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Longitudinal Study of Some Bacterial, Parasitic, and Viral Enteric Pathogens isolated from Diarrheic Calves from Dairy Herd in Egypt

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Abstract

Neonatal calf diarrhea remains one of the most important problems faced by livestock, causing great economic losses. Fecal samples were collected from 100 diarrheic calves in Al-Fayoum governorate, Egypt during 2021, and 2022, to investigate the prevalence of Enterotoxigenic *Escherichia coli* (ETEC), *Salmonella* Typhimurium, *Klebsiella pneumoniae*, *Clostridium perfringens*, bovine rotavirus, bovine coronavirus, and *Cryptosporidium parvum* which are the major enteropathogens associated with neonatal calf diarrhea, the prevalence of enteropathogens were 58%, 29%, 34%, 14%, 35%, 8%, and 65% respectively. Molecular characterization was performed to confirm the *E. coli*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, *Clostridium perfringens*, and *Cryptosporidium* isolates and to detect some virulent genes associated with their pathogenicity. All the bacterial isolates gave a clear band with 16S rRNA. In *E. coli*, virulent genes (*K99*, *F41*, *phoA*) were detected, also; *Salmonella* strains were found positive for the *invA* and *sopB* gene, while all *Clostridium perfringens* strains were tested positive for Alpha and Beta toxin but negative for Epsilon toxin. On the other hand, all *Klebsiella pneumoniae* isolates were tested positive with *iutA* and *fimH* genes. Also, the *in-vitro* antibiotic sensitivity testing of bacterial isolates was applied. Statistical analysis was carried out to determine the potential influence of age factor on the reported prevalence of concurrent infections, which revealed that the animals age significantly affected the infection prevalence in all pathogens inversely excepts those infected by *Klebsiella* was affected by age directly, and those infected by *E. coli*, or *Cryptosporidium*, were not affected by age at all. Good hygienic management and good vaccination program are very important to overcome acute diarrhea in neonate calves and the misuse of antibiotic revealed the presence of multidrug resistance isolates of some enteropathogenic bacteria.

KEYWORDS
Neonatal calf diarrhea, Bovine rotavirus, Bovine coronavirus, *Cryptosporidium* spp., *E. coli*, *Salmonella* spp., *Clostridium* spp., *Klebsiella*, Epidemiology, Pathogens.

INTRODUCTION

Neonatal calf diarrhea (NCD) is a well-known worldwide cattle disease that causes large economic losses due to high morbidity, mortality, growth delay, and treatment costs (Lojkić *et al*., 2015). NCD is caused by a variety of enteric pathogens, including those that are viral (bovine rotavirus (BRV), bovine coronavirus (BCoV), and bovine viral diarrhea virus), parasitic (*Cryptosporidium parvum*, *Giardia duodenalis*, and *Eimeria* species), and bacterial (*Escherichia coli K99* (ETEC), *Salmonella* species, and *Clostridium*) (Gillhuber *et al*., 2014; Scharnböck *et al*., 2018).

Neonatal calf diarrhea (NCD) is still the leading cause of calf mortality in Egypt, where it affects between 27.4% and 55% of the total deaths in young calves (Younis *et al*., 2009). Diarrhea affects neonatal calves due to a variety of infectious (bacteria, viruses, parasites) and non-infectious reasons. According to earlier research, enterotoxigenic *E. coli* (ETEC), bovine coronavirus (BCoV), bovine rotavirus (BRV), and *Cryptosporidium parvum* (CP) are the most significant infectious agents either singly or in combination (Foster and Smith, 2009). Other agents, including the Bovine Torovirus, Parvovirus, Pestivirus, Calicivirus, Astrovirus, Adenovirus, *Eimeria* spp., *Giardia*, *Clostridium perfringens*, *Campylobacter*, *Proteus*, and *Klebsiella*, can nevertheless contribute to enteric illnesses.

Regarding age factor, BRV, BCoV, ETEC, and *Cryptosporidium parvum* were the most frequently recognized causes of diarrhea in calves during their first 30 days of life. Newborn calves are mostly sensitive to ETEC infections during their first four days of life. While BCoV infections are more likely from day five to twenty, BRV infections are more common in the first to second week of life. Furthermore, calves are particularly vulnerable to *Cryptosporidium parvum* infections between the first and third weeks of life (Santín *et al*., 2008; Brunauer *et al*., 2021).

According on its virulence scheme, *Escherichia coli* can be divided into six pathogroups: enterotoxigenic *E. coli*, shiga toxin-producing *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enteroaggressive *E. coli*, and enterohaemorrhagic *E. coli* (Kaper *et al*., 2004). The most common cause of neonatal diarrhea is ETEC strains that produce the *K99* (*E. coli K99*) (F5) adhesion antigen referred to heat-stable enterotoxin (Nataro and Kaper 1998). Enterotoxins produced by the *E. coli* trigger epithelial cells to secrete fluids into the lumen resulting in severe diarrhea (Zhang *et al*., 2022). Enterotoxigenic colibacillosis is a major cause of diarrhea in newly born calves less than 3 days of

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age without an outbreak of diarrhea in calves older than 3 days (Radostitis *et al*., 2007). It was reported that low colostrum fed to young calves were significantly associated with *E. coli* isolation (Ashenafi and Tesfaye, 2016).

Salmonellosis in cattle is most frequently caused by the serovars Typhimurium (*S.* Typhimurium) and Dublin (*S.* Dublin). *S.* Typhimurium is the most common serotype that affects calves. Salmonellosis is most severe in calves under a month of age (Bicnell, and Noon, 1993). *Salmonella*-related diarrhea is watery and mucoid with blood and fibrin present (Fossler *et al*., 2005). *Salmonella* pathogenicity island SPI-1 and SPI-5 including *invA* and *sopB* genes are known to influence the type III secretion system and are mainly responsible for *Salmonella* induced diarrhea in calves (Treuer and Haydel, 2011).

Clostridium perfringens is a Gram-positive, spore- forming anaerobic bacterium that causes a wide range of diseases in mammals and birds (Van Immerseel *et al*., 2004). Five different toxin kinds were categorized this bacterium as (A, B, C, D, and E) based on the production of four major toxins: alpha (α), beta (β), epsilon (ε), and iota (ι) (Petit *et al*., 1999). Type A strains produce α toxin alone, type B stains produce α, β, and ε toxins; type C type strains manufacture α and β toxins; type D strains secrete α and ε toxins; and type E strains produce α and ι toxins. Among these groups, type C has been frequently reported in conjunction with calf diarrhea (Rings, 2004). Other enteric pathogens as BCoV, BRV, *Salmonella* spp., *E. coli*, and *C. parvum* are considered as a main cause of diarrhea in calves under two weeks of age, although *C. parvum* has been reported in calves up to two months of age (Songer, 1999). *C. perfringens* type C can easily infect newborn calves since their digestive tracts don't produce a lot of proteolytic enzymes (e.g., trypsin) and β toxin is recognized as the main virulence factor responsible for clinical signs seen in animals affected by this bacterium. These infected animals' intestinal lesions are characterized by diffuse or multifocal hemorrhagic necrotizing enteritis with bloody fluid distension (Barker *et al*., 1993).

A Gram-negative coliform bacteria called *Klebsiella* sp. lives in both human and animal intestines. Diarrhea may be caused by the opportunistic pathogens *Klebsiella* sp. and Proteus sp. It is challenging to successfully control NCD because of its multifactorial nature. Identification of the etiological and predisposing variables for calf diarrhea is crucial in order to devise preventive measures and reduce losses during the initial months of life (Lorino *et al*., 2005). Molecular diagnostics, most often Polymerase Chain Reaction (PCR)-based tests, are becoming more common in diagnostic laboratories for detecting enteric pathogens and present their own challenges in interpretation (Espy *et al*., 2006). PCR methods are usually more sensitive and may detect lower levels of pathogens that may not be causing disease due to age resistance or those arise from cross-contamination from one calf to another. PCR methods also can detect the dead or stressed bacteria or even in the presence of antibody-antigen complexes (Blanchard, 2012).

BRV is classified into family *Reoviridae*, subfamily *Sedoreovirinae*, genus *Rotaviruse* genus. The virion is non-enveloped with a diameter of 65-70 nm. The Rotavirus genome consists of 11 double-stranded RNA gene segments encoding six nonstructural (NSP1–6) and six structural (VP1–4, VP6–VP7) proteins (Gichile, 2022; Uddin Ahmed *et al*., 2022).

BCoV is a pneumoenteric virus. It belongs to the family Coronaviridae, subfamily Coronavirinae within the genus Betacoronavirus, subgenus Embecovirus. BCoV particles are enveloped and pleomorphic, 100-120 nm in diameter. BCoV consists of a non-segmented, single-stranded, positive-sense RNA (ssRNA) (Wentworth and Holmes, 2007) The virus has five major structural proteins the nucleocapsid protein (N), the integral membrane (M), the small membrane/envelope protein (E), the haemagglutinin-esterase (HE) and the spike (S) and sixteen non-structural proteins (NSP 1-16) (Asadi *et al*., 2015; Vlasova and Saif, 2021).

Different diagnostic approaches are used to detect the BRV and BCoV that include electron microscope, virus isolation, immunochromatographic rapid diagnostic assays, Enzyme-linked immunosorbent assay (ELISA) (de Mira Fernandes *et al*., 2018; Abouelyazeed *et al*., 2020)

Cryptosporidium parvum is a protozoan parasite that is usually related to gastrointestinal tract illness of neonatal calves. Infected neonatal calves might be asymptomatic or develop severe diarrhea and dehydration (Chalmers *et al*., 2011). Diagnosis of cryptosporidiosis can be carried out by using fecal smears stained by the modified Ziehl–Neelsen technique, Sheather's sucrose flotation solution (Singh *et al*., 2006). To identify different *Cryptosporidium* species in cattle feces we needed to molecular methods involving polymerase chain reaction (PCR) assays (Mirhashemi *et al*., 2016).

The objectives of this study were: (i) to perform longitudinal analysis to estimate the overall prevalence across the tested herd in Egypt, of the most common enteric pathogens; (ii) to determine the statistical influence of potential influencing factors on the reported prevalence of concurrent infections; (iii) to analyze the chance that one of the enteric pathogens occur in the presence of BRV; (iv) to determine the expected prevalence of mixed infection in calves with diarrhea, assuming that both considered causative agents occur independent from each other; (v) to model the detected prevalence of mixed-infection depending on the age class of sampled animals; and (vi) to establish a successful and true line of treatment based on sensitivity test to detect the suitable antibiotic for each field strain isolate and to avoid bacterial resistance.

MATERIALS AND METHODS

Ethical statement

Sampling was performed as non-experimental clinical work and registered veterinarians collected all samples. The study protocol and experimental design was approved by the institutional animal care and use committee (ARC-IACUC) at Animal health research institute (AHRI), agricultural research center (ARC), Egypt (ARC-AHRI-23-25) following the animal welfare act of the Egyptian Ministry of Agriculture.

Sample collection and preparation

Fecal samples were collected from 100 calves (Crossbred Holstein cows and Buffaloes, undefined), born in dairy herd with median size of 500 dairy animal located in Al-Fayoum governorate, between June 2021 and August 2022. Samples were collected from calves suffering from diarrhea and aged between one day to 45 days. No vaccine was used in dams or in the newborns to prevent calf scours. Fecal samples were collected directly from the rectum and transferred directly to the laboratory in a separate clean sterile plastic bag in an ice box and kept in retail package under complete aseptic condition at 4°C until bacteriological and parasitological examination, and at −20°C until virological examination.

Ziehl-Neelsen Staining

The fecal samples were examined by ordinary direct smear

method and saline smear method for detection of *Cryptosporidium parvum*. The fecal samples were stained with Modified Ziehl-Neelsen staining technique. It was performed according to Casemore *et al*. (1985). The stained slides were examined by the light microscope using high power (40x) and oil immersion lens (l00x). The Measurements were made by ocular micrometer calibrated against a stage micrometer slide (OIE, 2008).

Bacterial isolation

Isolation and identification of *E. coli*, *Klebsiella* species, *Salmonella* species and, *Clostridium perfringens* among the collected samples were confirmed based on their morphology, cultural and biochemical tests using standard bacteriological procedures described in Murray *et al*. (2003) and Octavia and Lan (2014).

Antimicrobial susceptibility testing

In vitro susceptibility of all *E. coli*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, and *Clostridium perfringens* isolates to commonly used antimicrobial drugs was tested by the Kirby-Bauer standard agar disk diffusion technique as described earlier (Bauer *et al*., 1966), using Mueller Hinton agar and commercial antibiotic disks (Oxoid, Basingstoke, Hampshire, England, UK). The tested antibiotics were as following: amoxicillin (AMX), amikacin (AK), ceftriaxone (CTR), tetracycline (TE), sulfamethoxazole/trimethoprim (SXT), Cefepime (CPE), gentamicin (CN), Erythromycin (ERY), Ampicillin (AMP), Amoxicillin / Clavulanic acid (AMC) and ciprofloxacin (CIP). The millimeter-sized inhibitory zones were tested twice and scored as sensitive, moderate, and resistant in accordance with the critical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

Detection of BRV and BCoV

BRV and BCoV were detected in the collected faces samples separately using indirect antigen-capture sandwich ELISA Kit (Monoscreen Ag Elisa®, Bio-X Diagnostics, SA., Belgium) according to the manufacture instruction. The optical density (OD) for each well was measured at 450 nm. Results were interpreted according to manufacturer's instruction.

Molecular characterization of NCD pathogens

DNA extraction

DNA extraction from fecal samples was done using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Briefly, 220 mg of the samples were added to 1.4 ml buffer ASL, and then incubated at 70˚C for 5 min. Then, samples were homogenized for 6 min. using the QIAGEN Tissue-Lyser and then centrifugated at 14000 rpm for 1 min. to pellet the stool. One Inhibit Ex tablet was added to 1.2 ml of the supernatant and vortexed, incubated for 1 min. at room temp. The samples were centrifuged at 14000 rpm for 3 min. and 200 µl of the supernatant were added to 15 µl of proteinase K and 200 µl of lysis buffer AL and incubated at 70˚C for 10 min. After incubation, 200 µl of absolute ethanol was added to the lysate. The lysate was transferred to the column, centrifugated at 14000 rpm for 1 min., then washed and centrifuged. Nucleic acid was eluted with 100 µl of elution buffer AE provided in the kit.

PCR amplification

Conventional PCR was performed for detection of *Cryptospo-*

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions used for detection of *Salmonella* spp, *C. perfringes, K. pneumonia, E. coli* and *Cryptosporidium parvum.*

Target bacteria	Target gene	Primers sequences	Amp. segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Salmonella	*invA*	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	Oliveira *et al*. (2003)
	sopB	TCA GAA GRC GTC TAA CCA CTC TAC CGT CCT CAT GCA CAC TC	517			58˚C 40 sec.	72˚C 45 sec.		Huehn *et al*. 2010
	S. Typhimurium *STM4495*	GGT GGC AAG GGA ATG AA CGC AGC GTA AAG CAA CT	915			50˚C 1min.	72˚C 1 min.		Liu *et al*. (2012)
C. perfringens	*16S rRNA*	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279			53˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Wu *et al*. (2009)
	Alpha toxin	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	YOO *et al*. (1997)
	Beta toxin	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236						
	Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541						
K. pneumoniae	*16S-23S ITS*	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Turton *et al*. (2010)
	iutA	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	94˚C 5 min.	94˚C 30 sec.	63˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Yaguchi *et al*. (2007)
	fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ghanbarpour and Salehi (2010)
E. coli	*phoA*	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Hu *et al. (*2011)
	F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Franck *et al*. (1998)
	K99	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
Cryptosporidium	*COWP*	GGACTGAAATACAGGCATTATCTTG GTAGATAATGGAAGAGATTGTG	553	94˚C5 min.	94˚C 30 sec.	55˚ 40 sec.	72˚C 45 sec.	72˚C10 min.	Feltus *et al*. (2006)

ridium spp., and bacterial agents, all reactions were performed in a T3 Biometra thermal cycler. All Primers were supplied from Metabion (Germany).

For Bacteriological examination; Uniplex PCR Primers for each of 3 genes of *Salmonella* spp., *Klebsiella pneumoniae*, and *E. coli*; were utilized in a 25µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol concentration), 5.5 µl of water, and 5 µl of DNA template. Also, Multiplex PCR for *C. perfringens* toxins was performed, primers were utilized in a 50µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol concentration), 12 µl of water, and 5 µl of DNA template. Details of primers sequences, amplicon size, and reaction conditions are listed in Table 1.

COWP gene of *Cryptosporidium* was amplified as previously described by Feltus *et al*. (2006). A 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of forward primer, 1 µl of reverse primer, 5.5 µl of water, and 5 µl of DNA template. Details of primers sequences, amplicon size, and reaction conditions are listed in Table 1.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (AppliChem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gene ruler 100 bp DNA Ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Sequencing and phylogenetic analysis

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Lasergene DNA Star version 12.1 Thompson *et al*. (1994) and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura *et al*., 2013).

Statistical analysis

Calves were grouped according to their age as follows: 1–7, 8–14, 15–21, and 36–42 days old or 1, 2, 3, and 6 weeks. The Pearson chi-square test was used at 95 % significance to estimate the relation between the proportion of fecal samples that tested positive for the enteropathogens studied and the age group of animals with diarrhea using SPSS program (Ver. 20, IBM Co.). The relative risk for the development of diarrhea in association with the enteropathogens was estimated with 95 % confidence interval.

RESULTS AND DISCUSSION

Diarrhea in newborn calves is a multifactorial disease, and a variety of infectious and non-infectious criteria play a role in its development (Gomez and Weese, 2017).

Calf rearing in dairy cattle herds has high costs and high mortality rates. The results presented here in this longitudinal study revealed a wide variation in the prevalence of considered mixed infection and their significant influencing factors. The considered causative agents in the presented study cover A BRV, BCoV, *Salmonella* spp., Enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens*, *Klebsiella pneumoniae*, and *Cryptosporidium parvum* in diarrheic calves up to 45 days old, which were reared on a dairy farm in Al-Fayoum governorate using management practices notably similar to those practiced in large dairy cattle herds in Egypt; therefore, the results obtained in this study can be extended to other herds. The results demonstrate the importance of diarrhea in calves within the first 6 weeks of age and that improved management practices during this time are necessary to reduce calf scours and its consequences. In Egypt, there is no longitudinal studies for enteropathogens in young claves, interestingly; there are only few studies worldwide also (Coura *et al*., 2015).

In our research the prevalence of microorganisms isolates from 100 diarrheic calves were 35% BRV, 8% BCoV,65% *Cryptosporidium*, 58% *E. coli*, 34% *Klebsiella pneumoniae*, 29% *Salmonella* Typhimurium and 14% *Clostridium perfringens*, the high incidence of bacterial isolates is due to secondary bacterial infection and mixed infection as showed at Table 2. Calves that developed diarrhea within the first day of life had significantly higher concentrations of aerobic bacteria, anaerobic bacteria, Enterobacteriaceae, and *E. coli* in the feces, the high levels of these bacterial groups may be related to poor hygiene during or immediately after birth (Schwaiger *et al*., 2022). Results of *E. coli* infection in diarrheic calves appear to be similar to those of Olaogun *et al*. (2016) who isolated highest prevalence rates of *E. coli* (63.2%) and higher than previous result recorded by Yue et al. (2021) who detected 28.8% *E. coli* isolates from diarrheal fecal samples. Also our results higher than those reported by studies carried out by Algammal *et al*. (2020) and Wang *et al*. (2023) where a percentage of 20% *E. coli* was detected. In the present study, *Salmonella* spp. was identified as a very important causative agent of diarrhea (29%), this result is closed to Olaogun *et al*. (2016) (52.6%) and near to that reported by El-Seedy *et al*. (2016) (18.1%). Ezzat *et al*. (2023) reported a low incidence of *Salmonella* pathogen (3.7%) recorded in diarrheic calves. However, Lee *et al*. (2019) reported only one case of *Salmonella* spp. (0.6%) as a very rare causative agent in single suckle beef calves. *Clostridium perfringens* has also been identified as a cause of enteric diseases in calf diarrhea (Tamara, 2022). Prevalence of *C. perfringens* recorded in this study (14%) came in agreement with the results of Ngeleka *et al*. (2019) and Ferrarezi *et al*. (2008) who reported the detection of 12.4% and 12.06%, respectively. Additionally, Ezzat *et al*. (2023) recorded that the prevalence of *C. perfringens* was 8.8%. *Klebsiella* sp. is considered as an opportunistic pathogen that potentially could cause diarrhea. *Klebsiella pneumoniae* was

Table 2. Total microbial organisms isolated from diarrheic calves.

Total samples	Viral infection (n.= 43)		Parasitic Infection (n.= 65)	Bacterial infection (n.= 135)			
	BRV	BCoV	*Cryptosporidium*	*E. coli*	*Klebsiella* spp.	*Salmonella* spp.	*Clostridium* spp.
100	35	8	65	58	34	29	14

detected as 34% in the current study, this result appears to be similar to the findings (29.79%) reported by Rajat *et al*. (2022). Fecal shedding and gastrointestinal carriage of *Klebsiella* spp. has also been reported from animals including cattle (Munoz *et al*., 2006; Zadoks *et al*., 2011; Bandyopadhyay *et al*., 2021). The results of antimicrobial susceptibility were recorded in Table 3, showed that Amikacin and Gentamycin are the most effective antibacterial against *E. coli* although it has immunosuppression effect on newly born calves. Combination between Amoxicillin with Clavulanic acid, Cefotaxime, and Cefepime are the most effective against *Salmonella* Typhimurium. *Clostridium perfringens* isolates were highly sensitive to Amoxicillin with Clavulanic acid, Ciprofloxacin, Enrofloxacin. Amikacin, Gentamycin, Cefepime and Cefotaxime were the most effective antibiotics against *Klebsiella pneumoniae*. while all *E. coli*, *Salmonella* Typhimurium, *Klebsiella pneumoniae* isolates were resistant to Amoxicillin, Erythromycin, and Sulphamethoxazole with Trimethoprim. On the other hand, *Clostridium perfringens* isolates showed high resistances against Sulphamethoxazole with Trimethoprim and Amikacin. The incidence of resistance to Trimethoprim-sulfamethoxazole may be related to the widespread usage of this drug (Ezzat *et al*., 2023). Antibiotics applied in animal feed stuff as growth promoters are the critical causes of bacteria evolution, specifically *C. perfringens* resistance patterns as the bacteria become adapted due to recurrent antibiotic use (Bendary *et al*., 2022). This result is similar to those of Haque *et al*. (2022), who showed that all *E. coli* isolates were found 100% resistant to Amoxicillin, Cefuroxime, Cephalexin, Erythromycin, and Tetracycline, whereas 94.4%, 86.1%, and 77.8% isolates were resistant to Doxycycline, Moxifloxacin, and Gentamycin, respectively. In case of *Salmonella* isolates, all were found 100% resistant to amoxicillin, cephalexin and erythromycin. Our results agreed with El-Azzouny *et al*. (2020) in antimicrobial susceptibility profiles of *E. coli* isolates pattern with Amikacin, Ciprofloxacin, Sulphamethoxazole with Trimethoprim and Amoxicillin but disagreed with Cefotaxime, Gentamycin and Tetracycline pattern while in case of *Salmonella* spp. agreed with Cefotaxime, Amikacin and Ciprofloxacin and disagreed with Sulphamethoxazole with Trimethoprim, Gentamycin, Tetracycline and Amoxicillin pattern. Zhang *et al*. (2022) studied antimicrobial use and antimicrobial resistance in pathogens associated with diarrhea and pneumonia in dairy calves and recorded the similar result with our research in resistance of *Salmonella* spp. with Ampicillin, Gentamycin, Ciprofloxacin, and Tetracycline. Zhang *et al*. (2022) reported the numbers of *E. coli* isolates included in the studies resistant to Cefalexin and Erythromycin. The resistance rates for most of the antimicrobials in *Salmonella* spp. were higher than those in *E. coli*. Future studies focusing on factors associated with antimicrobial use in calves and elucidation of the modes of transmission of antimicrobial resistance genes are needed in order to curb antimicrobial use and antimicrobial resistance. Rajat *et al*. (2022) recorded that very high percent of *Klebsiella* isolates were resistant to Tetracycline (75%), followed by Gentamicin, and Ceftriaxone (50%), while lesser percent of isolates were resistant to and Enrofloxacin (37.5%) whereas least percent of isolates were resistant to Amikacin (25%), these result matched well with our study in case of Tetracycline, Enrofloxacin and Amikacin resistance to *Klebsiella* strain of the present study. Bandyopadhyay *et al*. (2021) concluded that the high level of antibiotic resistant in the *Klebsiella* isolates might be due to the presence of capsule that gives some level of protection to the cells, presence of multidrug resistance efflux pump, easy spread of organism, efficient at acquiring and disseminating resistance plasmid. Our records agreed with Wu *et al*. (2022) in resistance of *Klebsiella pneumoniae* to Ampicillin and the resistance rate to Gentamicin, Tetracycline, and Sulfamethoxazole/trimethoprim which was between 20.08% and 47.28%.

PCR amplification of all genes was carried out using specific oligonucleotide primers and reaction cycling conditions, in our study as in Figure 1, we detected *Salmonella* spp. for three genes *invA*, Typhimurium *STM4495* and *sopB*. PCR was applied on four isolates of *Salmonella* spp. to determine the virulence *invA* gene that encodes a protein in the inner membrane of bacteria, which is responsible for invasion to the epithelial cells of the host (El-azzouny *et al*., 2020) while Malorny *et al*. (2003) mentioned that *invA* gene has been recognized as an international standard for detection of *Salmonella*. The obtained results revealed that all tested isolates harbored *invA* gene (100%). These results were similar to those obtained by Soliman (2014). Regarding to PCR screening of *sopB* gene in *Salmonella* species isolates, we found that all isolates had *sopB* gene, consistent with Rahman (2006). This study revealed that there was association between virulence genes and antimicrobial resistance of the tested *E. coli* and *Salmonella* spp. isolates, similar to previous study performed by Wagdy *et al*. (2016). All *E. coli* isolates were positive for *phoA* gene and *K99* while two isolates were positive only for *F41* (Figure 2).

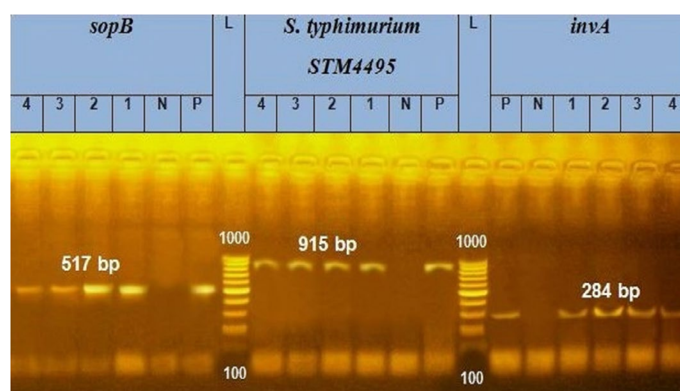

Fig. 1. Detection of three genes of *Salmonella*. On the right: Molecular typing of the *Salmonella* using *Salmonella invA* primers giving specific band at 284bp. L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1,2,3 and 4 positive clinical samples. On the intermediate: Molecular typing of the salmonella using *Salmonella* Typhimurium *STM4495* primers giving specific band at 915bp L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1,2,3 and 4 positive clinical samples. On the left: Molecular typing of the *Salmonella* using Salmonella *sopB* primers giving specific band at 517bp. L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1, 2, 3 and 4 positive clinical samples.

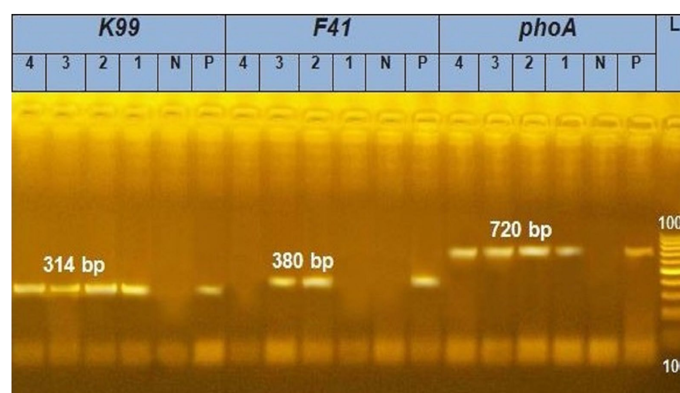

Fig. 2. Detection of three genes of *E. coli* (*K99, F41* and *PhoA*.) On the right: Molecular typing of the *E. coli* using *E. coli PhoA* primers giving specific band at 720bp. L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1,2,3 and 4 positive clinical samples. On the intermediate: Molecular typing of the *E. coli* using *E. coli F41* primers giving specific band at 380 bp P-positive control, N-Negative control. 2 and 3 positive clinical samples. On the left: Molecular typing of the *E. coli* using *E. coli K99* primers giving specific band at 314bp. P-positive control, N-Negative control. 1, 2, 3 and 4 positive clinical samples.

E. coli isolates from diarrhaeic calves had the main virulence genes, these results were similar to the results of previous studies (de Verdier *et al*., 2012; Yue *et al*., 2021). The most important adhesions involved in *E. coli* host colonization are fimbriae. Well characterized fimbriae of *E. coli* isolated from animals include F4 (*K88*), F5 (*K99*), F6 (*987P*), *F41* and *F18*, are associated with *E. coli* pathotypes. *K99*, *F41*, *STa*, *Stx1* and *Stx2* were considered the most common virulence gene markers of *E. coli* strains isolated from calves with diarrhea (Ok *et al*., 2009, Maciel *et al*., 2019; Nguyen *et al*., 2011). In our study we detected *Clostridium perfringens*, alfa, beta and epsilon toxin as described in Figures 3 and 4. The recovered *Clostridium* isolates were confirmed with PCR technique for (16S rRNA) and all *Clostridium perfringens* isolates were found positive for Alpha and Beta toxin while negative for epsilon toxin. Among *Clostridium perfringens*, type A is the most common of all types. *Clostridium perfringens* type C may result in severe bloody diarrhea, although often times calves die before diarrhea develops (House *et al*., 2014). Goossens *et al*. (2017) reported that alpha toxin, which is produced by all *Clostridium perfringens* strains, is essential for necro-hemorrhagic enteritis. The alpha toxin encoded by CPA gene is the main lethal toxin of type A, and it is also produced by all other genotypes of *Clostridium perfringens* type B and C which produce beta-toxin have been frequently reported in conjunction with calf diarrhea (Rings, 2004). *Clostridium perfringens* has been recently reported and postulated to have a synergistic function with enterotoxin (Gurjar *et al*., 2008). In young calves, severe abomasitis associated with *Clostridium perfringens* type A is characterized by sudden onset of disease, abomasal tympany, abdominal pain, and hemorrhagic diarrhea (Schlegel *et al*., 2012). In addition, the recovered *Klebsiella* isolates were confirmed with PCR technique for the species specific (16S-23S ITS) gene of *Klebsiella pneumoniae* isolates. Also, *iutA* and *fimH* virulence genes were assigned to detect the virulence traits of all isolates (Figure 5) for the virulence factors of *Klebsiella* spp., they own several virulence traits encompassing fimbriae, capsular polysaccharides, lipopolysaccharides, and siderophores that had been encoded by *fimH*, *rmpA*, *maga*, *uge*, *wabg*, *iutA* ,*fimH* , and other virulence genes (Remya *et al*., 2019). Moreover, *Klebsiella pneumoniae* infectivity and pathogenicity was found to be correlated with the ability to express their virulence genes. A proficient pathogen is virulent, resistant to antibiotics, and epidemic. The interplay between resistance and virulence is poorly understood, and is receiving great (Xu *et al*., 2022).

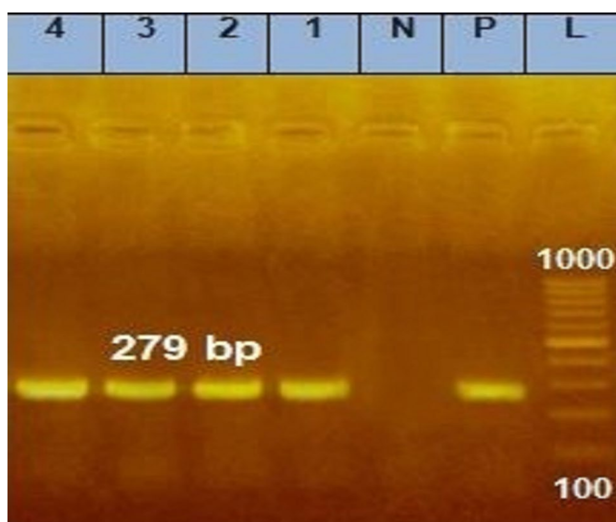

Fig. 3. PCR amplification for detection of *Clostridium perfringens* 16s rRNA. four DNA extracted from diarrhea culture showed positive for *Clostridium perfringens* 16s rRNA at 279 bp in lane 1, 2, 3, 4 positive samples. Lane P: Control Positive. Lane N: control Negative Lane L: Marker 100-1000 bp.

Cryptosporidium is a major parasite causing diarrhea in neonatal calves globally (Foster and Smith, 2009). Neonatal morbidity and mortality due to *Cryptosporidium* is also a major economic concern for the livestock industry (de Graaf *et al*., 1999). In addition, infected calves have been identified as significant sources of environmental contamination with zoonotic eggs of *C. parvum*, a fact with serious public health implications (Wielinga *et al*., 2008). Therefore, accurate identification of *Cryptosporidium* species is important not only for the adoption of appropriate treatment and preventive measures to reduce the burden of *Cryptosporidium* on the farm, but also to reduce the zoonotic risks involved (Hoek *et al*., 2008; Kinross *et al*., 2015, and Suler *et al*., 2016).

Cryptosporidiosis is typically caused by four *Cryptosporidium* species, including *C. parvum*, *C. bovis*, *C. andersoni*, and *C. ryanae* (Xiao and Feng, 2008; Ryan *et al*., 2014), although *C. felis* (Cardona *et al*., 2015), *C. hominis* (Smith *et al*., 2005), *C. suis* (Fayer *et al*., 2006; Bodager *et al*., 2015), and *Cryptosporidium* pig genotype II (Langkjaer *et al*., 2007) have also been sporadically reported infecting cattle. Among them, *C. parvum* shows a marked host age-related susceptibility and has been identified as the most dominant *Cryptosporidium* species infecting pre-weaned calves, in different studies worldwide (Geurden *et al*., 2007; Santín *et al*., 2004; 2008; Wielinga *et al*., 2008; Rieux *et al*., 2013). This was indeed the results of our present study, where *C. parvum* was successfully detected in sixty-five (65) samples out of one hundred (100) fecal samples from calves in the 1–45 days' age group, which came in agreement with previously reports in similar screening-based epidemiological surveys (23%-84%) (Ouchene *et al*., 2014; Benhouda *et al*., 2017).

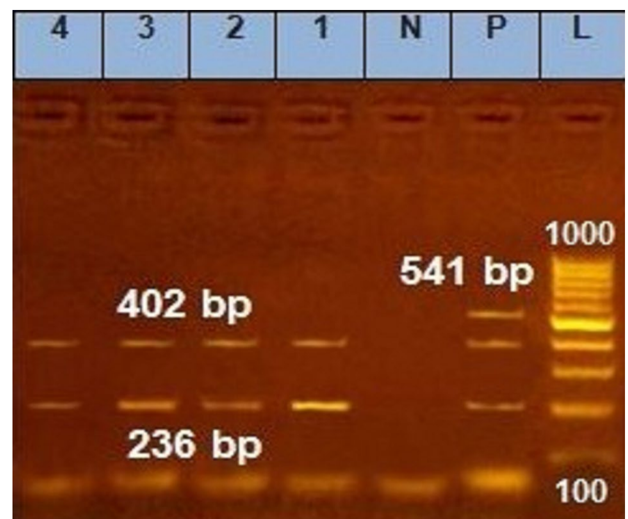

Fig. 4. Multiplex PCR for Toxino typing of 4 *Clostridium perfringens* isolates. Lane L: Marker 100-1000 bp Lane P: Control Positive *Clostridium perfringens* alfa, beta and epsilon toxin at 402bp, 236bp and 541bp respectively, Lane N: control Negative and lane 1, 2, 3, 4 showed positive for *Clostridium perfringens* alfa toxin at 402bp, beta toxin at 236bp and negative epsilon toxin at 541bp.

Using multiple diagnostic methods, bovine cryptosporidiosis (mainly in calves); has also been identified in 32% in Egypt (Helmy *et al*., 2015), 29% in Madagascar (Bodager *et al*., 2015), 52% in Nigeria (Ayinmode *et al*., 2010), and 87% in Tunisia (Soltane *et al*., 2007).

Furthermore, *C. parvum*, was the most prevalent species in cattle, as it was only identified in calves from 1 to 45 days of age. This contrasts with available molecular data in young calves (<2 months old) from Batna Province in Northeastern Algeria (Benhouda *et al*., 2017), where *C. bovis* (n = 14) was the most common *Cryptosporidium* species. were found, followed by *C. ryanae* (n = 6) and *C. parvum* (n = 4). In addition, we confirmed the presence

of *C. ryanae* and *C. andersoni* in an asymptomatic calf and cow, respectively. This is consistent with previous surveys showing that *C. parvum* causes sub-clinical infection in cattle 1 day of age and older than 1 month (Santín *et al*., 2004; 2008).

Our research findings revealed that *Cryptosporidium*-associated diarrhea primarily affects neonatal calves up to 1 month of age, with older animals often harbouring sub-clinical infection. This is consistent with previous studies showing that newborn calves are highly susceptible to *Cryptosporidium* infection due to their immature immune system and higher exposure to manure or equipment contaminated with parasite eggs (Foster and Smith, 2009; Bjorkman *et al*., 2015).

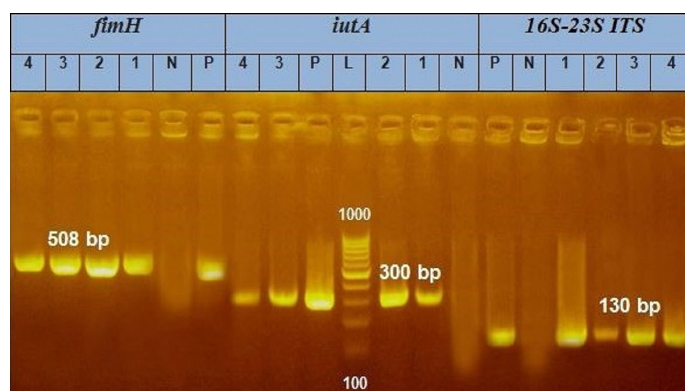

Fig. 5. Detection of 3 genes of *Klebsiella pneumoniae* (16S-23S *ITS, iutA* and *fimH*). On the right: Molecular typing of the *Klebsiella pneumoniae* using *Klebsiella pneumoniae* 16S-23S *ITS* primers giving specific band at 130bp. L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1,2,3 and 4 Positive clinical samples. On the intermediate: Molecular typing of the *Klebsiella pneumoniae* using *Klebsiella pneumoniae iutA* primers giving specific band at 300bp L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1,2,3 and 4 Positive clinical samples. On the left: Molecular typing of the *Klebsiella pneumoniae* using *Klebsiella pneumoniae fimH* primers giving specific band at 508bp. L- 100-1000bp DNA marker, P-positive control, N-Negative control. 1, 2, 3 and 4 Positive clinical samples.

Cryptosporidium was successfully identified by the modified Ziehl-Neelsen staining method in the tested samples (Figure 6). PCR was performed on five representative samples for molecular confirmation of *COWP* gene, specific positive amplification of 553 bp was detected in all tested samples as described in Figure 7. One PCR positive sample was selected for sequencing and was submitted in the GenBank as with accession no. (OP494720). Phylogenetic analysis of the *NAD1* gene showed nucleotide sequence identity range of 98.7-100% with *C. parvum* reference strains on the Genbank (Table 4), with clustering of the tested strain with *C. Parvum* strains apart from other *Cryptosporidium* Species as shown in Figure 8. When comparing results with PCR as the gold standard, enzyme immunoassays typically achieved diagnostic sensitivities in the range of 98.7–100%, significantly higher than those provided by modified Ziehl-Neelsen microscopy (Chalmers *et al*., 2011; Kuhnert-Paul *et al*., 2012).

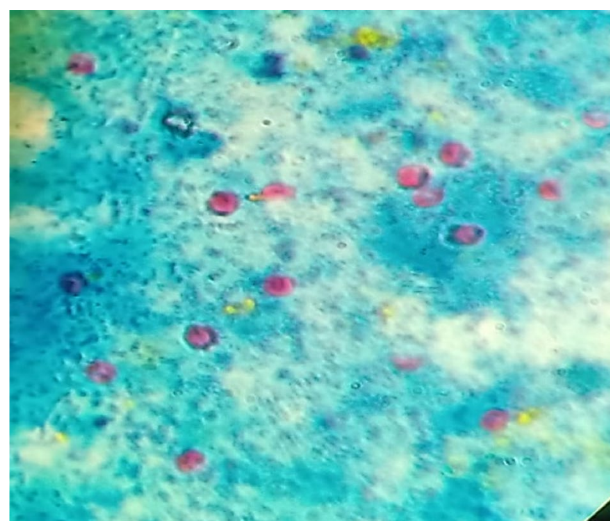

Fig. 6. Detection of *Cryptosporidium* with Ziehl-Neelsen Staining.

Moving to the viral causative agent; BRV, and BCoV diagnosis by ELISA is considered a crucial tool for detection from clinical samples because of its high sensitivity, simplicity, speed, low cost, and applicability without the requirement for experienced workers or expensive equipment (Abouelyazeed *et al*., 2020; Moussa *et al*., 2021). As a result, ELISA was chosen as the primary test for detecting the viral etiology of enteric diarrhea in calves. According to the findings of the present study, the overall detection rate of BRV was thirty-five samples from diarrheic calves (35%). This was substantially similar to the rate recorded in previous research, 32.5% (Ali *et al*., 2011; Abouelyazeed *et al*., 2020) and 36% (Hassan *et al*., 2014). These results disagreed with Barua and his

Table 3. Antibiotic sensitivity of enteropathogens bacterial isolates *(Salmonella spp, C. perfringes, K. pneumonia, and E. coli).*

Antibiotics	Bacterial isolates							
	E. coli (n.=58) No (%)		*Salmonella* Typhimurium (n.=29) No (%)		*Clostridium perfringens* (n.=14) No (%)		*Klebsiella pneumoniae* (n.=34) No (%)	
	S	R	S	R	S	R	S	R
Tetracycline (OXT 20 µg)	32(55)	26(45)	16(55)	13(45)	8(57)	6(43)	24(71)	10(29)
Amikacin (AK 30 µg)	56(97)	2(3)	21(72)	6(28)	2 (14)	12(86)	28(82)	6(18)
Cefotaxime (CTX 30 µg)	42(72)	16(28)	25(86)	4(14)	9(64)	5(36)	31(91)	3(9)
Ciprofloxacin (CIP 30 µg)	35(60)	23(40)	22(76)	7(24)	13(93)	1(7)	17(50)	17(50)
Amoxicillin (AMX 10 µg)	2(3)	56(97)	6(21)	23(79)	8(57)	6(43)	3(9)	31(91)
Sulphamethoxazole + Trimethoprim (SXT 25µg)	28(48)	30(52)	9(31)	20(69)	2(14)	12(86)	12(35)	22(65)
Amoxicillin + Clavulanic acid (AK 30 µg)	42(72)	16(28)	28(97)	1(3)	14(100)	0(0)	27(79)	7(21)
Ampicillin (AM 10 µg)	3(5)	55(95)	4(14)	25(86)	2(14)	12(86)	3(9)	31(91)
Cefepime (CFM 30 µg)	46(79)	12(21)	27(93)	2(7)	10(71)	4(29)	32(94)	2(6)
Gentamycin (CN 10 µg)	51(88)	7(12)	19(66)	10(34)	7(50)	7(50)	32(94)	2(6)
Erythromycin (E 30 µg)	6(10)	52(90)	9(31)	20(69)	9(64)	5(36)	9(26)	25(73)
Enrofloxacin (ENR 30 µg)	29(50)	29(50)	23(79)	6(21)	13(93)	1(7)	20(59)	14(41)

co-workers who have reported a very lower prevalence of 5.1% in similar study (Barua *et al.*, 2019).

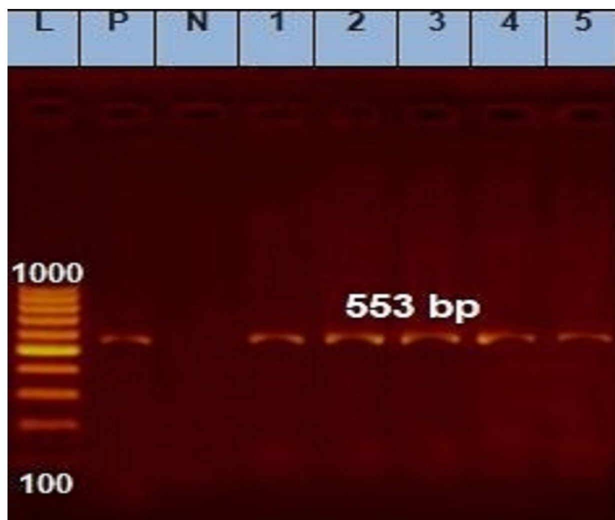

Fig. 7. PCR results for *Cryptosporidium COWP* gene showing positive amplification of 553 bp in 5 tested samples. L: 100 bp ladder (100-1000 bp), P: Positive control, N: Negative control.

We detected BRV in the feces of calves with a mean age of 8.5 days (range from 6 to 11). Different results were recorded by Lucchelli *et al.* (1992) and Garcia *et al.* (2000) who found a mean age of 18.6 days, and McNulty and Logan (1983) who found a mean age of 6.1 days. However, all these studies collected feces from calves with less than 30 days old, unlike the present study, therefore, resulting in a higher mean age. These findings suggest calves are exposed to infection soon after birth and infection also occurring in older calves.

A significant difference in the frequency of BRV in diarrheic calves was found among those in the first and second weeks of age. Same results were reported by Alfieri *et al.* (2006) and Mayameei *et al.* (2010). In accordance with our results, Bartels *et al.* (2010) detected the highest prevalence of BRV infection in animals within the second week of age; however, virus infection was associated with diarrhea in animals within the first week. Passive immunity may reduce the frequency of diarrhea caused by rotavirus in calves aged 1 week, and as calves get infected throughout calf rearing, the increased natural resistance reduces infection in calves older than 4 weeks (Alfieri *et al.* 2006).

Also, BCoV was detected in calves at mean age of 11 days (range from 8 to 14), which came in line with Torres-Medina *et al.* (1985) who showed that BCoV occurs mainly in animals less than 1 month old and generally affects calves at 7 to 10 days of age. Also, Coura *et al.* (2015) detected BCoV in the feces of diarrheic calves at mean age of 13.8 days. While Stipp *et al.* (2009) detected BCoV in calves from 1 to 60 days old, and the cases were concentrated in calves 16 to 30 days old. Generally, BCoV can infect animals up to 60 days old, and those within the second and third weeks of age develop diarrhea, due to the decrease of colostral antibodies present in the first week of age and the natural resistance acquired after infections, rendering the calf susceptible to the infection with this virus during this age.

Generally, Coronaviruses are conditional pathogens that are rarely detected when good management conditions are ensured but can invade the body when environmental conditions become poor. Therefore, coronaviruses generally have a very low detection rate (Lanz *et al.*, 2008; Reiten *et al.*, 2018). The prevalence of BCoV infected calves was 8% only from all over collected samples tested with ELISA. Previous studies have reported a lower or higher BCoV prevalence rates in diarrheic calves' feces. The percentage was 1.12% (Çabalar *et al.*, 2007), 1% (Erdogan *et al.*, 2003) and 0.7% (Jor *et al.*, 2008) while a high proportion had previously been documented, 30% (Moussa *et al.*, 2021), 38.9% (Abraham *et al.*, 1992) and 25.6% - 37.1% (Hasöksüz *et al.*, 2005). Worth to mention that our study did not evaluate respiratory infections; therefore, it is not possible to assert if the BCoV detected in feces originated from respiratory or enteric infections. BCoV can infect both the respiratory and the enteric tracts, and reinfection can occur in calves. Calves inoculated with BCoV orally or intranasally shed the virus in the respiratory and enteric tracts and are protected from winter dysentery, diarrheic, and respiratory BCoV strains, demonstrating that respiratory BCoV plays an important role in the epidemiology and transmission of diarrhea caused by BCoV (Saif *et al.* 1986; Heckert *et al.* 1990; Cho *et al.* 2001).

Table 4. Sequence distance of the *NAD1* gene of the tested *Cryptosporidium* strain (generated by lasergene software) showing identity range of 98.7-100% with *C. parvum* strains.

Percent Identity (upper triangle); Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		
1		99.8	96.4	97.9	98.1	97.9	90.4	90.4	86.2	86.2	82.8	85.5	86.0	85.7	77.6	92.5	98.7	98.7	97.5	98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.7	1	MK033061 C. hominis W8
2	0.2		96.2	97.7	97.9	97.7	90.1	90.1	86.4	86.4	83.0	85.7	86.2	86.0	77.4	92.7	98.5	98.5	97.3	98.5	98.5	98.5	98.5	98.5	98.5	98.3	98.5	2	KX365890 C. hominis
3	3.7	3.9		97.3	95.8	95.6	90.4	90.4	85.7	85.7	83.2	85.5	86.0	85.7	78.2	93.1	96.4	96.4	95.2	96.4	96.4	96.4	96.4	96.4	96.4	96.2	96.4	3	JX568159 C. meleagridis AmIR27
4	2.1	2.4	2.8		97.3	97.1	91.2	91.2	86.2	86.2	83.0	86.0	86.8	86.6	78.2	92.9	97.9	97.9	96.6	97.9	97.9	97.9	97.9	97.9	97.9	97.7	97.9	4	AF266271 C. wrairi
5	1.9	2.1	4.3	2.8		99.8	90.1	90.1	86.0	86.0	83.0	84.9	85.7	85.5	76.9	92.5	98.5	98.5	97.3	98.5	98.5	98.5	98.5	98.5	98.5	98.3	98.5	5	MH912965 C. tyzzeri 23228
6	2.1	2.4	4.6	3.0	0.2		89.9	89.9	86.2	86.2	83.2	85.1	85.5	85.3	76.7	92.2	98.3	98.3	97.1	98.3	98.3	98.3	98.3	98.3	98.3	98.1	98.3	6	MH912964 C. tyzzeri 30389
7	10.4	10.7	10.4	9.4	10.7	10.9		100.0	86.2	86.2	83.2	86.0	86.2	86.0	78.4	90.6	90.8	90.8	89.7	90.8	90.8	90.8	90.8	90.8	90.8	90.6	90.8	7	XM_029019345 C. ubiquitum EB
8	10.4	10.7	10.4	9.4	10.7	10.9	0.0		86.2	86.2	83.2	86.0	86.2	86.0	78.4	90.6	90.8	90.8	89.7	90.8	90.8	90.8	90.8	90.8	90.8	90.6	90.8	8	HM209385 C. ubiquitum goat2
9	15.5	15.2	16.0	15.5	15.7	15.5	15.5	15.5		100.0	81.8	94.3	88.3	88.1	76.9	86.0	86.2	86.2	85.1	86.2	86.2	86.2	86.2	86.2	86.2	86.0	86.2	9	MN065794 C. ditrichi Swec1089
10	15.5	15.2	16.0	15.5	15.7	15.5	15.5	15.5	0.0		81.8	94.3	88.3	88.1	76.9	86.0	86.2	86.2	85.1	86.2	86.2	86.2	86.2	86.2	86.2	86.0	86.2	10	MG266045 C. ditrichi 12667
11	19.6	19.3	19.0	19.3	19.3	19.0	19.0	19.0	21.0	21.0		80.7	80.7	80.5	79.7	82.2	83.4	83.4	82.6	83.4	83.4	83.4	83.4	83.4	83.4	83.2	83.4	11	DQ060432 C. baileyi Tcb
12	16.3	16.1	16.3	15.8	17.1	16.9	15.8	15.8	5.9	5.9	22.4		87.4	87.2	77.8	86.2	85.5	85.5	84.9	85.5	85.5	85.5	85.5	85.5	85.5	85.3	85.5	12	MG266046 C. apodemi 30405
13	15.7	15.5	15.7	14.7	16.0	16.3	15.5	15.5	13.0	13.0	22.4	14.0		99.8	75.9	86.0	86.0	86.0	84.7	86.0	86.0	86.0	86.0	86.0	86.0	85.7	86.0	13	LC503972 C. canis CDS-1284
14	16.0	15.7	16.0	14.9	16.3	16.5	15.7	15.7	13.2	13.2	22.7	14.3	0.2		75.7	85.7	85.7	85.7	84.5	85.7	85.7	85.7	85.7	85.7	85.7	85.5	85.7	14	LC503970 C. canis CDS-449
15	26.8	27.2	25.9	25.9	27.8	28.1	25.6	25.6	27.8	27.8	24.0	26.6	29.2	29.5		77.4	77.6	77.6	76.9	77.6	77.6	77.6	77.6	77.6	77.6	77.4	77.6	15	KJ917580 C. andersoni Y1
16	8.0	7.8	7.3	7.6	8.0	8.3	10.2	10.2	15.8	15.8	20.5	15.6	15.7	16.0	27.2		92.0	92.0	91.0	92.0	92.0	92.0	92.0	92.0	92.0	92.2	92.0	16	JX984441 C. viatorum Swec066
17	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5		100.0	98.7	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	17	BX538351 C. parvum
18	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0		98.7	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	18	AB089292 C. parvum
19	1.9	2.2	4.4	2.8	2.1	2.4	10.5	10.5	16.1	16.1	19.2	16.4	16.7	16.9	27.1	9.1	0.6	0.6		98.7	98.7	98.7	98.7	98.7	98.7	98.5	98.7	19	Z22537 C. parvum
20	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6		100.0	100.0	100.0	100.0	100.0	99.8	100.0	20	AB514045 C. parvum Sakha102
21	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0		100.0	100.0	100.0	100.0	99.8	100.0	21	DQ388390 C. parvum
22	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0	0.0		100.0	100.0	100.0	99.8	100.0	22	KY706489 C. parvum
23	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0	0.0	0.0		100.0	100.0	99.8	100.0	23	MK033059 C. parvum W6
24	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0	0.0	0.0	0.0		100.0	99.8	100.0	24	MK033058 C. parvum W5
25	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0		99.8	100.0	25	MG255789 C. parvum VANH9
26	1.5	1.7	3.9	2.4	1.7	1.9	10.2	10.2	15.7	15.7	19.0	16.6	16.0	16.3	27.1	8.3	0.2	0.2	0.9	0.2	0.2	0.2	0.2	0.2	0.2		99.8	26	OM817461 C. parvum FSA-1
27	1.3	1.5	3.7	2.1	1.5	1.7	9.9	9.9	15.5	15.5	18.8	16.3	15.7	16.0	26.8	8.5	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.2		27	OP494720 C. parvum AHRI_Alnady
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		

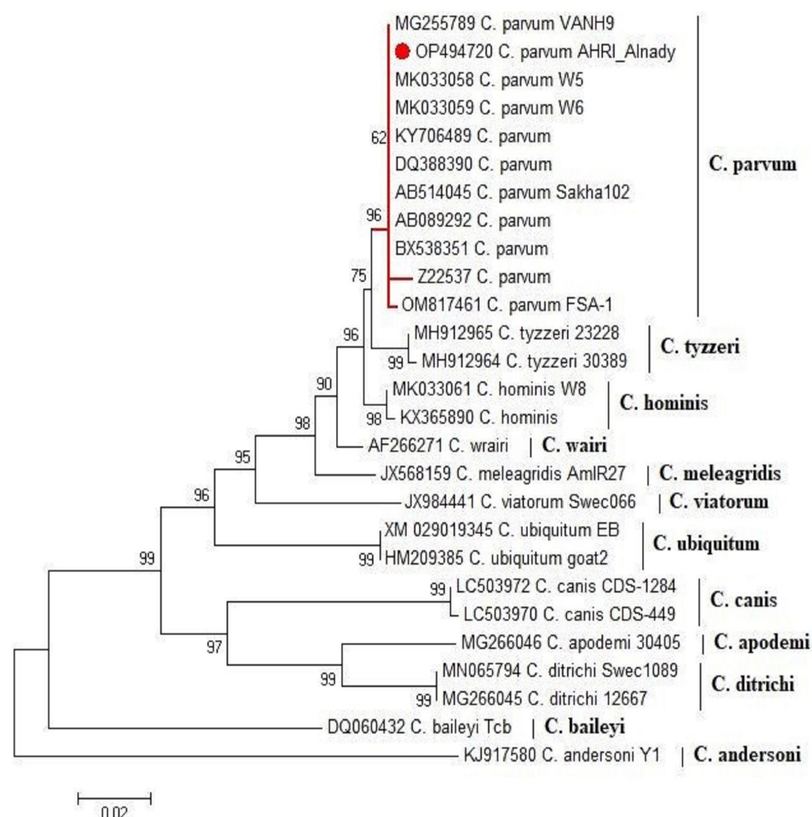

Fig. 8. Phylogenetic relatedness of th*e NAD1* gene. Maximum likelihood unrooted tree generated after 500 bootstraps indicated clustering of the tested strain with *C. Parvm* strains apart from other *Cryptosporidium* Species.

By statistical analysis, the Pearson chi square test was performed to set the relation between the proportion of positive animals for the enteropathogens studied and the age group of animals with diarrhea. The results revealed that all enteropathogens were significantly affected by the age inversely (P≤0.05), except *Klebsiella pneumoniae* infection is shown to be directly affected by age progression, also; *E. coli*, and *Cryptosporidium* infections were not affected significantly by age of animal (P<0.05).

Regarding the mixed infections, the results presented here revealed a wide variation in the prevalence of considered mixed infections and their significant influencing factors (Table 2). The considered causative agents in the presented study cover three (i.e., viruses, bacteria, parasites) of five classes of pathogens in different combinations which differ in their pathogenicity, virulence, infectivity, and environmental resistance (Kiehl *et al*., 2015). This might explain the heterogeneous distribution of the prevalence as well as why the factors differ regarding their significant influence and explained variance on the worldwide prevalence. It is not useful to discuss the detailed specific factors on the level of prevalence. For instance, the factor “geographical region” covered several country-specific factors such as average herd size, general law standards, typical husbandry systems, trading systems. All these factors might have a direct or indirect effect on the biosecurity level on farms. However, it has been described that those factors which influence biosecurity, such as herd size, can also have a direct influence on the incidence of infection (Frank *et al*., 1993). As an example, an accurate uptake of colostrum reduces infections with ETEC (Logan *et al*., 1977). Barry and colleagues showed that calves in smaller herds tend to have higher immunoglobulin G levels (Barry *et al*., 2019) which might be a consequence of better colostrum management and/or quality (Kehoe *et al*., 2007).

In this study, four cases of mixed infection with both BRV, and BCoV were detected at the age of 11 days. Barry *et al*. (2009) also detected both viruses and suggested that mixed infection could lead to a more severe outcome of the infection, resulting in a longer time to recover the intestinal villous. It could be related to the fact that both BRV and BCoV increasingly cause diarrhea in calves with failure of passive transfer (Durham *et al*., 1979), whereby weakening of the calf by one pathogen could also have a beneficial effect on other pathogens. It must be taken in account the fact that rotavirus infection induces important changes in the cytoskeleton which correlate with a decrease in apical expression of disaccharidase (Collins *et al*., 1988). This reduced disaccharidase activity on the cell surface, regardless of whether there is cell damage or not (Jourdan *et al*., 1998), could encourage the growth of bacteria, as described in several studies (Morin *et al*., 1976; Acres *et al*., 1985). Moreover, newborn calves have slow replacement of enterocytes, and those animals are more susceptible to diarrhea. There is competition between viral replication and enterocyte replacement, and highly virulent virus could cause diarrhea in older calves (Dodet *et al*., 1997). These results reinforce rotavirus as a primary pathogen causing acute diarrhea in neonatal calves and the importance of management and prophylactic programs to reduce rotavirus infection in calves within this age group.

Various pictures of mixed infections with one or both of BRV, and BCoV viruses and diarrheagenic *E. coli*, either alone or with other enteropathogenic bacterial spp. were detected also at different ages, (BRV-*E. coli* (6%), BRV- *E. coli*- *Salmonella* spp. (6%), BRV- *E. coli*- *Cryptosporidium* (3%), BRV- *E. coli*- *Cryptosporidium*- *Clostridium perfringens* (7%), BRV- *E. coli*- *Clostridium perfringens* (1%), BRV- *Cryptosporidium*- *Salmonella* spp. (8%), BRV- BCoV- *Klebsiella* spp.- *Clostridium perfringens* (4%), BCoV- *E. coli*- *Salmonella* spp. (2%), and BCoV- *Salmonella* spp.- *Cryptosporidium* (2%). These findings came in line with results of other surveys (Garcia *et al*. 2000; Ok *et al*., 2009; Lee *et al*., 2019; Brunauer *et al*., 2021; Dall *et al*., 2021; Wei *et al*., 2021). In addition to this, pre-

vious research conducted in South Korea by Jeong *et al*. (2012) reported that bacteria (45.4%) were the most common infectious agents, followed by viruses (39.0%), *Eimeria* spp. (0.4%), and mixed infection (10%) in diarrheal calves. In the present study, Bacterial and protozoan causative agents were found to be the predominant causes of diarrhea in claves.

CONCLUSION

This study elucidated the causative agents and epidemiological aspects of diarrhea in one of Egyptian provinces and may help broaden our understanding of calf diarrhea for the development of an effective strategy for disease prevention and control in whole country. Passive immunity reduces the frequency of diarrhea in calves within the first week of age. The increased natural resistance, as calves get infected throughout calf rearing, helps to reduce diarrhea in older calves. Rotavirus and coronavirus are common viruses among diarrheic calves under 1 month of age, and calves are exposed to infection right after birth. This study indicates the need for more standardized epidemiological studies to provide more robust conclusions regarding the importance of pathogens and their influencing factors.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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