent serum, but none with any correspondent monovalent or several reactions with various correspondent monovalent sera, it was categorized as untypeable. If an isolate showed no positive agglutination with any serum, it was categorized as seronegative.

4.4. Molecular Investigation

The molecular characterization of the *E. coli* isolates in the present study aimed at surface antigens, toxins and virulence factors. In all, 14 different target genes were of interest. Amongst were seven fimbrial genes F4, F5, F6, F17, F18, F41 and the outer membrane protein O157:H-. Further, two virulence genes were included, here adhesin intimin (eaeA), and enterohemolysin (ehxA). Finally, PCR targets coding for five toxins were screened, including heat-labile toxin (LT), heat-stabile toxin I (ST-I) and II (ST-II), Shiga toxin 1 (stx1) and stx2 (Table 4). Primer sequences were adopted from published protocols [9,39,40]. All 14 qPCR assays were performed applying a singleplex high resolution melting method, using AccuMelt HRM SuperMix (Quantabio, Beverly MA, USA) in 20 µL volumes according to the manufacturer's instructions. DNA was extracted after thermolysis. The primers were added in a concentration of 0.2 µM each, and 3 µL of template DNA was used. Polymerase chain reaction assays were conducted on a Stratagene MX3000P device (Agilent Technologies, Waldbronn, Germany). The cycling protocol comprised an initial single denaturation step for 10 min at 95 °C, followed by 40 cycles of annealing and polymerization for 30 s at 60 °C and 10 s at 95 °C. After completing amplification, the melting curve analysis was performed. Specific melting temperatures were determined for each molecular target and all tested isolates. Reference strains were used as positive controls and kindly provided from Prof. R. Bauerfeind (Justus-Liebig-Universität, Gießen, Germany), and purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) (Table 4).

Table 4. Targets and primers for the molecular characterization of *E. coli* isolated from calves.

Target Protein	Gene(s)	Primer	Oligo Sequence (5′ -> 3′)	Size (bp)	Melting Temperature (°C) ± 0.2 °C	Reference	Reference Isolate
Fimbria/outer membrane protein	F4	F4_F F4_R	GGTGGAACCAAACTGACCATTAC TCCATCTACACCACCAGTTACTGG	102	81.0	[9]	7156
	F5	F5_F F5_R	TTGGAAGCACCTTGCTTTAACC TCACTTGAGGGTATATGCGATCTTT	101	77.4	[9]	7159
	F6	F6_F F6_R	GCGGATTAGCTCTTTCAGACCA TGACAGTACCGGCCGTAACTC	102	83.2	[9]	7155
	F17	F17_F F17_R	ACTGAGGATTCTATGCRGAAAATTCAA CCGTCATAAGCAAGCGTAGCAG	83	79.7	[9]	5397
	F18	F18_F F18_R	CCTGCTAAGCAAGAGAATATATCCAGA AGAACATATACTCAGTGCCAACAGAGAT	82	73.3	[9]	7160
	F41	F41_F F41_R	CCTTTGTCATTTGGTGCGG TCAAATACTGTACCAGCAGAACCAC	101	81.5	[9]	7159
	O157 (rfbE)	O157_F O157_R	CGATGAGTTTATCTGCAAGGTGAT TTTCACACTTATTGGATGGTCTCAA	88	78.3	[39]	DSMZ 19206
Adhesin	intimin (eaeA)	Intimin_F Intimin_R	CCAGCTTCAGTCGCGATCTC GGCCTGCAACTGTGACGAA	91	86.1	[9]	7158
Hemolysin	enterohemolysin (ehxA)	ehec-F2 ehec-R	CGTTAAGGAACAGGAGGTGTCAGTA ATCATGTTTTCCGCCAATGAG	142	79.5	[40]	DSMZ 19206
Toxin	heat-labile toxin (LT)	LT_F LT_R	CTGCCATCGATTCCGTATATGAT CAGAACTATGTTCGGAATATCGCA	81	75.3	[9]	7157
	heat-stabile toxin (ST-I)	ST-I_F ST-I_R	TACCTCCCGTCATGTTGTTTCAC CCTCGACATATAACATGATGCAACTC	101	76.1	[9]	7155
	heat-stabile toxin (ST-II)	St-II_F St-II_R	TTTTTCTATTGCTACAAATGCCTATGC AACCTTTTTTACAACTTTCCTTGGC	101	75.9	[9]	7156
	Shiga toxin 1 (stx1)	Stx1_F Stx1_R	TCCCCAGTTCAATGTAAGATCAAC TTTCGTACAACACTGGATGATCTCA	81	79.0	[9]	7158
	Shiga toxin 2 (stx2)	Stx2_F Stx2_R	GAGTGACGACTGATTTGCATTCC CCATGACAACGGACAGCAGTT	82	84.6	[9]	7158

4.5. Statistical Analysis

All statistical analyses were performed using the free software R Studio version 1.2.5033 (RStudio, Inc., Boston, MA, USA). Resistance trends of three clinically relevant antimicrobials amoxicillin-clavulanate, enrofloxacin and trimethoprim-sulfamethoxazole were evaluated by calculating a logistic regression model. The respective year was set as a continuous variable. The resulting odds ratio (OR) > 1 indicated an increased resistance trend, whereat an OR < 1 indicated a decreased antimicrobial resistance. The Wald test was used to determine the statistical significance of the year-antimicrobial trend. A value of $p < 0.05$ was considered significant (Table 1).

5. Conclusions

We conclude that an extensive monitoring, characterization and the analysis of antimicrobial resistance regarding enteritis causing *E. coli* is crucial to determine the currently raging serotypes, virulent genotypes and most important, the resistance situation. It is then possible to calculate reliable tendencies and prognoses from data collected over long terms in routine diagnostics. This is an important premise for objective and professional treatment recommendations regarding humans and animals within the scope of One Health. A further goal should be a slowdown of the increasing antimicrobial resistance situation that constitutes a global public health threat.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11010023/s1, Table S1: data set for all 8713 isolates from 2015–2019. Table S2: data set for a subset of 108 isolates in 2019–2020.

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IV. DISCUSSION

1. Neonatal enteritis pathogens other than *E. coli*

A further aspect important for the effective treatment and the reduction of antibiotics is a comprehensive diagnostic algorithm. Within the scope of this study, we determined causative agents other than *E. coli* and responsible for diarrhea in young calves. As described in our publication, a subset of 66 samples from diarrheic calves was investigated more thoroughly for bacteria, viruses, and parasites, respectively (Table 2) (FEUERSTEIN et al., 2022). These included a collection of 108 *E. coli* isolates that was further investigated for virulence factors. The majority of the samples were positive regarding bovine rotavirus and bovine coronavirus (25.8 %; 17/66). A smaller proportion of cases revealed virulent *E. coli* isolates (19.7 %; 13/66), and some of the samples yielded positive results with the diagnosis parasitological infestation, here *Cryptosporidium* spp. or *Coccidia* spp. (13.6 %; 9/66) (Table 2). Virulent *E. coli* in combination with viruses or virulent *E. coli* in combination with parasites occurred in the same prevalence (9.1 %; 6/66). Viruses and parasites were detected in only four samples (6.1 %) and all three pathogens – viruses, parasites and virulent *E. coli* – were found in five samples (7.6 %). *Salmonella* spp. were not detected in any of these samples. In 11 samples (16.7 %), *Clostridium perfringens* was additionally detected (Table 2). The BVD virus was not detected in any sample.

Table 2: In 66 cases of calves' diarrhea, the following diagnoses regarding pathogenic *E. coli*, virus infection or parasitic infestation was determined

Infectious agents detected	**Number of cases**	**%**
virus*	17	25,8
virulent *E. coli*	13	19,7
Clostridium perfringens	11	16,7
parasites**	9	13,6
virulent *E. coli* + Virus	6	9,1
virulent *E. coli* + Parasites	6	9,1
virulent *E. coli* + Virus + Parasites	5	7,6
virus + parasites	4	6,1
Salmonella / BVD virus	0	0,0
samples with any pathogenic agent	60	90,9
no pathogenic agents detected	6	9,1
total number of samples	**66**	

*bovine rotavirus, bovine coronavirus
** *Cryptosporidium* spp., *Coccidia* spp.

In many cases, antibiotics are not necessary for treatment, however a hygienic management to suppress parasitic infestation.

A significant relationship between the calf's age and the detected pathogens was detected (Figure 4). At an age of one to two weeks, we found the highest detection rates for BRV and *Cryptosporidium* spp. In agreement with these findings, other authors also reported that BRV occurred mostly in calves with an age of one to two weeks (CHO & YOON, 2014) and *Cryptosporidium* spp. highest shedding rates were at an age of 2 weeks (FOSTER & SMITH, 2009). The highest detection rates of BCoV were at an age of under one week, which corresponds with previous

studies where the majority of BCoV infections occurred between the first and the seventh day of life (LANGPAP et al., 1979). *Coccidia* spp. were detected only in calves at the age of three to four weeks. Corresponding to our findings, *Eimeria* spp. are widespread in farm environments and calves may get infected soon after birth but develop moderate to potentially fatal hemorrhagic diarrhea at an age of three weeks to six months (DAUGSCHIES & NAJDROWSKI, 2005).

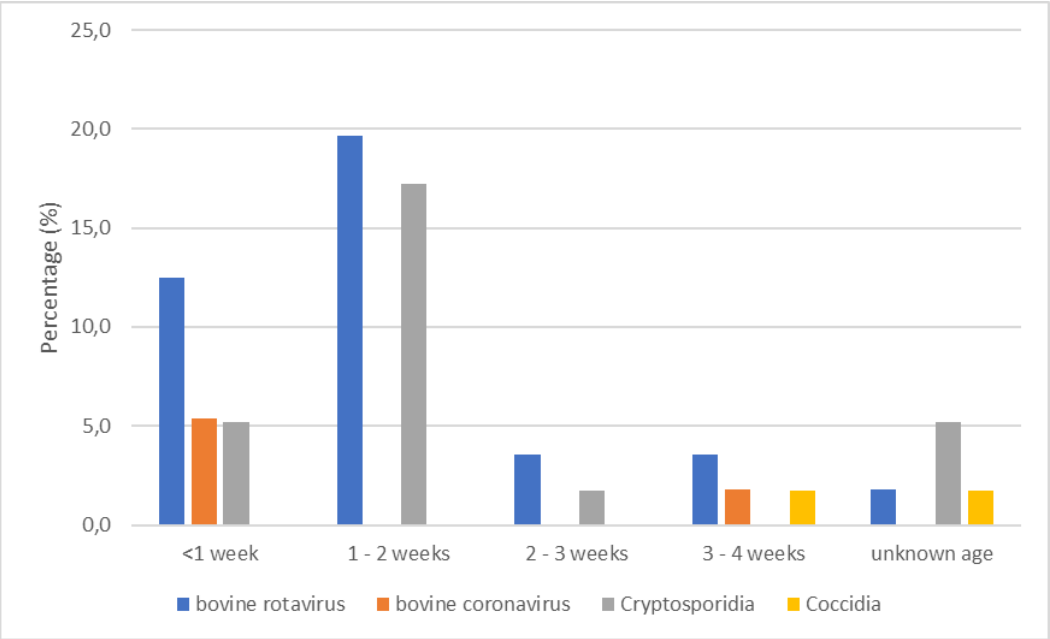

Figure 4: Age dependent occurrence of viruses and parasitic infestation in diarrheic calves

2. Treatment of bacterial enteritis - not always just antibiotics!

For the treatment of calves suffering from severe bacterial enteritis, the authors of the "Swiss Antibiotic Therapy Guidelines for Veterinarians" recommend parenterally administered amoxicillin as first line, sulfonamide-trimethoprim as second line, and fluoroquinolones (enrofloxacin) as a third line choice (VETSUISSE-FAKULTÄT et al., 2019). However, quinolones represent one of the highest priority critically important antimicrobial class for human medicine assessed by the World Health Organization (WHO), aside from 3rd, 4th and 5th generation cephalosporins,

macrolides and ketolides, glycopeptides and polymyxins (WORLD HEALTH ORGANIZATION, 2016). Thus, the application in animals is only justified if no alternative effective antimicrobial exists according to a pathogen evidence and additional susceptibility test (VETSUISSE-FAKULTÄT et al., 2019). A dosage for enrofloxacin, which is registered for gastrointestinal *E. coli* infections in calves, of 2.5 mg/kg IM or SC q24hr for 3 to 5 days (CLINIPHARM, 2021a). Other authors also recommend the treatment with broad spectrum β-lactams like ampicillin and amoxicillin, ceftiofur or sulfonamides such as trimethoprim-sulfadiazine (CONSTABLE, 2004; BERCHTOLD, 2009b; CLINIPHARM, 2021c, 2021b). Based on the bioavailability, the authors recommend the treatment with broad spectrum β-lactams like ampicillin and amoxicillin (10 mg/kg IM or SC q12hr for at least 3 days) (CLINIPHARM, 2021b), ceftiofur or sulfonamides such as trimethoprim-sulfadiazine (20 mg/kg sulfadiazine/5 mg/kg trimethoprim IV or IM q24hr for 5 days) (CONSTABLE, 2004; BERCHTOLD, 2009b; CLINIPHARM, 2021c). In contrast to this, the IPT recommendation cites cephalosporines as inacceptable due to a lack of therapeutic concentration within the gut (VETSUISSE-FAKULTÄT et al., 2019). In Europe, veterinarians prescribed polymyxins (44 %), (fluoro)quinolones (18 %), penicillins (13 %), aminoglycosides (9 %), and 3rd and 4th generation cephalosporins (8 %) in case of calf diarrhea according to a survey from De Bryine *et al.* (DE BRIYNE et al., 2014). Summarized, a total of 26 % critically important antimicrobials were prescribed based on the WHO criteria (DE BRIYNE et al., 2014). A recent study found that especially veal calves are commonly treated with antibiotics in groups and via the oral route. This was then associated with elevated antimicrobial resistance, compared to animals with targeted individual treatment. To prevent unnecessary antibiotic consumption and subsequent antimicrobial resistance, individual animal treatment should be favored (SCHÖNECKER et al., 2019). Regarding calves with normal appetite and without fever, close monitoring and oral rehydration is sufficient. In contrary, apathetic calves with signs of severe depression, coma, recumbency and no suckle reflex should be treated with intravenous fluid therapy to balance electrolyte and fluid losses (CONSTABLE, 2004; BERCHTOLD, 2009a). Moreover, the administration of non-steroidal anti-inflammatory drugs such as flunixin meglumine (2.2 mg/kg IM once) or meloxicam

(0.5 mg/kg IV or SC once) is beneficial, because it decreases abdominal pain and is requested in the context of calf welfare (CONSTABLE, 2009). A single dose of meloxicam improves the clinical signs such as rectal temperature, abdominal pain, feed intake and behavior in calves (PHILIPP et al., 2003).

3. Antimicrobial resistance and one health

Historically, antimicrobial resistance developed long time before the therapeutic use of antibiotics. Because of their extended application in human and veterinary medicine since the 1950s, the spread of antimicrobial resistance has clearly increased (VAN DUIJKEREN et al., 2018).

There are two types of resistance. Intrinsic or innate resistance means that target structures for antibiotics are absent, export mechanisms for antibiotic substances are existent or inactivating enzymes are produced, which is encoded on the bacterial chromosomes. Consequently, these bacteria are insensitive for these antimicrobial classes (QUINN et al., 2011; VAN DUIJKEREN et al., 2018). According to published data, *E. coli* possesses intrinsic resistance against penicillin (penicillins), erythromycin, and tilmicosin (macrolides) (AWOSILE et al., 2018). Extrinsic or acquired resistance arises when external resistance genes, e. g. encoded on a plasmid or likewise mobile genetic element, are collected or antimicrobial target encoding genes mutate. Thereof result enzymatic inactivation by depletion or chemical alteration, reduced intracellular aggregation or insensitivity due to modified target sites (QUINN et al., 2011; VAN DUIJKEREN et al., 2018). In contrast to intrinsic resistance, the properties of extrinsic resistance can only be found in several strains or subpopulations of a bacterial species (MURPHY et al., 2017). Extrinsic resistance augments, if antibiotics are applied inappropriately. Therefore, the European Centre for Disease Prevention (ECDC) and the Centers for Disease Control and Prevention (CDC) launched an international expert proposal for the definition of acquired resistance of Enterobacteriaceae. The occurrence of resistance to multiple antimicrobials has become a long-ranged public health concern, because it limits or denies antimicrobial therapy options in life threatening bacterial infections. The ECDC

classifies strains that are non-susceptible to at least one antimicrobial agent in more than three antimicrobial categories as multidrug-resistant, and we adopted this and further definitions for our study. Extensively drug-resistant isolates are non-susceptible in all agents but two or fewer categories. Finally, isolates non-susceptible to all agents in all antimicrobial categories are ranked as pandrug-resistant (MAGIORAKOS et al., 2012).

The development of resistance while using antimicrobials is inevitable, but a strong association was observed between the overall antimicrobial use und the occurrence of antimicrobial resistance of *E. coli* as an indicator microorganism in food-producing animals. Thus, the gut microbiome depicts a major source of mobile genetic antimicrobial resistance encoding elements (MURPHY et al., 2017). The antimicrobial use forces the process of selection, where an antimicrobial resistant bacterium gains the opportunity to reproduce in an exponential progression while the sensitive population of bacteria is suppressed or killed by an antimicrobial substance (MURPHY et al., 2017). Younger animals are often observed with higher levels of antimicrobial drug-resistant *E. coli* within the gut (KHACHATRYAN et al., 2004; DUSE et al., 2015; ASTORGA et al., 2019). This may occur due to a higher number of antimicrobial treatments of calves compared to adult animals or a potential correlation between resistance genes and genes that favor *E. coli* populating the calves' intestines. In the latter case, the reduction of the exposure to antimicrobials may not be able to reduce the resistances (KHACHATRYAN et al., 2004).

Previous data show that the prevalence of ESBL-producing *E. coli* in calves increased from 7 % to 29 % between 2006 and 2013 (EUROPEAN MEDICINES AGENCY (EMA)/CO-ORDINATION GROUP FOR MUTUAL RECOGNITION AND DECENTRALISED PROCEDURES-VETERINARY(CMDV), 2017). Concerns are kept of extended-spectrum β-lactamase (ESBL) -producing strains which often encode additional resistances to other veterinary antimicrobials (MURPHY et al., 2017). These *E. coli* bacteria constitute a resistance gene reservoir and may disseminate resistances by horizontal gene transfer which may impact animal and human health (MURPHY et al., 2017; ASTORGA et al., 2019). Key findings form the "European Food Safety Authority (EFSA) Scientific Opinion on the public health

risks of bacterial stains producing ESBL and/or AmpC beta-lactamases in food and food-producing animals" indicate that not only the use of cephalosporines but accordingly, general antimicrobial use forces resistances. This is, as ESBL-producing bacteria often carry additional resistance genes (MURPHY et al., 2017). As concluded in our publication, the use of cephalosporines for the therapy of *E. coli* caused diarrhea in calves is a malpractice, as the effective therapeutic concentration of the drug is not achieved within the gut (VETSUISSE-FAKULTÄT et al., 2019).

In consequence of high mortality rates within livestock farming, prophylactic antimicrobial use has been practiced since the 1950s. This includes antimicrobial treatment of healthy animals to prevent bacterial infection. Such practice is justified by reduced animal surveillance time, former disease outbreaks or management issues such as high stocking density, preventive treatment of newly introduced animals or covering critical stressful stages such as weaning, dehorning or castration. In contrast to this, metaphylaxis includes the treatment of healthy, but probably infected animals, as well as diseased animals within the same group. Prophylaxis and metaphylaxis enforce an extensive use of antimicrobials, because both include the treatment of mainly healthy animals and therefore should not be systematically used (AARESTRUP, 2005; MURPHY et al., 2017). A metaphylactical treatment may be beneficial by avoiding mass treatments. Targeted treatment of calves with a rectal temperature higher than 39.7 °C results in a lower number of prescribed antimicrobials and less stress due to handling of the young animal (MURPHY et al., 2017). To further reduce the need for antimicrobials in food-producing animals, the supply of diagnostics is one of many recommended strategies. Other factors, such as establishment of monitoring systems for antimicrobial resistance, improvement of animal housing and farm management practices regarding disease prevention and finally a promoted education for veterinarians need to be considered (MURPHY et al., 2017). New methods, such as whole genome analyses surely may propose comprehensive information regarding the presence of resistance genes in more than just one isolate per animal, but within the entire microbiome. This trend is currently followed in all disciplines of the "One Health" concept. However, it will still take some time to

reduce costs and establish evaluated analysis pipelines for the enormous amounts of data, generated within this approach.

A reduction of antimicrobial use to an absolute necessary minimum has been demanded governmentally (FEDERAL INSTITUTE FOR RISK ASSESSMENT, 2013). Nevertheless, regarding severe bacterial infections in animals, the antimicrobial agents represent indispensable tools. The curative treatment with antimicrobials is requested when the health and welfare is negatively affected by bacterial infectious diseases, for example in life threatening situations (MURPHY et al., 2017).

We therefore conclude that although antibiotics need to be applied in an utmost prudent way, these medications are not to be forbidden completely considering the welfare of animal health. Veterinarians should follow guidelines to optimize the antimicrobial treatment of animals and to minimize resistance selection (BUNDESTIERÄRZTEKAMMER, 2015). It is crucial that methods of quantification, monitoring, benchmarking and reporting antibiotic use are developed or operated so that antibiotic stewardship schemata can be established, and their benefits and failures analyzed.

4. Fighting microorganisms – prevention by vaccination

About 75 % of the currently emerging infectious diseases are zoonoses and that implies a major hazard to public health (LÜTTICKEN et al., 2007; SCHUDEL & LOMBARD, 2007). Since 2020, the world is challenged by a SARS-CoV-2 pandemic that most probably emerged from bats (WU et al., 2020). In human and animal health, vaccines probably play the most significant role in the prevention and control of emerging infectious diseases (LÜTTICKEN et al., 2007; MURPHY et al., 2017).

Regarding Shiga toxin producing *E. coli*, healthy cattle is a major reservoir for serotypes pathogenic for humans. Infection follows the uptake of contaminated food, such as raw milk, ground beef, or water (RILEY et al., 1983; MONTENEGRO et al., 1990; BEUTIN et al., 1993; BLANCO et al., 1994; GYLES, 2007; MARTIN &

BEUTIN, 2011). It was shown that the vaccination of cattle against EHEC significantly reduced the number of human disease outbreaks combined with improved hygiene practices (LÜTTICKEN et al., 2007; MARTIN & BEUTIN, 2011). Besides the public health concern, the national and regional economic damage produced by infectious diseases induces a major propulsion in vaccinating farm animals in veterinary medicine (MURPHY et al., 2017). There are different types of vaccines available with diverse advantages but as well disadvantages. On the one hand, live vaccines may induce long-term immunity, but can potentially return to virulence. Therefore, key virulence genes may be eliminated with the aid of deoxyribonucleic acid (DNA) technology to attain non-virulent modified pathogens, so called “attenuated live vaccines”. On the other hand, inactivated vaccines are unable to transmit diseases, but require an adjuvant to be able to produce adequate immunity (MURPHY et al., 2017). Besides modified live and inactivated vaccines, so-called autogenous vaccines are widely used in Europe (ATTIA et al., 2013; SALÉRY, 2017). These are inactivated vaccines and produced using pathogenic bacteria isolated from diseased animals in a specific locality. Therefore, the application is allowed only in the one epidemiological unit of origin for an individual flock to ensure their pathogen specificity. According to German law (FEDERAL MINISTRY OF JUSTICE AND CONSUMER PROTECTION, 2018) and the arriving Regulation (EU) 2019/6 on veterinary medicinal products (THE EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION, 2018), autogenous vaccines may only be applied, if the availability of registered vaccines is not given and/or if the protection by registered vaccines is not possible (EUROPEAN MEDICINES AGENCY (EMA)/CO-ORDINATION GROUP FOR MUTUAL RECOGNITION AND DECENTRALISED PROCEDURES-VETERINARY(CMDV), 2017; SALÉRY, 2017). These autogenous vaccines are tested in advance on a small population of the susceptible flock for adverse effects. Although some facts support the production and application of autogenous vaccines, there is few scientific evidence for significant effects in cattle (MURPHY et al., 2017). One previous study reported a significant reduction of calf mortality and morbidity when applying an oral inactivated autogenous vaccine (BALJER et al., 1976). Inactivated autogenous vaccines are allowed in 18 EU member states, under which only eight states allow

the use of viral autogenous vaccines and only one country permits parasitic inactivated autogenous vaccines (SALÉRY, 2017). As a principle, antiviral vaccines rather prevent immunosuppression and subsequent secondary infection triggered by many viruses. Whereas antibacterial vaccines rather reduce the bacterial load in an organism to a specific degree (MURPHY et al., 2017). As a main benefit, the prophylactic vaccination of farm animals specifically reduces the need for antibiotic treatment of these animals (EUROPEAN MEDICINES AGENCY (EMA)/CO-ORDINATION GROUP FOR MUTUAL RECOGNITION AND DECENTRALISED PROCEDURES-VETERINARY(CMDV), 2017; MURPHY et al., 2017).

Regarding the situation of the present study and ETEC infections in neonatal calves, parenteral vaccines for in cows are used commonly. As a result, the newborn calves receive protecting antibodies via colostrum (MOON & BUNN, 1993). In Germany, there are currently five registered combined vaccines for the active immunization of pregnant dams to administer some weeks pre calving (Table 1). Four out of these five are available on the market. The compounds contain inactivated ETEC with fimbrial antigen F5 and different strains of inactivated or live BRV and BCoV. Although, there is no transfer of immunoglobulins via the placenta (MOON & BUNN, 1993), the key point for the use of this kind of vaccines is a rich and early colostrum supply for the calves after birth. Then, only for the limited time of approximately 24 hours, the alimentary uptake of antibodies may be accomplished in the small intestine by pinocytosis (BROUGHTON & LECCE, 1970). After these few hours, the absorption of macromolecules in the gut is terminated, the so-called “closure”. As published, this process can be prolonged up to 36 hours after birth, if feeding has occurred belated (STOTT et al., 1979).

Table 1: Registered vaccines (status quo 2022) for the active immunization of pregnant cattle in Germany, Federal Institute for Vaccines and Biomedical Drugs (Paul-Ehrlich-Institut, Langen, Germany)

Designation	**Manufacturer**	***E. coli* characteristics**	**Rotavirus**	**Coronavirus**
Trivacton*	Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany	serotype O101: capsular antigen 99, fimbrial antigen 5, inactivated; serotype O117 with Y-Antigen, inactivated; serotype O78 with 31 A-antigen, inactivated; serotype O101 with fimbrial antigen 41, inactivated	strain "RoI", inactivated	strain "CR1", inactivated
Scourguard 3	Zoetis Deutschland GmbH, Berlin, Germany	strain "NADC 1471", O101 with fimbrial antigen 5 (K99), inactivated	strain "Lincoln", live	strain "Hansen", live
Bovilis Rotavec Corona	Intervet Deutschland GmbH, Unterschleißheim, Germany	fimbrial antigen 5 /capsular antigen 99, inactivated	strain "UK-Compton", serotype "G6P5", inactivated	strain "Mebus", inactivated
Lactovac C	Zoetis Deutschland GmbH, Berlin, Germany	fimbrial antigen 5, inactivated	strain "1005/78", inactivated, strain "Holland", inactivated	strain "800", inactivated
Bovigen Scour	FORTE Healthcare Ltd., Stamullen, Ireland	strain "EC/17", expresses F5 (K99) adhesins, inactivated	strain "TM-91", serotype "G6P1", inactivated	"C-197", inactivated

*currently not available for purchase

A second or alternative vaccination strategy is the oral application in calves. Unfortunately, there is no licensed vaccine for calves pertaining to the infection with *E. coli* in Germany. In this case, the Bavarian Health and Food Safety Authority is permitted to produce an autogenous inactivated oral vaccine. Analog to the protocol of Baljer *et. al.* (BALJER et al., 1976), one dose contains 10^{10} germs/ml in 0.9 % saline solution and 0.01 % Thiomersal solution for conservation. Pathogenic bacteria are collected directly from the young calves of the stable where infections have occurred. The bacteria are then cultured, and heat inactivated. The calves receive one ampule (3 ml) in 0,5 to 1,0 liter milk per day for ten consecutive days. In the years 2015 to 2019, the vaccine lab has produced almost 200.000 doses of inactivated autogenous vaccines for oral application on average per year requested by veterinarians (Figure 3). In Bavaria, there is another governmental institute producing such vaccines, the Tiergesundheitsdienst Bayern e.V., which highlights the importance and the demand of autogenous vaccines. They may stimulate the production of antibodies in the mucosa of the gut, which are transported to the luminal site (MOON & BUNN, 1993). Although autogenous live vaccines may be more effective than inactivated ones (EVANS et al., 1980), they are not permitted by Germany law (SALÉRY, 2017).

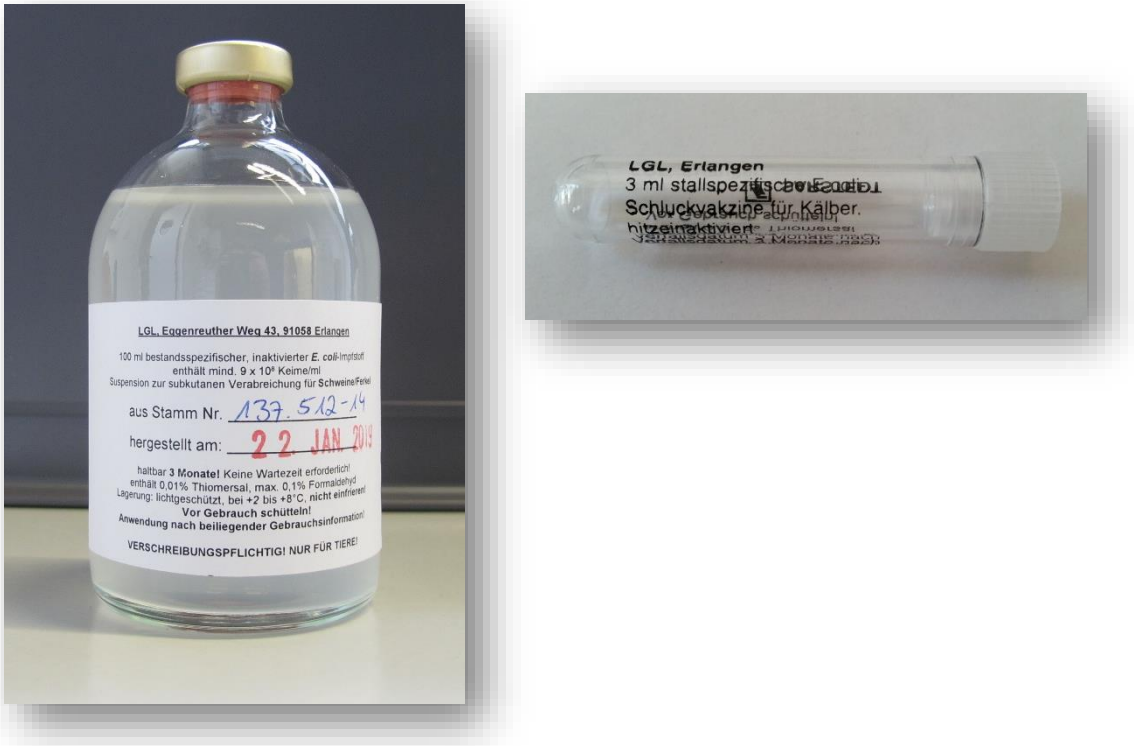

Figure 3: Examples of the autogenous vaccine, produced at the Bavarian Health and Food Safety

As a conclusion and for the improvement of the situation regarding severe or fatal enteritis in calves, we recommend the oral prophylaxis using inactivated autogenous vaccines in combination with improved animal husbandry and management practices. These measures for instance include regular diagnostic testing, all-in-all-out occupancy, frequent air exchange and control of adequate temperature, reduction of noxious gases and at last minimization of the on-farm stress level (MURPHY et al., 2017). Moreover, veterinary vaccinology should focus on the vaccination of animals that may transmit food-borne or zoonotic diseases (SCHUDEL & LOMBARD, 2007). Another focus should be laid on the harmonization of legislation in the European economic area regarding inactivated autogenous vaccines to cope with the fact that inactivated autogenous vaccines are a useful replenishment of registered vaccines (EUROPEAN MEDICINES AGENCY (EMA)/CO-ORDINATION GROUP FOR MUTUAL RECOGNITION AND DECENTRALISED PROCEDURES-VETERINARY(CMDV), 2017).

V. ZUSAMMENFASSUNG

Enterotoxische *E. coli* stellen weltweit eine der wichtigsten Erregergruppe der neonatalen Kälberdiarrhoe dar. In dieser Publikation wurden neun hauptsächlich auftretende Serotypen (O101:K28, O9:K35, O101:K30, O101:K32, O78:K80, O139:K82, O8:K87, O141:K85, O147:K89) und fünf häufig vorkommende Virulenzfaktoren (F17, F41, F5, ST-I, stx1) in unterschiedlichen Kombinationen detektiert. In zukünftigen Untersuchungen soll außerdem das Vorkommen der humanpathogenen Serotypen O26, O103, O111, O117, O128, O145, O146 und O157 erforscht werden, da Wiederkäuer eines der wichtigsten Reservoire für humanpathogene STEC darstellen.

Die Resistenzsituation der ersten, zweiten und dritten Wahl an klinisch relevanten Antibiotika, Amoxicillin-Clavulansäure, Enrofloxacin und Trimethoprim-Sulfamethoxazol, ist aktuell akzeptabel mit Resistenzraten von 28.1%, 37.9% und 50%. Trotzdem sollten kritische Antibiotika von höchster Priorität für die Humanmedizin, festgelegt durch die WHO, zurückhaltend eingesetzt werden. Dazu zählen unter anderem Fluoroquinolone (Enrofloxacin), Polymyxine (Colistin) und Cephalosporine der 3. und 4. Generation (Ceftiofur). Außerdem sollten die beachtlich hohen Resistenzraten berücksichtigt werden. Es wurden 84.2% multiresistente, sowie 15.8% erheblich resistente Isolate detektiert, was die bereits publizierten hohen Prävalenzen an resistenten Keimen bei Jungtieren bestätigt. Die Antibiotikaresistenzen sind immer noch eine große Sorge in der veterinärmedizinischen und humanmedizinischen Praxis, also im „One Health"-Ansatz. Dies berücksichtigt, dass in Veterinär- und Humanmedizin zu Therapiezwecken eingesetzte Antibiotika oft die gleichen bzw. ähnlichen Wirkstoffe beinhalten und die Entstehung von Antibiotikaresistenzen fördern, was eine globale Gefahr für das Gesundheitswesens darstellt. Die Verschreibung von Antibiotika sollte Kälbern vorbehalten sein, die Fieber haben, festliegen, keinen Saugreflex und Anzeichen einer Sepsis zeigen.

Für die Routinediagnostik und um spezifische Behandlungsoptionen aufzustellen, empfehlen wir, Kotproben auf Viren, Parasiten und Bakterien zu untersuchen sowie die Serotypisierung und das Erstellen von Antibiogrammen der potenziell

vorgefundenen *E. coli*. Das Asservieren der virulenten Stämme ist wichtig für die Herstellung von bestandsspezifischen Vakzinen.

In Zukunft sollte, auch im Kontext des „One Health“ Konzepts, eine Datenbank mit Vollgenomsequenzen etabliert werden. Der Inhalt sollte Antibiotikaresistenzdaten und Virulenzfaktoren berücksichtigen sowie die Rückverfolgbarkeit von Isolaten gewährleisten, z.B. über SNP Typisierung. Abschließend müssen weitreichende Resistenzmonitoring Methoden durchgeführt werden, um Langzeitdaten zu sammeln, damit in Zukunft Resistenz Trends rechtzeitig erkannt werden können.

VI. SUMMARY

Worldwide, enterotoxigenic *E. coli* organisms are a leading cause of diarrhea in neonatal calves (FOSTER & SMITH, 2009; KOLENDA et al., 2015). The present study revealed nine prevalent *E. coli* serotypes (O101:K28, O9:K35, O101:K30, O101:K32, O78:K80, O139:K82, O8:K87, O141:K85, O147:K89) and five leading virulence factors (F17, F41, F5, ST-I, stx1) in various combinations.

The resistance situation against the 1st, 2nd and 3rd line antimicrobials for the treatment, amoxicillin-clavulanic acid, enrofloxacin and trimethoprim-sulfamethoxazole (VETSUISSE-FAKULTÄT et al., 2019), is currently acceptable with non-susceptibility rates of 28.1%, 37.9% and 50%. Nevertheless, attention should be paid to the application of highest priority critically important antimicrobial class for human medicine assessed by the WHO, such as fluoroquinolones (enrofloxacin), polymyxins (colistin) and 3rd and 4th generation cephalosporines (ceftiofur). Furthermore, the considerably high rates of 84.2% multidrug-resistant and 15.8% extensively drug-resistant isolates should rise awareness of highly resistant *E. coli* and underlines the current high prevalence of antimicrobial resistance in young animals (KHACHATRYAN et al., 2004). Antimicrobial resistance remains a major concern in clinical veterinary medicine and “One Health”. This includes, that antimicrobials used for therapeutic purposes in human and animal health are frequently the same or closely related and trigger the development of antimicrobial resistances which constitutes a global public health threat. Thus, the prescription of antimicrobials should be reserved for calves with fever, recumbency, no suckle reflex and signs of sepsis.

For routine diagnostics and specific treatment options, we recommend initial sample investigation for viruses, bacteria and parasites. Further laboratory diagnostics should include antimicrobial resistance testing and serotyping of *E. coli*. Preservation of bacterial isolates is crucial for autogenous vaccine production.

For further analyses, we intend to investigate the major human pathogenic serogroups O26, O103, O111, O117, O128, O145, O146 and O157, because ruminants are known to be a major reservoir of human pathogenic STEC (MONTENEGRO et al., 1990; BEUTIN et al., 1993; BLANCO et al., 1994;

GYLES, 2007).

As an outlook, and again to follow the “One Health” idea, a database originating from whole genome data should be established and published. Contents should provide information about antimicrobial resistance, virulence factors, relevant for human and veterinary medicine, and finally trace-back options, respectively. We conclude that an extensive monitoring of antimicrobial resistance must be continued to collect long-term data for reliable prognoses and tendencies.

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