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Investigation on some bacterial and fungal causes of buffaloes calves diarrhea with emphasis on suitable treatment

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ABSTRACT

A total of 50 fecal samples of diarrheic buffalo calves aged 0–3 months collected from private farms in Giza Governorate, were examined for detection of the most common bacterial pathogens causing diarrhea. The results revealed that, 24 samples (48 %) were positive for *Escherichia coli,* 7 (14%) for *Salmonella Typhimurium;* 6 (12 %) for *Staphylococcus aureus*, 5 (10 %) for *Streptococcus faecalis;* 5 (10%) for *Pseudomonas aeruginosa,* 3 (6%) for *Klebsiella pneumoniae* and 2 (4%) for *Proteus vulgaris.* Serological diagnosis showed that, 16 strains belonging to five O-serogroups which included O111 (5 strains), O26 (4 strains), O125 (4 strains), O103 (2 strains) and O157 (1 strain). With respect to *Candida* spp. participate 60% of the total yeast isolated from diarrheic calves. *C. albicans* was the most frequently recovered spp. (22%) followed by *C. glabrata* (16%), *Candida krusei* (10%), *C. guilliermondii* (4%) and *Rhodotorula* spp. (8%).

Antibiotic sensitivity test proved that, *E. coli* isolates showed resistances against the majority of antimicrobials, it showed high resistances against amoxicillin followed by ampicillin erythromycin, cefotaxime and trimethoprim sulfamethoxazole. On contrary, isolates were showed high sensitivities against levofloxacin and ciprofloxacin followed by gentamicin, neomycin and colistin sulfate. On the other hand, *S. Typhimurium* showed high resistance against ampicillin followed by amoxicillin, erythromycin and trimethoprim-sulfamethoxazole, while isolates were showed high sensitivities against levofloxacin and ciprofloxacin followed by colistin sulfate, gentamicin, Cefotaxime and neomycin. Currently, sensitivity to commercial antifungal against *C. albicans* indicated that, it was highly resistant for fluconazole followed by ketoconazole and clotrimazole, while, it was completely susceptible to nystatin followed by amphotericin B.

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Polymerase chain reaction was applied for detection of some virulence genes and antibiotic resistance genes and results revealed that, among (5) *E. coli* isolates, *eae*A and *omp*A genes were successfully amplified in all isolates, while only O111 and O26 were harbored *sta* gene. On the other hand all 4 *S. Typhimurium* isolates harbored virulence factor *inv*A gene, while (2) *S. Typhimurium* possessed *adr*A and *mgt*C gens. With concern to PCR screening for detection of resistant genes *bla*TEM and *sul*1 showed that all isolates were positive for both genes. Two *C. albicans* isolates were screened using PCR analysis to determine some virulence genes and found that, the isolates were positive for *sap* 4 gene, while negative for both *als*1 and *hwp*1 genes. With respect to antibiotic resistance gene *erg*11, one strain was positive, while the other was negative.

Prevention of diarrhea requires particular attention to nutrition, environment, sanitation and care of the newborn calf. Development of prophylaxis protocols including the use of specific vaccines remain important aspects of scours prevention, in addition to enhancing calf disease resistance and minimizing exposure of calf to infectious agents are important.

INTRODUCTION

Diarrhea in newly born calves stays one of the most important economically life threatening diseases, mainly through high mortality and adverse effect on health status in the herd and the future productive performance in recovered buffalo calves as well as need for treatment. The disease depends mainly on incorrect management systems including, overfeeding, cold temperature, overcrowding, artificial feeding, bad hygiene and colostrums deprivation, close association with adults ،all these are predisposing factors of the disease also stress, low temperature and inadequate ventilation play a role in lowering the defense mechanism in the early age of calf. In Egypt, calf diarrhea is the first cause of calf mortality, which varies between 27.4% and 55% of the total deaths in young calves **(Younis et al. 2009 and Ali et al. 2019).**

Signs of diseased calves varied from laxation to severe watery diarrhea with offensive odor, dehydration, depression, sunken eyes, decreased milk intake to complete refuse of suckling, fever and may have subnormal temperature at late stages in severe cases, finally recumbency and death may occur. In some cases may be associated with endotoxemia and sometimes pneumoenteritis developed **(Gunn et al. 2009).**

Calf diarrhea is a multifactorial syndrome including non-infectious factors linked to the animal either nutritional or immunological status, the management or the environment as well as infectious pathogens. According to the age of the calf, the prevalence of the infectious etiologic agents differs. *E. coli* infection appeared to be the major reason for diarrhea in buffalo calves mostly in the first week of life while Salmonellosis happened around 21 days of life. Also diarrhea caused by other bacteria such as, *Clostridium perfringens*, *Pasteurella* spp., *Klebsiella* spp., *Proteus* spp. and *Pseudomonas aeruginosa* **(Diwakar et al. 2014).**

The major distinguishing factor between pathogenic and non-pathogenic strains of *E. coli* is the presence of virulence factors which include ability to adhere, colonize, and invade the hosts' cells. Further to these are the secretion systems, production of cell surface molecules, transport and siderophore formation. Differentiation and pathogenesis of *E. coli* pathotypes based on presence of genes encoding these virulence factors, seven pathotypes of *E. coli* have been described as enteric pathogenic *E. coli* including enteropathogenic *E.coli* (EPEC); enterohemorrhagic *E.coli* (EHEC); enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), entero-aggregative *E.coli* (EAEC), diffusely adherent *E. coli* (DAEC) and adherent invasive *E. coli* (AIEC), causing mostly diarrhea and intestinal disorders. Nevertheless, the EHEC has been involved in extraintestinal diseases such as hemolytic uremic syndrome (HUS). Many of these pathotypes constitute public health concerns and cause several fatal outbreaks **(Pakbin et al. 2020).**

S. enterica serovar *Typhimurium* is the most common causative agent of salmonellosis in calves causing acute diarrheal disease. The le-

sions observed in the intestinal mucosa as well as the mesenteric lymph nodes enlarged. Infected calf considered a source of infection either through direct contact or food borne routes where shedding the organism for variable periods of time and intermittently depending on the degree of infection. Diarrhea due to *salmonella* infection is watery and mucoid with the presence of blood and fibrin calves can shed salmonella for variable periods of time and intermittently depending on the degree of infection (**Molossi et al. 2021).**

Yeast and molds are sometimes associated with lesions in the stomach or intestines of scouring calves. These organisms are not considered a primary cause of scours, but rather secondary invaders. Often they are found when scouring calves are subject to overuse of antibiotics and very little was done to counteract dehydration by using fluids and electrolytes. Calves with fore stomach candidiasis have watery diarrhea, anorexia, and dehydration; with gradual progression to prostration and death *C. albicans* is influenced by a great quantity of virulence factors, including filamentous growth which is important for the adhesion and invasion of organism, secreted aspartyl proteases (Sap) for cell surface adhesion, also agglutinin (Als) factor is essential for endothelial and epithelial cells adherence **(Bu et al. 2022).**

Antimicrobial agents are considered popular to fight diarrhea in calves. The frequent use of antibacterial agents, the practice of under dosing or over dosing as well as dis-continuation or incomplete course of treatment developed bacterial strains that resist many antibiotics also adverse side effects as it results in failure of microbial response to standard treatment, leading to prolonged illness, higher expenditures for health care, and an immense risk of death. Therefore, it is important that sensitivity of different bacteria isolated from diarrheic calves needs to be studied from time to time in order to formulate appropriate therapeutic measures **(Okello et al. 2021**).

Therefore, the objectives of the current study were thus to isolate and identify various bacteria and yeast from fecal samples of buffalo calves suffering from diarrhea and detect virulent and antibiotic resistant genes related to *E. coli* (*eae*A, *omp*A , *sta, bla*TEM and *sul*1) *S. Typhimurium* (*inv*A, *adr*A, *mgt*C, *blaTEM and sul*1) and *C. albican* (*als*1, *hwp*1, *sap*4 and *ERG*11*)* in addition to antibiotic susceptibility patterns of them.

MATERIAL AND METHODS

1-Samples: Using sterile swabs, a total of 50 fecal samples were collected from diarrheic buffalo calves up to 3 months old from farms at Giza Governorate, and then transferred in an ice box to the laboratory as soon as possible.

2-Isolation and identification of bacterial pathogens: Fecal sample was diluted in sterile saline; a loopful from the diluted specimens was inoculated onto Eosin Methylene Blue agar, MacConkey agar, Barid-Barker media, Mannitol salt agar, Salmonella Shigella agar and Xylose Lysine Deoxycholate (XLD) agar media. The inoculated plates were incubated aerobically at 37°C for 24-48 hours. Suspected colonies were subjected to morphological and biochemical characters according to **Quinn et al. (2002).**

3-Serological identification:

A-Serological identification of *Salmonella*: It was carried out using Kauffman-White scheme as described by **Grimont and Weill (2007)** using *Salmonella* antiserum (DENKA SEIKEN CO., LTD, Japan) at the serological department in Animal Health Research Institute, Dokki, Giza, Egypt.

B-Serological identification for *E.coli*: Serological identification of the isolates was carried out as described by **Lee et al. (2009)** by using polyvalent and monovalent antisera (DENKA SEIKEN CO., LTD, Japan) at the serological department in Animal Health Research Institute, Dokki, Giza, Egypt.

4-Mycological examination of samples:

A-For isolation of Yeast and Mould: Fecal swabs were placed in Sabouraud's dextrose broth tubes at pH 5.6, and then incubated at 37°C during 2 days. Then, 50µl of each broth culture was inoculated on Sabouraud's dextrose agar (SDA) with chloramphenicol (0.05 mg/ml). The plates were incubated at 37°C and

examined for growth at 24, 48 and 72 hours and at weekly intervals for 2-6 days, after which the plates showing no growth were considered negative. The colonies were picked up and re-streaked on another SDA plate to get the pure cultures **(Washinton et al. 2006).**

B-Identification of moulds and yeasts: Morphological examination was carried by studying the macroscopic and microscopic characters of the isolates. The colonies characteristics were described including the rate and pattern of growth, their size, consistency and surface color. Vegetative reproduction was studied on corn meal agar, urease test and Germ tube test was performed according to **Pitt and Hocking (2009).**

5- Antimicrobial susceptibility testing: Antimicrobial susceptibility patterns were performed by using the disk diffusion method. The *E. coli* and *S. Typhimurium* isolates suspension was inoculated onto Muller-Hinton agar (Oxoid, UK). The plates were incubated aerobically at 37°C for 24h using different antimicrobial disks: Levofloxacin (5µg), gentamicin (10 µg), colistin sulfate (10µg), amoxicillin (10µg), cefotaxime (30µg), erythromycin (15µg), ciprofloxacin (5µg), neomycin (30µg), ampicillin(10µg) and trimethoprim sulfamethoxazole (25µg). The diameters of the inhibition zones around the disks were measured to the nearest millimeter using calibrated rulers according to **CLSI (2021).**

6-Antifungal sensitivity testing: The in vitro susceptibility of the *C.albicans* isolates was determined by mixing of loopful from pure culture of *C.albicans* with 9 ml of sodium chloride solution, then spreading over the surface of SDA plate. Five antifungal discs: fluconazole (10 µg), clotrimazole (10 µg), ketoconazole (10 µg), amphotericin B (20 µg) and nystatin (100 units), were spread on the surface of the inoculated plate. Plates were incubated at 37^{o}C for 24hrs. The diameter of the inhibition zone of each disc was measured and judged according to **CLSI (2020)**.

7. Detection of some virulence genes and antibiotic resistance genes by PCR: According to **Sambrook et al. (1989)** PCR assays were developed with specific primers for the detection of different virulent genes and antibiotic resistant genes (*eae*A, *omp*A *,sta, bla*TEM and *sul*1) of (5) *E. coli* isolates (one of each serogroup), (*inv*A, *adr*A, *mgt*C, *blaTEM* and *sul*1) of (4) *S. Typhimurium* isolates and (*als*1, *hwp*1, *sap*4 and *ERG*11*)* of (2) *C. albican* isolates applied at Reference laboratory for veterinary quality control on poultry production in Animal Health Research Institute, Dokki, Giza, Egypt..

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100 % ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from **Metabion (Germany)** are listed in **Table** (1).

For PCR: 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 concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products(Alpha Innotech, Biometra) and the data was analyzed through computer software **:** The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. A Generuler 100 bp ladder (Fermentas, Thermo) and Genedirex 100-3000 bp DNA ladder H3 RTU (Genedirex, Taiwan) were used to determine the fragment sizes. The gel was photographed by a gel documentation system

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target agent	Target gene	Primers sequences 5' -3'	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles) Secondary denaturation	Annealing	Extension	Final extension	Reference
S. Typhimurium	*inv*A	GTGAAATTATCGCCAC GTTCGGGCAA	284	94˚C 5min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7min.	**Olivera et al. (2003)**
		TCATCGCAC-CGTCAAAGGAACC							
	*adr*A	ATGTTCCCAAAAA-TAATGAA	113	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	**Bhowmick et al. (2011)**
		TCATGCCGCCAC-TTCGGTGC							
	*mgt*C	TGAC-TATCAATGCTCCAGTG AAT	677	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Huehn et al. (2010)**
		ATTTACTGGCCGC-TATGCTGTTG							
	*als*1	GACTAGTGAAC-CAACAAATACCAGA	318	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 7 min.	**Inci et al. (2013)**
		CCAGAAGAAACAG-CAGGTGA							
C. albicans	*hwp*1	ATGACTCCAGCTGGTT C	572	94˚C 5 min.	94˚C 30 sec.	45˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
		TAGATCAA-GAATGCAGC							
	*sap*4	GCTCTTGC-TATTGCTTTATTA	394	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Sikora et al. (2011)**
		TAGGAAC-CGTTATTCTTACA							
E. coli	*eaeA*	ATG CTT AGT GCT GGT TTA GG	248	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Bisi-Johnson et al. (2011)**
		GCC TTC ATC ATT TCG CTT TC							
	ompA	AGC-TATCGCGATTGCAGTG	919	95˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Ewers et al. (2007)**
		GGTGTTGCCAGTAAC-CGG							
	sta	GAAACAACATGACGG-GAGGT	229	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Lee et al. (2008)**
		GCACAGGCAGGAT-TACAACA							
Antibiotic resistant genes									
E. coli and *S. Typhimurium*	*bla*TEM	ATCAGCAATAAAC-CAGC	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)**
		CCCCGAAGAAC-GTTTTC							
	*Sul*1	CGGCGTGGGCTAC-CTGAACG	433	95˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Ibekwe *et al.* (2011).**
		GCCGATCGCGTGAAGT TCCG							
C. albicans	*ERG11*	CAAGAA-GATCATAACTCAAT	1641	94˚C 5 min.	94˚C 30 sec.	53˚C 1 min.	72˚C 1.5 min.	72˚C 12 min.	**Wang *et al.* (2015)**
		AGAACAC-TGAATCGAAAG							

RESULTS

Table 2. Bacterial isolates recovered from faecal samples of 50 diarrheic buffalo calves.

Isolated bacteria	Number	Percentage (%)
Escherichia coli	24	48
Salmonella Typhimurium	7	14
Staphylococcus aureus	6	12
Streptococcus faecalis	5	10
Pseudomonas aeruginosa	5	10
Klebsiella pneumoniae	3	6
Proteus vulgaris	2	4

%: percentage was calculated according to the total number of samples.

Table (2) revealed that, 24 (48%) were found positive for *E. coli*, 7 (14%) *S. Typhimurium,* 6 (12%) *S.aureus,* 5 (10%) *S. faecalis,* 5 (10) *P. aeruginosa,* 3 (6%) *K. pneumoniae* and 2 (4%) for *P. vulgaris.*

Table 3 Incidence of yeast isolates recovered from fecal samples of 50 diarrheic buffalo calves.

Fungal isolates	Number	%
C. albicans	11	22
C. glabrata	8	16
C. krusei	5	10
C. guilliermondii	2	4
Rhodotrula spp.	4	8
Total	30	60

%: percentage was calculated according to the total number of samples.
Candida species **(Table 3)** participate 60% of the total yeast isolated from diarrheic calves. Among *Candida* spp. isolates, *C. albicans* was the most frequently recovered species (22%) of all positive diarrheic samples followed by C. *glabrata* (16%), *C. krusei* (10%), *C. guilliermondii* (4%) and *Rhodotorula spp*. (8%).

Table 4. Results of single and mixed infection of *E. coli*, *S. Typhimurium* and *C. albicans* obtained from fecal samples of 50 diarrheic buffalo calves.

Isolated bacteria		Number
Single infection	*E.coli*	14
	S. Typhimurium	5
	C. albicans	3
Mixed infection	*E. coli + S. Typhimurium*	2
	E. coli +C. albicans	8

%: percentage was calculated according to the total number of samples.
Results in **Table (4)** showed that 8 samples had *E, coli* and *C. albicans* mixed infection, while 2 samples showed *E.coli* and *S. Typhimurium* mixed infection

Table 5. Serological identification of isolated *E.coli* (n= 24)

Antigenic structure of *E.coli* serogroups		
***E. coli* serogroups**	**Number**	**Total Number of serogroups**
O111	5	
O26	4	
O125	4	16
O103	2	
O157	1	
Untyped	8	8

Serological diagnosis **(Table 5)** showed that, 16 strains belonging to five O-serogroups which included O111 (5 strains), O26 (4 strains), O125 (4 strains), O103 (2 strains) and O157 (1strain), and 8 isolates were unidentified.

Table 6 Antibacterial disks used for antibiotic sensitivity test of *E. coli* isolates (n=24) and *S. Typhimurium* isolates (n= 7):

Antibiotics	***E.coli* isolates (n=24)**				***S. Typhimurium* isolates (n=7)**	
	Sensitive		**Resistant**		**Sensitive**	**Resistant**
	Number	**%**	**Number**	**%**	**Number**	**Number**
Levofloxacin (5 µg)	24	100	-	-	7	-
Gentamycin (10 µg)	20	83.33	4	16.67	6	1
Colistin sulphate (10 µg)	15	62.50	9	37.50	6	1
Amoxicillin (10 µg)	-	-	24	100	1	6
Cefotaxime (30 µg)	6	25.00	18	75.00	5	2
Erythromycin (15 µg)	5	20.83	19	79.17	1	6
Ciprofloxacin (5 µg)	23	95.83	1	4.17	7	-
Neomycin (30 µg)	17	70.83	7	29.17	4	3
Ampicillin (10 µg)	2	8.33	22	91.67	-	7
Trimethoprim-Sulfamethoxazole (25 µg)	8	33.33	16	66.67	3	4

With respect to in-vitro antibiotic sensitivity test, **Table 6** proved that, *E*. coli isolates showed high resistances against the majority of antimicrobials where showed resistances against amoxicillin, followed by ampicillin, erythromycin, cefotaxime, trimethoprim sulfamethoxazole. On contrary, isolates were showed high sensitivities against levofloxacin and ciprofloxacin followed by gentamicin, neomycin and colistin sulphate. On the other hand, *S. Typhimurium* isolates showed resistance against ampicillin followed by amoxicillin, erythromycin and trimethoprim sulfamethoxazole, while isolates were showed high sensitivities against levofloxacin and ciprofloxacin followed by colistin sulfate, gentamicin and Cefotaxime

Table 7. Inhibition zones diameter of antifungal drugs used for disc diffusion method of *C. albicans* isolates (n=11).

C. albicans	**Fluconazole**		**Clotrimazole**		**Ketoconazole**		**Nystatin**		**Amphotericin B**	
(11)	**(10 µg)**		**(10µg)**		**(10µg)**		**(100 unit)**		**(20µg)**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
Number	-	11	3	8	2	9	11	-	10	1
%	-	100	27.27	72.73	18.18	81.82	100	-	90.91	9.09

S: Sensitive **R** : Resistant

Among sensitivity to commercial antifungal against C. *albicans* (**Table 7)** indicated that, it was highly resistant for fluconazole followed by ketoconazole and clotrimazole, while, it was completely susceptible to nystatin followed by amphotericin B.

Table 8. Virulence genes and antibiotic resistant genes of 5 *E. coli* strains isolated from diarrheic buffaloes calves:-

E. coli	*omp*A	*sta*	*eae*A	*bla*TEM	*sul*1
O111	+	+	+	+	+
O26	+	+	+	+	+
O125	+	-	+	+	+
O103	+	-	+	+	+
O157	+	-	+	+	+

Polymerase chain reaction was applied for detection of some virulence genes and antibiotic resistance genes. **Table 8** and **Fig**. **1**, **4** and **5** revealed that, among 5 *E. coli* isolates showed that, *eae*A and *omp*A genes were successfully amplified in all isolates, while O111 and O26 only were harbored *sta* genes. With reference to PCR screening for detection of resistant genes *bla*TEM and *sul*1 showed that all isolates were positive for both genes

Table 9. Virulence genes and antibiotic resistant genes of 4 *S. Typhimurium* strains isolated from diarrheic buffaloes calves:-

S. Typhimurium	*inv*A	*adr*A	*mgt*C	*bla*TEM	*sul*1
1	+		-	+	+
2	+	+	+	+	+
3	+	+	-	+	+
4	+	-	+	+	+

Table (**9).** and **Fig 2**, **4** and **5**) showed that, all (4) *S. Typhimurium* isolates harbored virulence factor *inv*A gene, while 2 *S. Typhimurium* possessed *adr*A and *mgt*C gens**.** Also all isolates were positive for resistant genes *bla*TEM and *sul*1 genes..

Table 10. Virulence genes and antibiotic resistant genes of 2 *C. albicans* strains isolated from diarrheic buffaloes calves:-

C. albicans	*als*1	*hwp*1	*sap*4	*ERG*11
1	-	-	+	-
2	-	-	+	+

PCR analysis to determine some virulence genes in 2 *C. albicans* isolates and results in **Table 10** and **Fig 3** and **6** proved that, the isolates were positive for *sap*4 gene, while negative for both *als*1 and *hwp*1 genes. Relevant to antibiotic resistance gene *ERG11,* one strain was positive, while the other was

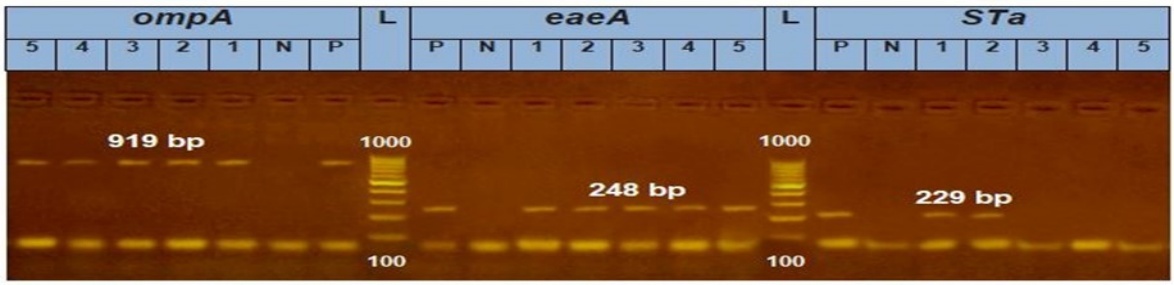


Fig.(1): Agarose gel electrophoresis showed results of PCR for detection of *omp*A, *eae*A and *sta* genes in *E. coli* isolates.
Neg :Negative control.
Pos: Positive control of eae gene (248 bp), *omp*A gene (919 bp) and *sta* gene (229 bp).
L: represents the molecular size marker. Lane 1(O111), 2(O26), 3(O125), 4(O103), 5(O157).

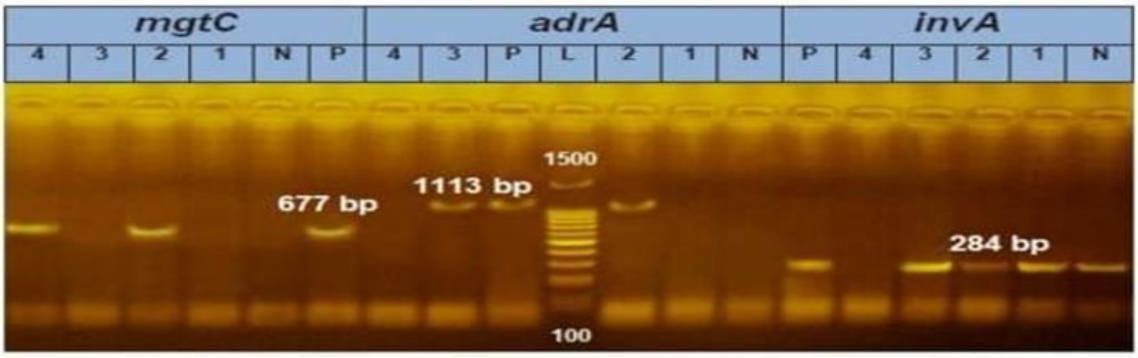
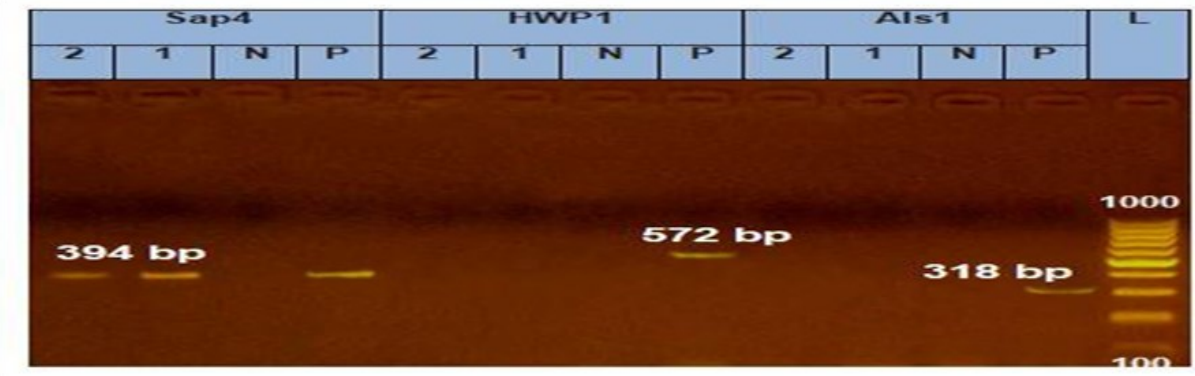
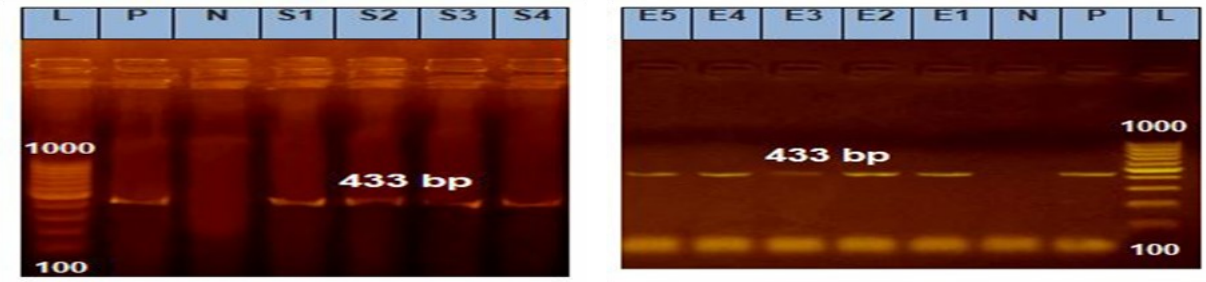


Fig.(2): Agarose gel electrophoresis showed results of PCR for detection of *mgt*C, *adr*A and *inv*A genes in *S. Typhimurium* isolates.
Neg : Negative control. Pos:Positive control of *mgt*C gene (677 bp), *adr*A gene (1113 bp) and *inv*A gene (284 bp). L: represents the molecular size marker. Lanes 1-4: *S. Typhimurium* isolates.



Fig(3) Agarose gel electrophoresis showed results of PCR for detection of *sap*4, *hwp*1and *als*1 genes in *C. albicans* isolates.
Neg: Negative control. Pos: Positive control of *sap*4 gene (394 bp), *hwp*1 gene (572 bp) and *als*1 gene (318 bp) L: represents the molecular size marker. Lanes 1,2: *C. albicans* isolates.



Fig(4) Agarose gel electrophoresis showed results of PCR for detection of *sul*1 gene in *S. Typhimurium* and *E. coli* isolates.
Neg : Negative control. Pos: Positive control of *sul*1 gene (433 bp) L: represents the molecular size marker.
Lanes 1-4: *S. Typhimurium* isolates.
Lane 1-5: *E.coli* isolates, 1(O111), 2(O26), 3(O125), 4(O103), 5(O157).

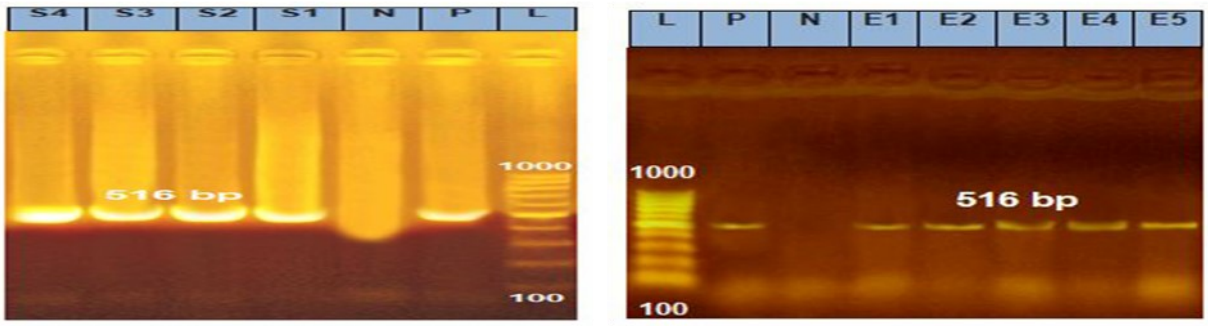


Fig (5): Agarose gel electrophoresis showed results of PCR for detection of *bla*TEM gene in *S.Typhimurium* and *E. coli* isolates.
Neg: Negative control.
Pos: Positive control of *bla*TEM gene (516 bp) L: represents the molecular size marker
Lanes 1-4: *S. Typhimurium* isolates.
Lane 1-5: *E.coli* isolates, 1(O111), 2(O26), 3(O125), 4(O103), 5(O157).

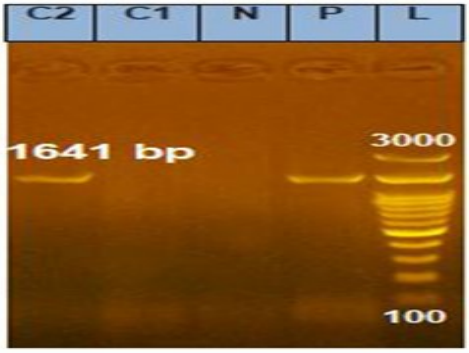


Fig (6): Agarose gel electrophoresis showed results of PCR for detection of *ERG*11 gene in *C. albicans* isolates.
Neg: Negative control.
Pos: Positive control of *ERG*11 gene (1641 bp) L: represents the molecular size marker.
Lanes 1,2: *C. albicans* isolates.

DISCUSSION

Diarrhea considered fatal to neonatal calves as it may cause acidosis and dehydration which could result in anorexia and ataxia. Numerous infectious agents causing diarrhea in animals are zoonotic and have been associated with food-borne diseases. Gastrointestinal infections caused by *E.coli* and *Salmonella* spp. are significant causes of morbidity and mortality worldwide. These pathogens cause various intestinal and extraintestinal diseases by means of virulence factors. These virulence induced infections usually involve complex mechanisms with interdependent interactions between hosts and pathogens. Identification of the possible causative agent in outbreaks of diarrhea is important to allow targeted preventative measures, such as vaccination, and identification of possible risk factors or sources of infection **(Izzo et al. 2011).**

During the last years fungal diarrhea, in calves have received much attention. *C. albicans, C. tropicalis, C. krusei, C. glabrata* and other yeast species have been associated with the syndrome. Various diagnostic criteria have been suggested to differentiate between normal calves shedding yeasts and calves diseased with noninvasive fungal diarrhea. The presence of hyphae in the stools is not sufficient as cases of diarrhea in which only budding yeast cells were observed in the feces and which were successfully treated with antimycotic drugs **(Azimpour and Pourtaghi, 2016).**

The present research work was conducted to isolate, identify and characterize the bacterial pathogens causing calf diarrhea. Our findings in **Table (2)** indicate that, the prevalence of *E. coli* is considered the most predominant isolates from neonatal diarrheic buffalo calves followed by *S. Typhimurium, S.aureus, S. faecalis, P. aeruginosa, K.pneumoniae.*and *P.vulgaris.* This result was comparable with **Diwakar et al. (2014)**. The prevalence of *E. coli* in the current study was in harmony to that recorded previously with **Gebru et al. (2022)**.

The high rate of *E. coli* in the present study could be attributed to many reasons, such as the mixing of different age groups, poor environmental and hygienic conditions, or the poor quantity and/ or quality of colostrum. The prevalence of *S. Typhimurium* was nearly agreed with **El-Seedy et al. (2016)**. Clearly **Patra et al. (2021)** added that, *S. Typhimurium* was described as major etiological agents of infectious diarrhea. The basic mechanism of virulence includes adhesions, invasions, fimbriae, hemagglutinins, exotoxins, and endotoxins. These factors singly or in combination with others allow the *Salmonella* to colonize its host through attaching, invading, surviving, and bypassing the host's defense mechanisms. The organism invades intestinal epithelial cells, surviving within macrophages, and causing enteric pathogenicity. The prevalence of *S.aureus* nearly coincided with that recorded by **Gebru et al. (2022)** and **Abdulridha and Ibrahim (2018),** while incidence of *P. aeruginosa* was nearly similar to that obtained by **Abd El-Tawab et al. (2017).** This variation in incidences could be due to the difference in study area, age of calves, sample size, farm size, the climatic conditions, feeding managements and hygiene measurements, where poor hygiene often allows buildup of pathogenic strains and diminish immunity of young calves.

Our study indicated that, *Candida* spp. in table (3) participate 60% of the total yeast isolated from diarrheic calves. Among *Candida* spp. isolates, *C. albicans* was the most frequently recovered species which was nearly agreement to those obtained by **Nayel et al. (2014)**. A higher percentage of *C. albicans* isolation (86.7%) had been reported by **Hassan et al. (2021).** Percentage of *C. glabrata* detected in our study correlated to that recorded by **Nayel et al. (2014). Sebryokov et al. (1984)** added that, total *Candida* isolates were the most common isolates of yeasts recovered from cases of calf diarrhea

In addition to, results in table (4) showed 2 samples had mixed infection between *E. coli* and *S. Typhimurium* as that observed by **Elhady et al. (2020),** while 8 samples showed *E. coli* and *C. albicans* mixed infection, that nearly coincidence with **Azimpour and Pourtaghi (2016)** who concluded that, yeasts are not a primary cause of diarrhea in calves, it sometimes associated with lesions in the stomach or intestines of scouring calves, they are possibly opportunistic pathogens that proliferate and invade the intestinal mucosa following antibiotic therapy. Reducing the incidence of calf diarrhea is based on: maintenance of disease resistance which include providing the calf with a passive immune protection obtained from colostrum, reducing of exposure to infection by improved environmental management, also good feeding practices and reduction of stress factors, special care is required to reduce environmental risk factors closely associated with calving season.

Serogrouping of 24 *E. coli* isolates in table (5) showed that, 16 strains belonging to five O-serogroups which included O111, O26, O125, O103 and O157. These serogroups were closely similar to that recorded by **Algammal et al. (2020)** and **Abed and Menshawy (2019)** who identified O26, O103, O111 and O157 from diarrheic calves in different Middle Egypt Governorates, and the most common serogroups was O26. Serogroups O26, O111 belonged to STEC that have previously been found to be associated with diarrhea and enteritis in calves in many countries. STEC strains belonging to serogroups O103 and O157 have previously been found to be associated with diarrhea and enteritis in calves **(Nguyen et al. 2011).**

In the present study, the results of in-vitro antibiotic sensitivity test in table (6) showed widespread multidrug resistance patterns among *E. coli* and *S. Typhimurium* isolated from diarrheic calves. Mostly, *E. coli* isolates

showed high resistances against the majority of antimicrobials where showed resistances.

against amoxicillin followed by ampicillin, erythromycin, cefotaxime, trimethoprim sulfamethoxazole. On contrary, isolates were showed high sensitivities against levofloxacin and ciprofloxacin. On the other hand, *S. Typhimurium* showed resistance against ampicillin followed by amoxicillin, erythromycin and trimethoprim sulfa-methoxazole, while isolates were showed high sensitivities against levofloxacin and ciprofloxacin followed by colistin sulfate and gentamicin. Our results consistence with that obtained by **Abdulridha and Ibrahim (2018)** that most of the *E. coli* and *S. Typhimurium* were resistant to ampicillin, amoxicillin, erythromycin, gentamicin and trimethoprim/ sulfamethoxazole with varying percentages, and susceptible to ciprofloxacin, also **Hagag et al. (2022)** found that, all the tested *E. coli* and *S. Typhimurium* were resistant to erythromycin, moreover most of *E. coli* isolates were resistant to cefotaxime, sulfamethoxazole and sensitive to ciprofloxacin and gentamicin. This high resistance of isolates may be attributed to either indiscriminate use of antibiotics at recommended doses or at subtherapeutic doses or used as feed additives to promote growth, and as chemotherapeutic agents irrespective of etiological agents.

Currently, sensitivity to commercial antifungal against *C. albicans* in table (7) indicated that, it was highly resistant for fluconazole followed by ketoconazole and clotrimazole, while it was completely susceptible to nystatin followed by amphotericin B, which was close agreement with that explained by **Hassan et al. (2021)** and **Monroy-Pérez et al. (2016)** who clarified that; C. albicans was highly resistant for fluconazole and highly sensitive to nystatin.

PCR screening for some virulent genes among *E. coli* isolates (Table 8 and Fig. 1) showed that, *eae*A and *omp*A genes were successfully amplified in all isolates, while O111 and O26 only were harbored *sta* gene. Similarly, **Elfaky et al. (2022)** found that, the majority of *E. coli* isolates harbored *omp*A gene. In *E. coli*, *omp*A participates in pathogenesis through its association in the adherence of EHEC to the intestinal cells; also *omp*A is known to be important for outer membrane stability. **Torres and Kaper (2003)** added that, when deleting of *omp*A, the adhesion of the bacteria to intestinal epithelial cells was decreased. Intimin (eae) protein is considered one of the most important virulence factors in *E. coli* strains. Enteropathogenic *E. coli* (EPEC) or Attaching and effacing *E. coli* (AEEC) produces an outer membrane protein, intimin, which facilitates the adherence of pathogen to intestinal villi producing attaching and effacing lesions. Intimin encoded by *eae* is an important virulence factor in many STEC strains, which plays a critical role in intestinal colonization. Study of **Hua et al. (2020)** referred to that most clinical STEC strains possessed *eae* revealed association between *eae* subtypes and disease severity. Moreover, the presence of *eae* was significantly associated with severe human disease, especially HUS. Our findings nearly agreed with **Abed and Menshawy (2019)** who discussed that O26, O103, O111 and O157 isolated from diarrheic calves were carrying *eae* gene. Another one of the most important bacterial virulence factors is Heat-stable enterotoxin which is able to cause severe diarrhea in calves. After colonization of the gut epithelium, heat stable toxin production induced by ETEC causes damage to the epithelial cells and development of secretory diarrhea in calves **(Foster and Smith 2009).** Results obtained were in agreement with **Abed and Menshawy (2019)** who recorded that, O26 and O111 were harbored *sta* genes.

Obtained results in table (9) and fig. (2) demonstrated that the invA gene was successfully amplified in all 4 investigated *S. Typhimurium* isolates. Our results confirmed the findings reported by **El- Ashmawy et al. (2016).** *inv*A gene has been recognized as an important for attachment and invasion of the pathogen to host epithelial cells and highly specific for salmonella serotypes, so it has been used as a gold marker for detection of Salmonella **(Sharma and Das 2016).** With respect to adhesion related gene *adr*A, obtained results said that, 2 *S. Typhimurium* isolates harbored that gene. Importance of *adr*A gene was explained with **Zeich et al. (2016)** where the presence of *adr*A gene is the most im-

portant to *Salmonella* biofilm formation which facilitates host colonization and promotes the survival of the bacteria and make it more tolerant to antimicrobial agents and disinfectant and even for host immune system, resulting in difficulty in the treatment of diseases and consequently chronic infection and the development Salmonella carrier state also causing many problems in food industry as it becomes a persistent of source of contamination. Moreover the review by **Jamal et al. (2018)** suggests that, most chronic infections are associated with the biofilm formation of microorganisms. Regarding to *mgt*C gene which act as virulence factor by supporting bacterial invasion and proliferation in macrophages, 2 *S. Typhimurium* isolates were positive**.** Moreover **Retamal et al. (2009)** suggested that, *mgt*C is a key factor in most pathogenic Salmonella serovars.

Concerning Molecular characterization of *E. coli* and *S. Typhimurium* for detection of resistant genes *bla*TEM (encoded for β-lactamases) and *sul*1 (encoded for sulfonamide), results in table (8, 9) and fig (4, 5) showed that, all investigated isolates were positive for both genes. These results revealed that, there was close association between virulence genes and antimicrobial resistance of the tested *E. coli* and *S. Typhimurium* isolates. Presence of resistant genes *bla*TEM and *sul*1 in all examined isolates explained widespread multidrug-resistance patterns among *E. coli* and *S Typhimurium* especially against ampicillin, amoxicillin, cefotaxime and trimethoprim sulfa- methoxazole. The prevalence of *bla*TEM found in our study matches results obtained by the previous studies **Algammal et al. (2020)** who recorded that, pathogenic *E. coli* incriminated in calf diarrhea showed presence of sul1 and *bla*TEM resistance genes.

The high prevalence of the *bla*TEM gene in *S Typhimurium,* also corresponded with **Osman et al. (2012)**. **Schroeder et al. (2002)** found from his study that, antimicrobial resistance is wide spread among *E. coli* O26, O103 and O111. Bacterial resistance to antibiotics may occur by an impulsive mutation in the target gene due to using antibiotics in therapy or enhancing growth in animals. Extended spectrum β-lactamase producing enterobacteriaceae (*E. coli* and *S. Typhimurium*) in newborn dairy calves may indicate the transmission of AMR genes from dams to calves.

In the present study, two strains previously identified morphologically as *C. albicans* were tested using molecular characterization to distinguish virulence and antifungal resistant genes (Table 10 and Fig. 3,6), and results revealed that, isolates were negative for both *als*1 and *hwp*1 genes (encoding adhesions), while positive for *sap*4 gene. Regarding to *erg*11 gene antibiotic resistance gene one strain was positive, while other was negative. Screening for the *als*1 and *hwp*1 genes among *C. albicans* isolates using PCR method was carried out by **Hamady and Marei (2021).** Furthermore, **Monroy-Pérez et al. (2016)** suggested that, the Sap proteins play an important role in the pathogenesis of infection. **Hamady and Marei (2021)** explained the pathogenicity of *C. albicans* is attributed to specific virulence factors that assist the fungus to invade the host tissues such as adhesion and biofilm formation in *C. albicans* which are mediated through the hyphal wall protein 1(Hwp1) present on the surface of the hyphae, the adhesion and colonization of host cells by *C. albicans* is also mediated by agglutinin-like sequence 1 gene (*als*1). Presence of *sap*4 was investigated by **Pawar et al. (2022)**. Several mechanisms of resistance to the azole antifungal agents directly involving *erg*11 have been described in *C. albicans.* One major mechanism involves accumulation of point mutations in the gene encoding lanosterol demethylase (*ERG*11), these mutations have been producing changes that prevent effective binding between the azoles and their target while not affecting the function of this enzyme in ergosterol biosynthesis.

CONCLUSION

Calf diarrhea is a commonly reported disease in calves and still a major cause of productivity and economic loss to producers. *E. coli* and *S. Typhimurium* infections remain the major bacterial diseases causing diarrhea in calves. *C. albicans* is the most common microorganisms among the mycotic agents in diarrhea. Presence of virulence genes played an important role in its pathogenicity, thus there is need for more reliable and faster methods. PCR is con-

sidered as a reliable technique for the determination of virulence and resistant encoding genes of *E. coli*, *S. Typhimurium* and *C. albicans*. Testing for antimicrobial and antifungal susceptibility are important to formulate a suitable treatment against *E. coli*, *S. Typhimurium* and *C. albicans*, to apply effective treatment to reduce or prevent the losses. Effective control of calf diarrhea should be based on clear understanding of pathogen and its characteristics. Prevention of diarrhea requires particular attention to nutrition, environment, sanitation and care of the newborn calf. Development of prophylaxis protocols including the use of specific vaccines remain important aspects of scours prevention, in addition to enhancing calf disease resistance and minimizing exposure of calf to infectious agents are important.

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