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Relationship between virulence and antibiotic resistant genes in some Gram negative bacteria causing diarrhea in calves

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ABSTRACT

Diarrhea in calves is a multifactorial disease. It is the main cause of high morbidity and mortality in calves during the first three weeks of life, resulting in negative economic effects on the livestock industry worldwide. In the current study a total of 80 fecal samples from diarrheic calves were collected and bacteriologically examined. The results revealed that 72 out of 80 samples were positive for a variety of Gram negative bacteria in percentage of 90%. *Enterobacteriaceae* were predominant, with the isolation of *E. coli* (37.5%) which serotyped to (O164:K, O125:K70 ,O86:K61, O111:K58 , O119:K69, O78:K80 and O44:K70) and *Klebsiella oxytoca* (33.3%), while *Salmonella* were in percentage of 9.7% and serotyped to *S.* Typhimurium, *S.* Montevideo, *S.* Mississippi and *S.* Infantis, other isolates included *Proteus* sp., *Pseudomonas aeruginosa*, *Citrobacter freundii* in percentage of 9.9%, 5.6% and 4.2%, respectively .There was a high level of antimicrobial resistance among isolates. All tested isolates; *Salmonella* and *E. coli* were multiple antimicrobial resistance. Virulence genes (*inv*A, *stn*, *bcf*C) were detected in all tested isolates of *Salmonella* while (*omp*T (100%), *pap*C (71.42%), *ast*A (42.85%) were detected in tested *E. coli* isolates. Resistance genes (bla_{TEM} (100%) and, *TetA*(A), *Sul1*) were detected in most tested isolates. This study highlights the significant level of multiple resistance to antimicrobials and its high relation with virulence in these pathogens, emphasizing the importance of surveillance and targeted treatment and stressing the dangers associated with the use of antibiotics on a random basis.

Introduction

Calf diarrhea is recognized as one of the most critical diseases affecting newborn calves less than one month of age globally. It presents a serious threat to the livestock sector due to direct losses, fatalities, stunted growth, and high treatment costs (Wei *et al*., 2021).

Calf diarrhea is a disease caused by viruses, bacteria, and protozoa. Among calves under 21 days old, the primary bacterial culprits are enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella enterica* subsp. Enterica serovars (Bhat *et al*., 2015).

Bacterial pathogens continue to be responsible for over 50% of neonatal calf diarrhea cases, with *E. coli*, which isolated during cultural examinations of the intestinal contents of calves during their first three weeks of life (Subhash Malik *et al*., 2012). *E. coli* is particularly significant due to its role in causing severe watery diarrhea within the first four days of a newborn calf's life, potentially leading to death within 24 hours (Cho *et al*., 2010). Additionally, *Salmonella* spp., *E. coli* K99, and *Clostridium* species have been identified as bacterial agents in calves under two months old (Acha *et al*., 2004; Smith, 2009).

E. coli pathogenicity genes, such as iutA, iroN, iucD, *pap*C, *hly*F, *omp*T, and *ast*A, have been linked to diarrhea in both humans and animals (Fröhlicher *et al*., 2008; Huehn *et al*., 2010).

Salmonella is an enteric pathogen found in the intestinal tracts of animals, excreted in feces, and spread through water, soil, plant surfaces, and dairy farms (Halimi *et al*., 2014). Calves under three weeks old are particularly susceptible to *Salmonella* infections, which can lead to systemic infections characterized by diarrhea and septicemia, potentially resulting in death in severe cases (Berge *et al*., 2008).

Salmonella produces various virulence determinants, including hemagglutinins, adhesins, invasins, fimbriae, exotoxins, and endotoxins (Lee *et al*., 1996). Among these, the invasion A (*inv*A) gene is one of the most studied virulence factors and serves as a biomarker for *Salmonella* detection due to its unique sequences (Ramatla *et al*., 2024). The *Salmonella* enterotoxin (*stn*) gene is a virulence factor responsible for enterotoxic activity inducing significant intestinal fluid loss, leading to diarrhea (Chopra *et al*., 1999). The *bcf*C (Bovine colonization factor) gene codes for bacterial fimbriae involved in surface adhesion and gut colonization (Barrow *et al*., 2010).

Klebsiella belongs to the *Enterobacteriaceae* family and colonizes both human and animal gastrointestinal tracts. As an opportunistic pathogen, it can cause a variety of diseases in humans and animals (Davis and Price 2016). Antimicrobial resistance has emerged as a significant problem in human and veterinary medicine in recent years, primarily due to the widespread misuse of antimicrobials in farm animals (Suojala *et al*., 2011).

Antibiotic resistance is multifactorial and *comp*lex and constitutes a major problem in the treatment of calf diarrhea. Perhaps, one of the most critical causes of antibiotic resistance in Egypt is the administration of antibiotics without doing the appropriate laboratory tests. Resistance can develop within and between bacteria through gene mutations and horizontal gene transfer (Buller *et al*., 2014).

Klebsiella species might acquire genes for antimicrobial resistance (AMR). *Klebsiella* species derived from animals may play a significant role in the trafficking of drug resistance genes.

The emergence of antimicrobial resistance among pathogens is an increasing concern in veterinary medicine, posing a threat not only to animals but potentially to humans as well (Pomba *et al*., 2017). Thus, this study aimed to identify the main bacterial causes of calf diarrhea, their virulence factors, and antibiotic resistance genes, while highlighting their relationships.

Materials and methods

Sampling

A total of 80 fecal samples were collected from neonatal calves less than 3 months old, from farms in Fayoum and Giza governorates and suffering from diarrhea were collected randomly from September 2022

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to October 2023. Seasons of samples collection (rainy and dry). The fecal samples were collected after the onset of diarrhea and before antimicrobial therapy was applied. Fecal samples were collected in a separate sterile bag and kept in an ice box. The samples were transported without delay to the lab of serology unit, Animal Health Research Institute for bacteriological investigation. The affected calves showed soft to watery and mucoid diarrhea with varying degrees of dehydration and some animals showed systemic responses.

Isolation of bacterial species from diarrheic calves

Isolation and identification of *Salmonella* according to ISO (2017)

The fecal samples (10 g) were pre-enriched in 90 ml buffered peptone water and incubated at 37±1°C for 18± 2 h. sample Then 0.1 ml of broth culture was inoculated in selective enrichment broth [Rappaport-Vassaliadis soya broth (RVS broth) (MERCK), Muller-Kauffmn Tetrathionate Novobiocin broth (MKTTn) (Oxoid)] and incubated at 41.5±1.0°C, 37.0±1.0°C for 24.0±3.0 h, respectively. A loopful from each broth culture was inoculated onto selective plating medium Xylose Lysine desoxycholate agar (XLD) (Oxoid) and Brilliant Green agar media (HiMedia) and incubated at 37.0±1.0°C for 24±3h. Pink colonies with or without black center were the typical colonies of *Salmonella*. After streaking one colony onto the nutrient agar, it was incubated for 24 hours at 37°C. It was then kept at 4°C until it was biochemically identified in accordance with the method outlined by Hammack *et al*. (2001).

Isolation and identification of *E. coli* isolates (Nolan *et al*., 2013)

Each fecal sample was inoculated into 10 ml buffered peptone water and incubated aerobically at 37°C for 24 h., a loopful from bacterial suspension were subcultured onto MacConkey agar (Oxoid, England) and incubated at 37°C for 24 h. The pink colonies were subcultured on Eosin methylene blue (EMB) agar (Oxoid, England). Suspected *E. coli* colonies were preserved for future testing. The isolates were subjected to biochemical tests, including the indole reaction, methyl red test, Voges Proskauer test, citrate utilization test, catalase test, sugar fermentation test, oxidase test, and urea agar test.

Isolation and identification of *Klebsiella*.

Each fecal sample was inoculated into 10 ml buffered peptone water and incubated aerobically at 37°C for 24 h, a loopful from bacterial suspension was subcultured onto MacConkey agar and Chromatic agar (Liofilchem) and incubated at 37°C for 24 h. A mucoid, light pink colony on MacConkey agar and a green-blue colony on Chromatic agar were selected for confirmation by biochemical tests. Biochemical tests for *Klebsiella* include indole production, citrate utilization, motility (Adkins and Middleton, 2017), other carbohydrate fermentation (glucose, sucrose, and lactose), and gas production, methyl red-Voges Proskauer, oxidase, and catalase also tested.

Serological identification of Escherichia coli and Salmonella

Biochemically confirmed *Salmonella* isolates were subjected to serological identification by monovalent antisera by slide agglutination test according to ISO (2014), part III. Diagnostic omnivalent A-67, polyvalent A-E, F-67 and monovalent *Salmonella* O and H (phase 1 and phase 2) antisera (Denka Seiken co., LTD- Japan).

Serotyping of biochemically confirmed *E. coli* isolates was done by agglutination test This was performed by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN CO., Japan) at the serological department in the Animal Health Research Institute, Dokki, Giza, Egypt as described by Kok, *et al*. (1996).

Antimicrobial susceptibility testing

The antimicrobial susceptibility test of isolated *E. coli*, *Salmonella* and *Klebsiella* was carried by disc diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020), using the following selected 9 antimicrobial discs; amoxicillin/clavulanic (AMC, 30 µg), Cefotaxime (CTX), 30 µg; Gentamycin (CN), 10µg; Trimethoprim sulfamethoxazole (SXT) 25 µg; Enrofloxacin (ENR), 5µg; ciprofloxacin (CIP) 5µg;Tetracyclin (TE), 30µg; chloramphenicol (C), 30µg; colistin (CT), 10µg. The diameters of the obtained inhibition zone were measured and categorized into sensitive or resistant according to Clinical and Laboratory Standards Institute (CLSI., 2020).*E. coli*, *Salmonella* and *Klebsiella* isolates

Table 1. Target gene, primers sequences, amplicon sizes and cycling conditions for detection of virulence and antibiotic resistance genes of tested bacterial isolates.

Bacteria	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Salmonella	*inv*A	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)
	*bcf*C	ACC AGA GAC ATT GCC TTC C TTC TGC TCG CCG CTA TTC G	467	94°C 5 min.	94°C 30 sec.	53°C 40 sec.	72°C 45 sec.	72°C 10 min.	Huehn *et al*. (2010)
	Stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617	94°C 5 min.	94°C 30 sec.	59°C 40 sec.	72°C 45 sec.	72°C 10 min.	Murugkar *et al*. (2003)
E. coli	*pap*C	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501	94°C 5 min.	94°C 30 sec.	58°C 40 sec.	72°C 45 sec.	72°C 10 min.	Wen-ji e *et al*. (2008)
	*ast*A	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	110	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min.	Piva *et al*. (2003)
	*omp*T	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	496	94°C 5 min.	94°C 30 sec.	63°C 40 sec.	72°C 45 sec.	72°C 10 min.	Johnson *et al*. (2008)
Salmonella, E. coli and K. oxytoca	bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 45 sec.	72°C 10 min.	Colom *et al*. (2003)
	Sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	94°C 5 min.	94°C 30 sec.	60°C 40 sec.	72°C 45 sec.	72°C 10 min.	Ibekwe *et al*. (2011)
	TetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	570	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	Randall *et al.* (2004)

that exhibited resistance to one antimicrobial agent in three or more classes were considered as multidrug resistant (MDR). Multidrug resistance index (MARI) was recorded (Number of antimicrobial agent showed resistance / Total number of the tested antimicrobial agents).

Detection of virulence and antibiotic resistance genes of tested isolates of E. coli, Salmonella and Klebsiella Spp.

DNA extraction

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations.

PCR amplification

Primers used were supplied from Metabion (Germany) are listed in Table 1. Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler.

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, 20 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, Thermofisher) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through *comp*uter software.

Results

Results are illustrated in Table 2-5 and Figs. 1-5.

Table 2. Prevalence of Gram negative species isolated from diarrheic calves.

Type of isolates	NO.	*%
E. coli	27	37.5
Salmonella spp.	7	9.7
Proteus sp.	7	9.7
Klebsiella oxytoca	24	33.3
Pseudomonas aeruginosa	4	5.6
Citrobacter freundii	3	4.2
Total	72	100%

* The percentage was calculated according to the No of total Gram –ve isolates (72).

Table 3. *Salmonella* serovars isolated from diarrheic calves.

Serovars of *Salmonella* isolates	Antigenic structure	No	%*
S. Typhimurium	1,4,[5],12:I,1,2	2	28.57
S. Infantis	6, 7, 14: r 1, 5	2	28.57
S. Montevideo	6, 7, 14:g, m, [p], s [1, 2, 7]	2	28.57
S. Mississippi	1,13,23:b:1,5	1	14.29
Total		7	100%

%* was calculated according to total no of *Salmonella* isolates

Table 4. *E. coli* serotypes isolated from diarrheic calves.

Serovars of *E. coli* isolates	No	%*
O164:K-	5	18.6
O125:K70	4	14.8
O86:K61	4	14.8
O111:K58	4	14.8
O119:K69	4	14.8
O78:K80	3	11.1
O44:K79	3	11.1
Total	27	100%

*The percent was calculated according to total no. of *E. coli* isolates (27)

Results of PCR for detection of some virulence genes

As shown in Fig. 1, the virulence characteristics of *Salmonella*e revealed that *inv*A, *stn* and *bcf*C virulence genes were observed on extracted DNA of 100% of 4 *Salmonella*e (*S.* Typhimurium, *S.* Infantis, *S.* Montevideo and *S.* Mississippi).

The virulence characteristics of *E. coli* serogroup (O44, O78, O86, O111, O119, O125 and O164) revealed that *omp*T, *pap*C, and *ast*A virulence genes were observed on extracted DNA of 100%,71.42% and 42.85% respectively for 7 *E. coli* serogroup as shown in Fig 2.

*Pap*C virulence gene were not observed on extracted DNA of O44 and O164 while *ast*A virulence gene not observed in *E. coli*O44, O78, O111 and O119 but was observed on extracted DNA of O86, O125 and O164.

Prevalence of *pap*C, *omp*T and *ast*A virulence genes were observed on extracted DNA of 100% of serogroupO86 and O125.

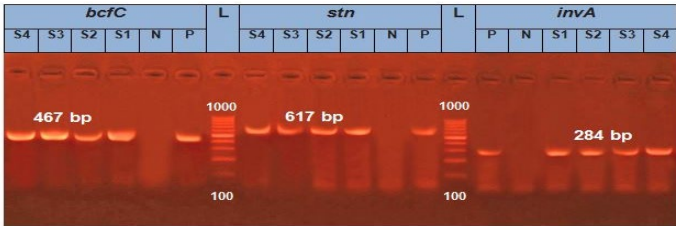

Figure 1. Agarose gel electrophoresis of PCR products after amplification of: *inv*A gene, *stn* gene and *bcf*C virulence genes for *Salmonella* strains, L: MWM-molecular weight marker (100 – 1000 bp DNA ladder), P: control positive N: control negative and strains of *Salmonella* species. (S1:*S.* Typhimurium, S2:*S.* Infantis, S3: *S.* Montevideo and S4: *S.* Mississippi) (*inv*A gene products at 284 bp, *stn* gene products at 617 bp and *bcf*C gene products at 467 bp).

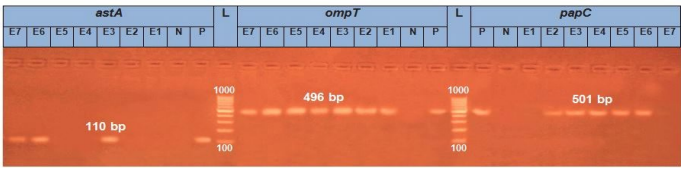

Figure 2. Agarose gel electrophoresis of PCR products after amplification of: *ast*A gene, *omp*T gene and *pap*C virulence genes for *E. coli* strains, L: MWM-molecular weight marker (100 – 1000 bp DNA ladder), P: control positive N: control negative and different strains of *E. coli* (E1: O44, E2: O78, E3: O86, E4:O111, E5: O119, E6: O125, E7: O164). *ast*A gene products at 110 bp, *omp*T gene products at 496 bp and *pap*C gene products at 501 bp.

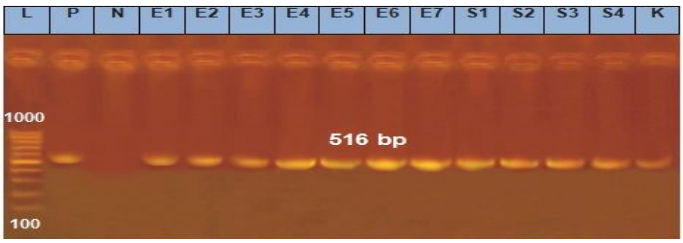

Figure 3. Agarose gel electrophoresis of PCR products after amplification of bla_{TEM} antimicrobial resistance gene for *E. coli* strains, *Salmonella* strains, *K. oxytoca* strains, L: MWM-molecular weight marker (100 – 1000 bp DNA ladder), P: control positive N: control negative and different strains of *E. coli* (E1:O44, E2: O78, E3:O86, E4:O111, E5:O119, E6:O125, E7:O164). *Salmonella* strains (S1: *S.* Typhimurium, S2: *S.* Infantis, S3: *S.* Montevideo and S4: *S.* Mississippi), K: *Klebsiella oxytoca* (bla_{TEM} gene products at 516 bp).

Results of PCR for detection of antimicrobial resistance gene

bla$_{TEM}$ were observed on extracted DNA of 100% of total 12 isolates (*Salmonella* n=4, *E. coli* n=7, *K. oxytoca* n=1) as shown in Fig 3. While *TetA*(A) were found in 100% of *Salmonella* isolates and *K. oxytoca* but it was 85.71% of *E. coli* (Fig. 4).

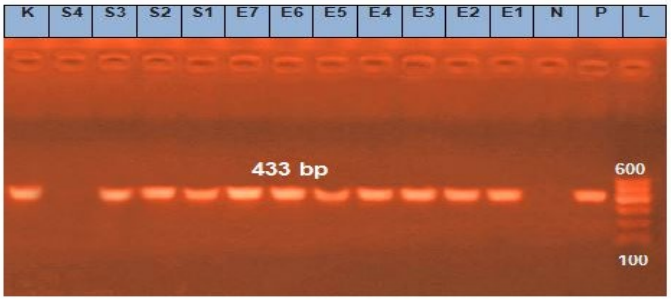

Figure 4. Agarose gel electrophoresis of PCR products after amplification of *Sul1* antimicrobial resistant gene for *E. coli* strains, *Salmonella* strains, *Klebsiella oxytoca* strains, L: MWM-molecular weight marker (100 – 1000 bp DNA ladder), P: Control positive N: Control negative and different strains of *E. coli* (E1:O44, E2: O78, E3:O86, E4:O111, E5:O119, E6:O125, E7:O164). *Salmonella* strains (S1:*S.* Typhimurium, S2: *S.* Infantis, S3: *S.* Montevideo and S4: *S.* Mississippi), K: *Klebsiella oxytoca* (*Sul1* gene products at 433 bp).

Sul1 were detected in 100% of *E. coli* and *K. oxytoca* but it was 75% in *Salmonella* (n.=4), as shown in Fig. 5.

bla$_{TEM}$, *TetA*(A) and *Sul1* were detected in *S.* Typhimurium, *S.* Infantis and *S.* Montevideo while *bla*$_{TEM}$ and *TetA*(A)only were detected in *S.* Mississippi.

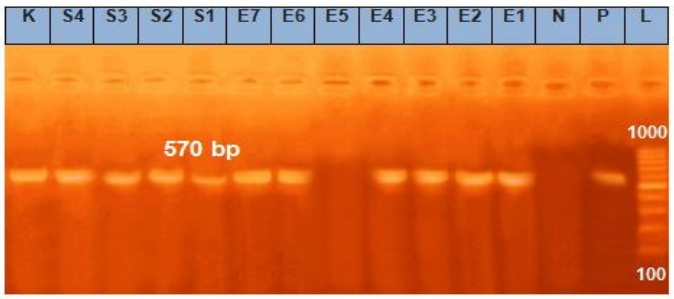

Figure 5. Agarose gel electrophoresis of PCR products after amplification of *TetA* (A) antimicrobial resistant gene for *E. coli* strains, *Salmonella* strains, *Klebsiella oxytoca* strains, L: MWM-molecular weight marker (100 – 1000 bp DNA ladder), P: Positive control; N: Negative control and different strains of *E. coli*, E1:O44, E2: O78, E3:O86, E4:O111, E5:O119, E6:O125, E7:O164. *Salmonella* strains (S1:*S.* Typhimurium, S2: *S.* Infantis, S3: *S.* Montevideo and S4: *S.* Mississippi), K: *Klebsiella oxytoca* (*TetA* (A) gene products at 570bp).

Discussion

Diarrhea remains a significant economic challenge for cattle producers, not only in Egypt but globally (Ibrahim., 2007).

E. coli and *Salmonella* are significant bacterial contributors to calf diarrhea, with Rotavirus and Coronavirus being the most common viral causes (Foster and Smith, 2009). In this study, the prevalence of *E. coli* and *Salmonella* was 37.5% and 9.7%, respectively. The prevalence of *E. coli* aligns with findings by Bekele *et al.* (2009) and Mousa. (2020), who reported isolation rate of *E. coli* from diarrheic calves 37% and 40%, respectively. However, this rate is lower than a previous Egyptian study, which found a high incidence of *E. coli* (66%) in diarrheal calves (Abdulgayeid *et al.*, 2015). In contrast, lower rates of *E. coli*-associated diarrhea in calves, at 18.9% and 13.5%, were reported by Luna *et al.* (2009) and Darsema. (2008) respectively. The incidence of *Salmonella* at 9.7% in this study is lower than the 45.53% reported by Moussa *et al.* (2010) and higher than the 4.09% reported by Younis *et al.* (2009). These variations could be attributed to differences in geographical location, climate, and management practices.

In present study, 33.3% *Klebsiella* isolated from diarrheic fecal cattle calves. Many researchers reported *Klebsiella* from calves diarrhea and

Table 5. Antimicrobial susceptibility pattern of *Salmonella*, *E. coli* and *Klebsiella oxytoca* recovered from diarrheic calves.

Antibiotic agents	*Salmonella* isolates (n=7)						*E. coli* isolates (n= 27)						*Klebsiella oxytoca* (n= 24)					
	Resistant		Intermediate		Sensitive		Resistant		Intermediate		Sensitive		Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Colistin sulfate (CT) 10µg	7	100	0	0	0	0	27	100	0	0	0	0	24	100	0	0	0	0
Amoxicillin/clavulanic acid (AMC) 30µg	7	100	0	0	0	0	27	100	0	0	0	0	24	100	0	0	0	0
Cefotaxim (CTX) 30 µg	6	85.7	0	0	1	14.3	15	55.6	7	25.9	5	18.5	0	0	24	100	0	0
Trimethoprim + Sulphamethoxazole (SXT)25 µg	7	100	0	0	0	0	27	100	0	0	0	0	24	100	0	0	0	0
Gentamicin (CN) 10µg	7	100	0	0	0	0	23	85.2	0	0	4	14.8	24	100	0	0	0	0
Chloramphenicol (C) 30 µg	4	57	0	0	3	43	22	81.5	0	0	5	18.5	24	100	0	0	0	0
Ciprofloxacin (CIP) 5 µg	0	0	4	57	3	43	14	51.9	0	0	13	48.1	0	0	0	0	24	100
Enrofloxacin (ENR) 5 µg	4	57	2	28.7	1	14.3	14	51.9	0	0	13	48.1	0	0	24	100	0	0
Tetracycline (TE) 30µg	7	100	0	0	0	0	27	100	0	0	0	0	24	100	0	0	0	0

% calculated according to the total number of isolates of each species

other sources with varying degree. Okela *et al*. (2010) reported only 2.6%. Montso *et al*. (2019) found *Klebsiella* species in 32% samples of cattle feces which agree with our findings.

E. coli isolates were serotyped to O164: K-, O125:K70, O86:K61, O111:K58, O119:K69, O78:K80 and O44:K70 have shown frequency in most previous studies that concerned calf diarrhea. The most predominant isolated serotypes of *E. coli* were O164: K- in percentage of 18.5% followed by O125:K70, O86:K61, O111:K58, O119:K69 were 14.8% respectively, while O78:K80 and O44:K70 were 11.1% for each. In addition, other serotypes were detected by Mohamed and Farag (2019) and included O26, O119, O125, O126 and O127 while Mohamed *et al*. (2017) obtained a twelve different serotype of *E. coli*.

Salmonella isolates in this study were serotyped as *S.* Typhimurium, *S.* Montevideo, and *S.* Infantis, each accounting for 28.57% of the isolates, while the less common *S.* Mississippi made up 14.29% of the samples. This frequency of *S.* Mississippi is lower than the 24.6% reported by Moustafa *et al*. (2020). *S.* Typhimurium remains one of the most common serovars, as noted in previous studies by Youssef and El-Haig (2012) and El-Tawab *et al*. (2017). Elhady *et al*. (2020) reported *S.* Typhimurium as 61.1% isolation rate and *S.* Infantis 11.1%. The presence of *S.* Typhimurium and *S.* Infantis in diarrheic calves supports the idea that isolating these organisms in primary culture can suffice for diagnosing the disease, as suggested by Ngeleka *et al*. (2019). *S.* Montevideo isolation rate was 9.7% as recorded by Sato *et al*. (2001). The variation in frequency of *Salmonella* serovars may be due to difference in geographical and country region.

Antimicrobial resistance of *Salmonella* and *E. coli* is particularly worrying in view of its potential to extend into the human food chain, posing a challenge to public health. The data from the present study showed widespread resistance of *Salmonella* species isolates 100% against the tested antibiotics especially Colistin sulphate, Gentamicin, Tetracycline, Amoxicillin/clavulanic acid and Trimethoprim + Sulphamethoxazole. While 85.7% of the isolates were resistant to Cefotaxime. The lowest resistance rate was observed against Chloramphenicol and Enrofloxacin 57%. These results indicate the extensive and frequent use of these antibiotics in animals.

Resistance of all *Salmonella* isolates to gentamycin (resistant rate 100%) in this study nearly agree with Moustafa *et al*. (2020). On the other hand, *E. coli* isolates obtained from diarrhea were shown to be (100%) resistant to Colistin sulphate, Amoxicillin/clavulanic acid, Trimethoprim + Sulphamethoxazole, and Tetracycline while 85.2% of the isolates were resistant to Gentamicin, 81.5% of isolates were resistant to Chloramphenicol and 55.6% of isolates were resistant to Cefotaxime. The lowest resistance rate was observed against Ciprofloxacin and Enrofloxacin 51.9%. The current investigation found that (multi drug resistant) MDR *E. coli* strains are 67%. Additionally, in India Khawaskar *et al*., (2022) reported a significant incidence of MDR *E. coli* isolates in a percentage of 49.6%. MDR *E. coli* strains were discovered in several reports, and it was shown that resistance to routinely used antibiotics.

In this study, generally *E. coli* and *Salmonella* isolates showed high MDR against the tested antibiotics which being hand to hand with Elhady *et al*. (2020) and Pereira *et al*. (2011).

In this study, all tested *K. oxytoca* isolates (100%) were resistant to Colistin sulfate, Amoxicillin/clavulanic acid, Trimethoprim + Sulphamethoxazole, Gentamicin, Chloramphenicol and Tetracycline.

Arbab *et al*. (2021) found *Klebsiella* showing 95% resistance against ampicillin and tetracycline. Carvalho *et al*. (2021) reported that resistance rates for ciprofloxacin, gentamycin and tetracycline were 100%, 75% and 66.67%, respectively.

The present study revealed that all tested *Salmonella* isolates harbored the *inv*A, *stn* and *bcf*C virulence genes. These findings align with Soliman. (2014) for *inv*A but show higher percentage for the *bcf*C gene detection than that reported by Wagdy *et al*. (2016) and higher *stn* gene detection than Maysa and Abd-Elall. (2015) who clarified that *stn* gene was identified in *S.* Typhimurium and S. Enteritidis with incidence rates of 78.9% and 75%, respectively.

E. coli strains still have virulence factors that help with tissue invasion and colonization, which contribute to the development of calf colibacillosis (Zhao *et al*., 2005). According to our analysis of virulence genes of *E. coli*, it had been shown that *omp*T, *pap*C, and *ast*A virulence genes were detected as 100%, 71.42% and 42.85% respectively for the tested 7 *E. coli* serogroups. *E. coli* carrying the enteroaggregative *E. coli* heat-stable enterotoxin 1 (*ast*A) virulence gene is associated with diarrhea and the enhanced pathogenicity of other virulence factors (Awad *et al*., 2020). Our results of *ast*A gene disagree with the result of El Refaey *et al*. (2023) who showed high occurrence of *ast*A (78.8%). However, lower result of *omp*T gene occurrence than our findings was recorded by Mbanga and Nyararai, (2015) and El Refaey *et al*., (2023) as 2.2% and 50% respectively, somewhat lower *pap*C gene carriage rates of 64.4% was obtained by El Refaey *et al*. (2023). The *pap*C gene is one of the most important virulence factors in *E. coli* (Paixao *et al*., 2016).

Sul1 genes are those genes responsible for conferring resistance to sulfonamide drugs. in this study the results showed that the resistance of the tested *E. coli* isolates to sulphamethoxazole/ trimethoprim was 100% and these results was c*omp*letely agreed with Shekhar and Singh (2014) who found that the maximum resistance was observed against sulphamethoxazole was 100%. *TetA* (A) gene was detected in all tested *Salmonella* isolates which agree with Adesiji *et al*. (2014) who detected *TetA* (A) gene at a percentage of 100%. Similar to previous findings (Abd El-Twab *et al*., 2016), the *Sul1* gene was detected at a 433 bp in all tested *Salmonella* strains.

The detection of β–lactamases encoding genes among isolated strains of *E. coli* and *Salmonella* strains showed that all isolates contain β – lactamase encoding genes. These results indicate that *TetA* (A) and bla_{TEM} genes seriously contribute to the rise in antibiotic resistance (Ahmed and Shimamoto, 2013). Up to 90% ampicillin resistance in *E. coli* is attributed to bla_{TEM} production with most isolates likely sharing resistance to amoxicillin and tetracycline. due to intensive use of β–lactam drugs as therapeutic agents led to the development of β–lactams resistance.

Conclusion

E. coli and *Salmonella* continue to be the primary bacterial agents causing calf diarrhea, with the emergence of multi-drug resistant strains making treatment increasingly challenging and with high risk to human health, as they can transmit antibiotic resistance genes through the food chain. This study confirms the presence of virulent and multidrug-resistant *Salmonella* and *E. coli* serotypes in diarrheic calves as they harbored virulence and antibiotic resistance genes. Also, our study proved presence of close relationship between virulence and antibiotic genes resistance in the tested strains of *E. coli*, *Salmonella* and *Klebsiella*, this emphasized the need for targeted antimicrobial therapy based on sensitivity testing for treating *Salmonella* and *E. coli* infections and other pathogens. Strict hygiene measures should be implemented, especially during the rainy season and for young calves, to reduce infection rates by periodical application of powerful disinfectants in farms.

Conflict of interest

The authors have no conflict of interest to declare.

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