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Marbofloxacin Influence on Haemato-biochemical Alterations in Diarrheic Calves Infected with *Salmonella* spp.

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Abstract

Salmonella spp.; a major zoonotic pathogen worldwide could cause serious diseases that hinder the productivity in calf farms. This study discussed the antibacterial efficacy of the Marbofloxacin drug in calves affected with *Salmonella* spp. and how it impacted the animal body's haemato-biochemical changes. *Salmonella* spp. was isolated from diarrheic cases (18.8%) along with other bacteria which were also isolated in varying degrees. *Salmonella* isolates exhibited multiple serovars' identities. Also, they exhibited a multidrug resistant (MDR) pattern. Moreover, PCR approach confirmed both genotypic and phenotypic resistance traits of highly virulent MDR *Salmonella* isolates. In a trial to evaluate the anti-*Salmonella* effect of the Marbofloxacin drug, three groups of calves were divided; the first group (G1) was diarrheic without any drug administration, the second (G2) was diarrheic but intramuscularly administered with Marbofloxacin (2ml/50 Kg B.W.) while the control (healthy) group was (G3). The haemato-biochemical results of *Salmonella* infected group recorded significant increases in RBCs count, PCV, Hb, and leukocytes with marked neutrophilia, monocytosis, and lymphopenia. Moreover, high rates of AST, ALT, urea and creatinine were recorded as well as serum globulin ($P<0.05$) was extremely increased. Also, a high increase of serum potassium but with notably limited levels of both glucose and sodium was stated. Meanwhile, after treatment, all haemato-biochemical and mineral parameters in G2 were completely improved. In conclusion, the Marbofloxacin drug impacted positively on animal health and in vitro, it completely overcomes calf *Salmonella* infection which in turn enhances the growth curves of these animals and considerable financial profits.

KEYWORDS
Diarrheic calves, *Salmonella*, Marbofloxacin, Multidrug resistant, MDR, Haemato-biochemical changes.

INTRODUCTION

Neonatal calf diarrhea (NCD); gastrointestinal or enteric disease that could affect young pre-weaned calves and it might be fatal due to insufficient immunization. Diarrhea had been always associated with hypovolemia, acidosis, anorexia and ataxia (Maier *et al*., 2022). It could be implicated in severe losses in bovine industry, high calf mortality, impaired growth rate and performance, replacement of the herd capacity, high treatment costs and subsequent chronic illness (Priyadarshini, 2021; Strockbine *et al*., 2015).

Salmonella spp. is Gram-negative bacilli; involved in enteritis, diarrhea and other syndromes in both animals and humans. Asymptomatic or infected animals with no apparent symptoms regarded as a major source of microbe dissemination via feces not only on the level of animal herd but also for its environment (Overton *et al*., 2022). The microbe had been then proliferated and multiplied in the intestine invading the mucosa and adhering to the epithelial cells conquering the host defense mechanisms and disease occurred (Ezzat *et al*., 2022). Mild to severe symptoms (fever, dull mentation, inappetence, dehydration, emaciation and scours with high mucus content or tinged with blood) could be developed. An electrolyte imbalance, metabolic acidosis and hypovolemia disorders could be also, raised and resulted in kidney failure and heart block due to hyperkalemia (Strockbine *et al*., 2015).

The unwise use of antibiotics or antimicrobials in animal agriculture all over the world could facilitate the emergence of multidrug resistant (MDR) *Salmonella* strains. Restricting or combating the antimicrobial resistance (AMR) included figuring out how to speed up basic and applied research as well as how to create and produce new, potent antibiotics (CDC, 2022).

PCR based technology is a crucial sensitive diagnostic tool could aid in the general diagnosis of multiple diseases. More specifically, a phenotypic expression of the effector proteins in the bacterial cell could play an essential role in the pathogenicity of *Salmonella*e spp. Also, the aggressiveness of *Salmonella*e spp. could be affected by the diversity of its virulence variables (Abdeltawab *et al*., 2016).

Marbofloxacin is a 3rd generation fluoroquinolone broad spectrum antibacterial drug. It is extensively approved and applied in the veterinary field in the treatment of gastroenteritis disorders. It is highly efficient against wide range of bacteria (Fernández-Varón *et al*., 2021). It is primarily inhibit the bacterial DNA-gyrase and topoisomerase IV (El-Sayed *et al*., 2019). The Marbofloxacin pharmacokinetics had been studied previously in

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different animal species including cows in many reports (Birhanu *et al*., 2020). The antibacterial efficacy of this drug is mainly depending on the drug concentration and the minimum inhibitory concentration (MIC) of the selected bacterium since the group of fluoroquinolones exhibited a concentration-dependent killing effect (Fernández-Varón *et al*., 2021). In previous experimental infection in mice and calves; the lower doses of fluoroquinolones could achieve clinical cure, and potentially decrease microbiological load especially in the early stage of the disease targeting lower levels of bacterial infections (Ferran *et al*., 2011; Lhermie *et al*., 2015; Lhermie *et al*., 2016).

Therefore, this work was planned to evaluate experimentally the antimicrobial efficacy of a recent treatment "Marbofloxacin" on some virulent MDR *Salmonella* spp. isolates along with a study of its effects on most essential hematological and biochemical parameters in the diarrheic newly borne calves in Egypt farms.

MATERIALS AND METHODS

Ethical approval

This study was conducted under the Animal Ethics of Institutional Animal Care Committee (ARC-IACUC) regulations at the Agriculture Research Center, Egypt (Approval Number: ARC-AH-23-10).

Animal study design

The first part of this study was surveying for the bacterial causes of diarrhea with special regard to Salmonella spp. isolation in the diseased cases of selected local and crossbred calves' ≤ 2 months. These calves were raised in an intensive management system at different farms in Ismailia and Sharkia Governorates, Egypt. It was investigated during the period from June 2021 to September 2022 for their health status housing and sanitation conditions. However, the second part of the study included the estimation of some hematobiochemical parameters in blood samples without any treatment and then after the experimental treatment with intramuscular (I/M) injection of Marbofloxacin drug and it would be discussed later in detail.

Clinical examination

The clinical examination of the diseased calves in this study recorded that they exhibited major clinical signs of diarrhea (fever, depression, loss of weight, weakness, reduced suckling, rough hair coat, and soiling of the hind quarter and tail with diarrheic feces).

Drug

Marbox® 100 mg/ml is a trading name for the Marbofloxacin drug. Marbox® was examined experimentally in this study to inspect its role in the limitation of diarrhea in newly born calves. It was obtained from (Ceva Sante Animale, Egypt), and administered in an experimental dose of 1ml/50kg B.W. via intramuscular injection (I/M) for 3 successive days.

Bacterial investigation

For bacteriological investigation of *Salmonella* spp. as one cause of diarrhea in this study: about 260 rectal or fecal content samples were collected aseptically from the rectum of the examined and diarrheic calves. Then, all samples were transported to the AHRI microbiological laboratory in an ice box without any delay for further examination of bacterial causes of animal diarrhea with special regard to *Salmonella* spp.

Isolation and identification of Salmonella spp. of diarrheic calves

All rectal samples of 260 calves were bacteriologically examined using the traditional cultural isolation and identification scheme according to ISO-6579, 2002 (for isolation of *Salmonella* spp.) and Quinn *et al*., 2002 (for isolation of other bacteria). The recovered bacterial isolates were subjected to and identified with biochemical tests (Quinn *et al*., 2002). In addition, all identified *Salmonella* isolates were serotyped using slide agglutination testing with the aid of the commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions, at the serology unit, AHRI, Dokki, Giza.

Antimicrobial sensitivity testing

All recovered *Salmonella* isolates were tested for their antibiotic sensitivity via disc diffusion method using Mueller Hinton agar plates and commercial antibiotic disks (tetracycline, ampicillin, sulfamethoxazole/trimethoprim, enrofloxacin, amoxiclavulinic acid, streptomycin, colistin, norfloxacin, amikacin, gentamicin, ciprofloxacin and marbofloxcin) of different antibiotic groups (Oxoid, Basingstoke, Hampshire, England, UK) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2022).

Genotypic detection of virulence and antibiotic resistance attributes

The DNA of selected *Salmonella* isolates was extracted in accordance with the manufacturer's guidelines using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). This extracted DNA of the recovered salmonella isolates were amplified using specific oligonucleotide primers of selected target antibiotic resistant genes (*sul1*, *tetA*, and bla_{TEM}) and virulence genes (*invA*, *Stn*, *sopB*, *pefA* and *spvC*). The PCR cycling conditions were programmed according to the reference of each primer as tabulated in Table 1. The reaction mixture was adjusted to be (25 µl) in volume which formed from 12.5 µl of Emerald Amp GT PCR master mix (Takara), 1 µl of each set of forward and reverse primers (20 pmol), (Eurofins Pvt. Ltd., Bangaluru), 6 µl of DNA as a template and 4.5 µl of nuclease-free (grade) water. This reaction was performed in an applied biosystem 2720 thermal cycler and the amplified PCR products were resolved later with agarose gel electrophoresis system (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. The gel was prepared and then inoculated with 20 µl of amplified PCR product and loaded in each gel slot separately with Gelpilot 100 bp (Qiagen, Germany, GmbH) to determine the amplicon and fragment sizes. Finally, the gel was photographed using a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Animal groups and the experimental design for investigation of Marbofloxacin drug

Thirty crossbred calves (1-15 days old) of both sexes were used in the present study obtained from Salhya dairy farm at Sharkia Governorate of the same age range. These calves were divided further into three groups (n= 10); the first one; the diarrheic group (G1) without any treatment, the second group (G2);

Table 1. PCR cycling conditions, target genes, amplicon sizes and primers sequences for *Salmonella* spp. isolates.

Target gene	*Primers sequences*	*Amplified segment (bp)*	*Reference*
Virulence genes			
invA	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	Oliveira *et al*. (2003)
Stn	F: TTG TGT CGC TAT CAC TGG CAA R: ATT CGT AAC CCG CTC TCG TCC	617	Murugkar *et al*. (2003)
sopB	F: TCA GAA GTC TAA CCA CTC R: TAC CGT CCT CAT GCA CAC TC	517	Huehn *et al*. (2010)
pefA	F: TGTTTCCGGGCTTGTGCT R:CAG GGC ATT TGC TGA TTCTTC C	700	
spvC	F: ACCAGAGAC ATT GCC TTCC R: TTC TGA TCG CCG CTA TTC G	467	Huehn *et al*. (2010)
Antibiotic resistant genes			
sul1 (Sulfonamides)	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433	Ibekwe *et al*. (2011)
tetA (Tetracycline)	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	576	Randall *et al*. (2004)
bla_{TEM} (*B-lactams*)	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516	Colom *et al*. (2003)

the infected group from which *Salmonella* spp. was isolated and then injected with Marbofloxacin intramuscularly with a dose of 1ml/50 kg B.W., for three successive days and the third group (G3) of clinically health calves was considered a control group (they were administered saline only).

Sampling for haemato-biochemical study

The whole blood samples were collected aseptically from the jugular vein once from the examined diarrheic group (G1), then once from infected group (G2), after treatment with Marbofloxacin and finally from a control group (G3). The first type of collected blood sample was the (non-coagulated sample); it was collected on EDTA for haemogram estimation. However, the second type of blood samples was without anticoagulant and these samples were placed in plain centrifuge tubes, then were centrifuged at 3000 rpm for 15 minutes and the clear serum was carefully aspirated into chemically free and clean tubes and stored at –20°C until assayed for the biochemical parameters. All blood samples were placed in a bed of crushed ice and taken immediately to the laboratory for further analysis.

Moreover, re-isolation and identification of *Salmonella* spp. were also performed in fecal samples after experimental treatment with Marbox in the G2 and also the G3 groups to examine the in vitro antibacterial effect of this challenged drug (Marbox®).

Haemato-biochemical estimation

The haemogram parameters of the calves under experiment in this study were estimated and included: red blood cell count (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV), total leukocytic count (TLC) and differential leukocytic counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) according to the method that was adopted (Feldman *et al*., 2000). Moreover, the biochemical parameters were also studied: serum total proteins according to Doumas *et al*. (1981), albumin and globulins according to Batavani *et al*. (2006) with cellulose acetate electrophoresis using Helena system (Helena France). Also, ALT, AST were estimated as described previously by Kachmar and Moss (1987) and Bergmeyer and Harder, (1986), respectively. Urea and creatinine were analyzed also with the aid of commercial test kits as mentioned by Rock *et al*. (1987) and Young (1995), respectively. In addition, serum glucose, sodium (Na), magnesium (Mg), potassium (K), chlorine (Cl), calcium (Ca), phosphorus (P), zinc (Zn) and copper (Cu) were also analyzed with an atomic absorption spectrophotometer (A Analyst 100, Perkin Elmer).

Statistical analysis

Serum biochemical and blood parameters were statistically analyzed with one-way ANOVA using program SPSS software (version 23.0) for Windows (IBM Corp. Armonk, NY, USA). The results were presented as mean ± SE and $p<0.05$ that were assumed to reflect statistical significance.

RESULTS

Clinical examination of diarrheic animals

The examined calves in the current study exhibited the clinical signs of diarrhea including dullness, sunken eyes, lethargy, depression, reduction of appetite, odorous semisolid to watery faces of greenish to yellowish white colour, which sometimes it was tinged with blood and mild to moderate degrees of dehydration.

Bacterial isolation results in diarrheic calves

The bacterial cultural and biochemical identification of the examined 260 diarrheic claves (that exhibited the typical clinical signs of diarrhea); revealed that the total prevalence rate of *Salmonella* spp. in this study was 18.8% (49/260). It was isolated in pure culture in 3.85% only meanwhile, *Salmonella* spp. was detected in a mixed form with other bacterial pathogens (*Salmonella* + *E. coli* spp. and *Salmonella* + *Proteus* spp.) in percentages 10.8% (28/260) and 4.2% (11/260), respectively in all examined diarrheic animals (Table 2) whereas, other bacteria were also identified. The calves of age lower than 2 months were more susceptible to the infection than older age (> 2 months).

Phenotypic cultural characterization of Salmonella isolates

Based on the microscopical and cultural characteristics for all yielded isolates in this study, *Salmonella* spp. was identified in samples from diarrheic animals without treatment on XLD medi-

um as pink with black center colonies. Biochemically, they were catalase and methyl red positive while negative for indole, vogus prescour, oxidase test and urea tests. On TSI medium, they converted it to red slant and yellow butt with H2S production. However, other Gram negative bacteria (*E. coli* and *Proteus* spp.) were isolated and identified with its unique characteristic cultural morphology and interpretations on Macconkey`s and eosin methylene blue agar plates and also, they were biochemically confirmed.

The serotyping diversity of *Salmonella* isolates in the recent study identified *S.* Typhymurium (19/49), *S.* Enteritidis (9/49), *S.* Dublin (6/59), *S*. Anatum (4/49), *S.* Saintpaul (4/49), *S.* Stratford (3/49), *S.* muenchen (3/49) and S. Agona (1/49). However, *Salmonella* spp. couldn't be yielded on its specific medium from ten faecal samples of (G2) after the administration and treatment with Marbox® drug indicating the drug potent antibacterial effect and inhibiting *Salmonella* growth.

Phenotypic antimicrobial resistance pattern

The identified *Salmonella* isolates in this study revealed phenotypically high resistance level (100%) to tetracycline, ampicillin and sulfamethoxazole/trimethoprim drugs, while they showed a resistance range of (67.3-91.8%) for norfloxacin, streptomycin, amoxiclavulinic acid, enrofloxacin and colistin drugs. Moreover, these isolates were highly sensitive to Marbofloxacin, ciprofloxacin, gentamicin and amikacin (96%, 89.7%, 81.6% and 71.4%), respectively (Table 3).

Detection of antibiotic resistant and virulent genes

PCR genotyping confirmed the virulence traits of *Salmonella*

Table 2. The prevalence of bacteria causing diarrhea including *Salmonella* spp. in calves.

Bacterial species	No. of positive samples	%
Salmonella spp. (pure form)	10/260	3.85%
Salmonella + *E. coli* spp.	28/260	10.77%
Salmonella + *Proteus* spy	11/260	4.23%
Total *Salmonella* isolates	49/260	18.80%

Table 3. Antibiotic resistance profile of the recovered *Salmonella* spp. isolates.

Antibiotic disc	Antibiotic group	Abbreviation	Disc Conc. (μg)	*Antibiotic resistance*				
				S	S%	*I*	*R*	*R%*
Tetracycline	Tetracycline	TE	30	--	--	--	49/49	100%
Ampicillin	Aminopenicillin	AMP	10	--	--	--	49/49	100%
Sulfamethoxazole/trimethoprim	Combination	SXT	25	--	--	--	49/49	100%
Enrofloxacin	Fluoroquinolone (4th generation)	ENR		--	--	4/49	45/49	91.80%
Amoxicillin-Clavulanic Acid	Combination	AMC	3	--		5/49	44/49	89.80%
Streptomycin	Aminoglycosides	ST	10	--	--	6/49	43/49	87.80%
Norfloxacin	Fluoroquinolone (2nd generation)	Nor	10	--	--	16/49	33/49	67.30%
Colistin	Macrolides	C	30	13/49	22.44%	2/49	33/49	67.30%
Amikacin	Aminoglycosides	AK	30	35/49	71.40%	6/49	8/49	16.30%
Gentamicin	Aminoglycosides	GEN	10	40/49	81.60%	3/49	4/49	8.16%
Ciprofloxacin	Fluoroquinolone (2nd generation)	CIP	10	44/49	89.70%	3/49	2/49	4.08%
Marbofloxacin	Fluoroquinolone (4th generation)	MXF	5	46/49	96%	2/49	--	--

S: sensitive, S%: the sensitivity percentage, I: intermediate, R: Resistant, R%: the resistance percentage

Table 4. Hematological changes in *Salmonella* infected calves, treated calves with Marbox and control groups.

Parameter	Diarrheic calves without any treatment (G1)	Diarrheic calves after Marbofloxacin administration (G2)	Control group (administered with saline only) (G3)
RBCs (10^6 /μl)	10.46±0.20^a	8.60±0.24^b	8.38±0.26^b
HB Conc. (g/dl)	12.58±0.30^a	10.04±0.09^b	9.78±0.18^c
PCV (%)	39.00±0.29^a	31.62±0.42^b	30.82±0.72^b
MCV (fl)	37.42±0.59^a	36.78±0.77^a	36.86±0.54^a
MCH (pg)	12.05±0.24^a	11.69±0.34^a	11.72±0.22^a
MCHC (g/dl)	32.22±0.56^a	31.78±0.51^a	31.80±0.44^a
WBCs (10^3 /μl)	14.04±0.86^b	19.60±0.68^a	8.19±0.26^c
Neutrophiles (10^3 /μl)	8.036±0.39^a	6.55±0.52^b	2.8689±0.11^c
Lymphocytes (10^3 /μl)	3.277±0.37^c	11.309±0.31^a	4.719±0.16^b
Monocytes (10^3 /μl)	1.63±0.082^a	1.047±0.14^b	0.3587±0.018^c
Eosinophiles (10^3 /μl)	1.091±0.058^a	0.688±0.065^b	0.2426±0.012^c
Basophiles (10^3 /μl)	0.006±0.0007^a	0.006±0.0013^a	0.0008±0.0003^b

Data are presented as mean±SE. Values in the same column with the different superscripts are significantly different at P<0.05.

spp. isolates in this study; it cleared the presence of *invA*, *Stn* and *sopB* virulence genes in all ten isolates meanwhile, *pefA* and *spvC* virulence genes were shown in 60% of these isolates for each (Fig 1). In addition, the antibiotic resistance profile confirmed the multidrug resistance (MDR) of *Salmonella* isolates where bla_{TEM}, *tetA* and *sul1* resistant genes for β-lactam, tetracycline and sulphonamides drugs were recorded in 100% of isolates.

Hematological analysis

The results as shown in Table 4 declared a notable increase in the levels of RBC count, PCV value and Hb concentration in the diseased group (G1) (that hadn't been administered with any treatement) more than G2 (the group after Marbox administration) and also than G3 (control group which was administered

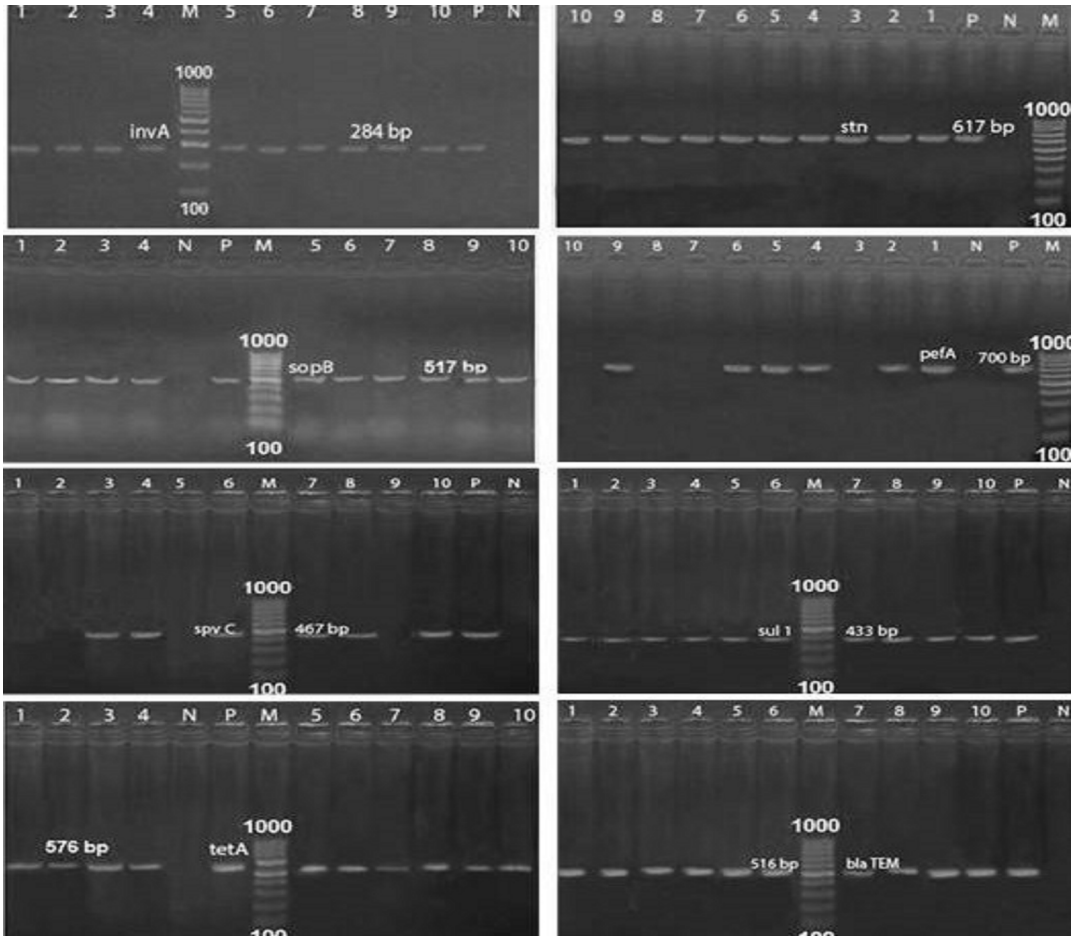

Fig. 1. PCR amplification of virulence genes *invA*, *Stn*, *sopB* (C), *pefA* and *spvC* and antibiotic resistant genes: *sul1*, *tetA*, and bla_{TEM}. Lane M: 100 bp molecular weight marker, Lanes 1-10: *Salmonella* spp. Lane P: positive control, Lane N: negative control.

Table 5. Serum biochemical changes in *Salmonella* infected calves, treated calves with Marbox and control groups.

Parameter	Diarrheic calves without any treatment (G1)	Diarrheic calves after Marbofloxacin administration (G2)	Control group (administered with saline only) (G3)
ALT(U/L)	73.54±1.35^a	59.3±1.45^b	38.52±2.12^c
AST (U/L)	153.24±1.21^a	131.67±0.94^b	94.25±1.98^c
Urea (mg/dl)	40.6±1.52^a	36.69±.91^b	28.27±1.67^c
Creatinine (mg/dl)	2.25±0.51^a	1.81±0.73^b	0.75±0.53^c
Total protein(g/dl)	6.15±0.52^b	7.08±0.38^a	7.56±0.36^a
Albumin (g/dl)	2.79±0.06^b	3.51±0.06^a	3.94±0.07^a
Globulin (g/dl)	4.06±0.08^a	3.77±0.08^b	3.12±0.09^c
Glucose (mg/dl)	52.29±0.42^c	61.36±0.36^b	72.26±0.18^a
Na (nmol/L)	131.58±2.52^c	139.97±2.01^b	142.21±3.24^a
K (nmol/L)	7.23±0.4^a	6.11±0.28^b	5.43±0.24^c
Cl (nmol/L)	87.52±0.61^c	98.11±0.73^b	109±0.85^a
Ca (mg/dl)	8.46±0.12^b	10.30±0.22^a	10.65±0.21^a
Phosphorus (mg/dl)	4.20±0.11^b	6.36±0.21^a	6.29±0.23^a
Zinc (µg/L)	103.22±4.2^c	165.18±3.6^b	170.22±3.2^a
Copper (µg/L)	40.28±0.31^c	63.70±0.34^b	74.43±0.15^a
Magnesium (nmol/L)	1.41±0.07^c	1.63±0.09^b	1.95±0.04^a

Data are presented as mean±SE. Values in the same column with the different superscripts are significantly different at P<0.05.

with saline only). Also, non-significant changes in the values of MCV, MCH and MCHC ($p< 0.05$) were estimated. Moreover, a marked leukocytosis with neutrophilia was recorded in G1 and G2 after treatment with Marbox. Also, leukocytosis along with remarkable decreases in the counts of monocyte and eosinophil were yielded in the treated group.

Blood biochemical profile

A marked elevation in AST, ALT, urea and creatinine levels; however, total proteins and albumin were significantly decreased ($p<0.05$) in the diseased group without any treatment (G1) and G2 after administration of Marbox drug when compared to the control group (G3). Also, the serum globulin was notably elevated in G1 (without treatment). Moreover, the diarrheic calves (G1) in this study recorded hyponatremia, hypocalcemia, hypomagnesemia with decreased levels of Cl, Zn, Cu and P ($p>0.05$) but potassium (K) level was increased in the diseased calves in comparison with G3 control animals. All estimated parameters in the treated animals (G2) with Marbox were significantly improved as shown in Table 5.

DISCUSSION

All over the globe, the newly born calves are regarded the crucial assets for cow replacement in animal farms for the sustainability of dairy and beef herds (Leliso *et al*., 2021). For several years, gastrointestinal illnesses continue as a global and public health menace. *Salmonella* infections are considered as serious threat to animal health; it is an insidious problem of diarrhea especially in young age (calves) that impaired animal performance.

By clinical examination of 260 diseased calves in this study, the inspected animals exhibited general signs of illness and variable degrees of diarrhea that sometimes were tinged with blood. The same clinical finding was consistent with Adeladlew (2020) and Vasconcelos *et al*. (2021). Moreover, identical colonial morphological cultural and biochemical features of the *Salmonella* isolates were found in corresponding with previous reports (Duenas *et al*., 2017; Manishimwe *et al*., 2021; Ezzat *et al*., 2022).

The total recovery rate of *Salmonella* spp. in all examined samples in this investigation was 49/260 (18.8%). Corresponding results reported that *Salmonella* spp. was recovered from diarrheic calves in 18.1% (23/127) and also, in 18.66% (42/255) in previous studies in Egypt farms by El-Seedy *et al*. (2016) and Youssef and El-Haig (2012). In India, a similar recovery rate (18.33%) of *Salmonella* spp. was detected in 80 diarrheic calves (Manickam and Ramasamy, 2017). Similar rates were recorded (15.8%, 13.2%, 13% and 11.42%) in Egypt, Bangladesh, Nigeria and North West Ethiopia from the faecal samples from calves showing the clinical signs of diarrhea (Marouf *et al*., 2016; Olaogun *et al*., 2016; Adeladlew, 2020) and also, in Egypt, Elhady *et al*. (2020) and Elsayed *et al*. (2020) estimated *Salmonella* spp. from (36/120 and 65/200) fecal samples of the diarrheic calves in higher levels (30% and 32.5%), respectively. On the other hand, lower isolation rates of *Salmonella* spp. (9.75% and 6.2%) were recovered from (59/646) and (8/129) fecal samples in diarrheic cow and buffalo calves (Elbehiry, 2014). This variation in the prevalence rate of salmonellosis in diarrheic calves might be caused as a result of many factors like stress, sex, age, transportation, starvation, overcrowding, fluctuant temperature, the geographical distribution, managemental practices of the farm and also, the farm size (Cho and Yoon, 2014).

The age is an important risk factor that was associated mainly with the incidence of NCD in calves especially within the first month of life (Duenas *et al*., 2017; Elhady *et al*., 2020). In the recent study, diarrhea frequently occurred in calves of age (that ranged from earlier weeks to ≤ 2 months) more than the older age (> 2 months). The same results were documented in many studies (El-Seedy *et al*., 2016; Elhady *et al*., 2020).

Of interest, asymptomatic animals might shed different serovars of *Salmonella* spp. such as *S.* Kentucky and *S.* Enteritidis (Van Kessel *et al*., 2013). The serotyping of yielded *Salmonella* isolates clarified different serovars in which *S.* typhimurium and S. Enteritidis were the most prevalent serotypes (19/49) types. Corresponding results of Elsayed *et al*. (2020) who detected eight serotypes of *Salmonella* species; of which *S.* Typhmurium and *S.* Anatum were highly detected. Moreover, *S.* Anatum, *S.* Dublin, *S.* Saintpaul and other types were also identified in calves and dairy cows (El-Seedy *et al*., 2016; HadİMlİ *et al*., 2017).

The worldwide problem of antimicrobial resistance (AMR) among *Salmonella* spp. and their resistance genes might be dispersed and horizontally transmitted within same or different bacterial species (Andino and Hanning, 2015). AMR *Salmonella* strains are disconcerting issue, especially for its potential to spread through the human food chain, constituting a public health challenge (Geletu *et al*., 2022). Also, MDR could reflect poor infection control practices within the animal environment (Abdeltawab *et al*., 2016).

The data in Table 4 displayed that the recovered *Salmonella* isolates from the diarrheic calves which were resistant to six or more of the tested antimicrobials; regarded as multi-drug resistant (MDR) strains. Highest resistance rates (100%) of the *Salmonella* strains in this study were documented to tetracycline, ampicillin and sulfamethoxazole/trimethoprim antimicrobials. Moreover, norofloxacin, streptomycin, amoxiclavulinic acid and enrofloxacin drugs recorded variable degrees of resistances that ranged between (67.3-91.8%) however, Marbofloxacin, gentamicin, ciprofloxacin and amikacin drugs were the most sensitive drugs in all examined *Salmonella* isolates.

In the same way, a study in African countries by Peruzy *et al*. (2020) stated the incidence of high level of *Salmonella* resistance against multiple of antibiotics which could be regarded as (MDR). Another study reported a high level of MDR of *Salmonella* spp. in cattle meat against tetracycline and ampicillin (Mthembu *et al*., 2019). Also, MDR strains of *Salmonella* spp. from diarrheic cattle calves in Egypt were reported (Abdel Aziz *et al*., 2018; Elhady *et al*., 2020; Elsayed *et al*., 2020) against ampicillin, streptomycin and sulphamethaxozle trimethoprim (100%). In addition, A high resistance rate of *Salmonella* spp. for ampicillin was also mentioned (Abdeltawab *et al*., 2016). In line with that Liu *et al*. (2021) revealed also, the incidence of MDR *Salmonella* in animal food-chain in China with high resistance to tetracycline, amoxicillin/clavulanic acid and ampicillin. Also, the cattle *Salmonella* isolates showed high resistance against streptomycin, tetracycline, nalidixic acid and vancomycin in Central Ethiopia (53.9%); however, gentamycin was found highly effective (85%) (Geletu *et al*., 2022). For *Salmonella* quinolones and beta-lactams antimicrobial resistances that were recorded; it could be considered as a major threat for public health since these antimicrobial classes are currently used as preferred drugs for the treatment of salmonellosis (Le Hello, 2014).

On the other side, similar to our finding, amikacin (100%), ciprofloxacin (77.7%) recorded highest sensitivity rates (Elhady *et al*., 2020) and gentamycin drug was highly sensitive drug for MDR *Salmonella* (Abunna *et al*., 2017). The recorded worldwide MDR could arise from the unwise use of several antimicrobials in the veterinary field like (tetracycline, streptomycin, penicillin, and sulfa) especially these drugs were widely available and could be obtained easily with no need for prescription from an authorized facility (Liu *et al*., 2021). This is a concern because these MDR strains might transmit their resistance traits to human by consumption of food carrying antibiotic-resistant bacteria that reflect directly or indirectly causing acquisition of antibiotic-resistant infections (Geletu *et al*., 2022).

PCR virulence determinants perform an essential elementary role in the enhancement of bacterial pathogenicity and concurrently bovine or calf salmonellosis. It was previously mentioned that *Salmonella* pathogenicity islands (SPIs) contain wide number of these virulence genes (Cheng *et al*., 2019). Though not

all these virulence genes were buried in the known twenty-four SPIs, they could found either on chromosomes or plasmids (Ilyas *et al*., 2017; Cheng *et al*., 2019). These SPIs have the potential to horizontally spread to other intestinal bacteria, which could turn theses already found non-pathogenic germs into pathogenic ones (Naidoo *et al*., 2022). Moreover, the pathogenesis process comprise the ability of virulence genes for boosting *Salmonella*'s adhesion, invasion, and intracellular survival and bypassing the hosts' defense mechanisms which lead to exhibiting of a variety of animal clinical symptoms (Cheng *et al*., 2019).

Salmonella is more complex heterogenic species as it could possess numerous virulence genes that involved in an organism invasion and adhesion (Siddiky *et al*., 2021). Prior studies stated that the main function of chromosomally located gene *invA* (invasion) was to detect and identify the *invA*sive and pathogenic serovars of *Salmonella* spp. It encoded for the protein that *invA*des the host epithelial cells (Naidoo *et al*., 2022) causing sever loss of intestinal fluids making bacterial colonization producing secretory diarrhea (Elhady *et al*., 2020) .

Notably, PCR detection of the *invA* gene in *Salmonella* spp. is the gold standard biomarker for its being since it is found only in this species (El gresly *et al*., 2021). Moreover, *Stn* gene is an enterotoxin virulence gene that induces more loss of intestinal fluids leading to an intense bacterial colonization and secretory diarrhea (Ezzat *et al*., 2022) and also, *sopB* virulence is present mainly in SPI1 and could have a main role in *Salmonella* induced diarrhea in calves. Moreover, *spvC* is also a plasmid mediated virulence gene of *Salmonella* spp. which it has a main role in vertical transmission (Siddiky *et al*., 2021). Also, many reports mentioned that Spv genes (spvB, *spvC*, and spvR) could play essential role in the virulence system of *Salmonella* strains (Liu *et al*., 2021).

PCR screening of *invA* and *Stn* virulence genes of *Salmonella* isolates in the current study discovered their presence in 100% of them however; other virulence genes (*pefA*, *spvC* and *sopB*) genes were also detected variably in this study. The results of *invA* and *Stn* virulence genes were consistent with many studies (Adeladlew, 2020; El-Seedy *et al*., 2016; El gresly *et al*., 2021; Marouf *et al*., 2016). However, Liu *et al*. (2021) reported that all examined S. Enteritidis isolates harbored pef encoding fimbriae genes. In addition, Elhady *et al*. (2020) reported *invA* and *sopB* in all MDR *Salmonella* strains that were isolated from calves with enteritis. In addition, the *spvC* gene showed their characteristic bands at its specific amplicon size of 467 bp in 92% and 100% of the tested *Salmonella* strains (100%) (Giacomodonato *et al*., 2014; Moustafa *et al*., 2020), respectively. From all above data, all recovered *Salmonella* isolates exhibited different virulence profiles, indicating the different potential severity of infection and pathogenicity in diarrheic calves.

The aforementioned PCR results confirmed the existence of antibiotic resistant genes (*sul1*, *tetA* and $bla_{TEM)}$ in the examined *Salmonella* isolates. In the same way, the prevalence of antibiotic resistance genes (bla_{TEM}, *tetA*, *aadA2* and *sul1*) in some MDR *Salmonella* isolates from cattle, poultry and human samples was 83.3%, 91.7%, 41.7% and 83.3%, respectively. However, *tetA* was detected by Elhady *et al*. (2020) and Adesiji *et al*. (2014) in 100% of the isolates. The wide distribution of *tetA* gene across Gram-negative bacteria, including *Salmonella* spp. indicates the horizontal transfer of tetracycline resistance genes among this family (Elhady *et al*., 2020). Similar previous findings of the *sul1* gene which was detected in all tested *Salmonella* strains by Abdeltawab *et al*. (2016). Moreover, it was documented that resistant genes encoding for drugs (streptomycin, sulfonamide, gentamicin, ampicillin and trimethoprim antibiotics) were the most common resistant genes in the diarrheic calves (Shahrani *et al*., 2014). In addition, the results of Adeladlew (2020) declared the incidence of high level of resistance against β-lacatmase than quinolones also, Liu *et al*. (2021) reported the variable detection rates of sul, tet A, bla_{TEM} and *qnrS1* resistant genes among MDR *Salmonella* isolates from animal food chain in China. The discrepancies between genotypic and phenotypic antimicrobial sensitivity was owing to the possibility of carrying multiple drug resistance genes or harboring many extra-chromosomal genetic elements or antimicrobial resistance by different resistance mechanism as efflux pump (Elhady *et al*., 2020).

For the other part of this study, the hematological changes in Table 4 identified the inflammatory responses that were linked to enteritis disorders. They aid in the early diagnosis of the condition and forecast the severity of the illness. The haemogram estimation of the *Salmonella* infected group (G1) before administration of Marbox drug stated significant increases in RBC count, PCV value and Hb concentration which might be due to the diarrheal haemoconcentration. Hypovolemia also occurred as a result of the extracellular fluid loss especially with insufficient milk and drink intake. The same results were concurred with prior research (Malik *et al*., 2013). However, non-significant changes in values of MCV, MCH and MCHC ($p< 0.05$) were recorded. This was on the contrary with the results by Arafa *et al*. (2008) who reported a significant decrease ($p< 0.05$) in total erythrocyte count, HB values, MCH and MCHC values in diarrheic calves when compared with the values of healthy ones. Moreover, significant decreases in hemoglobin content and packed cell volume (PCV) in the treated group with Marbox compared to the control one. This result was consistent with a study by Chauhan *et al*. (2017) in which a substantial decline in the values of Hb and PCV in a group pf animals that received only Marbox (5 mg/kg of body weight for 5 days) than the values of the pre-treated animals. The decrease in total erythrocytic count was linked to a variety of factors including lowering of the erythropoietin hormone and lead to a decline in PCV level (El-sayed *et al*., 2019) .

The leukocytosis in the diarrheic animals in this study might occur as a natural response of the animal body due to the defense mechanism against bacterial infections. These results were corresponding to that obtained by Brar *et al*. (2015). Leukocytosis was primarily estimated due to neutrophilia (Eddy and Pinsent, 2004; Shehta *et al*., 2022). Furthermore, a significant neutrophilia with lymphopenia could indicate the bacterial enteritis and intestinal infections (Malik *et al*., 2013). This finding was parallel with Sekhar *et al*. (2017) who indicated that an inflammatory mechanism might be linked with the leukocytosis, neutrophilia, monocytosis, and lymphopenia.

The therapeutic efficacy of Marbox drug in the *Salmonella* infected group was studied experimentally in this study. It was evaluated on the basis of recovery rate that was evidenced with the degree of resolution of clinical manifestations, profiles, hematological, biochemical and percent recovery of diarrheic calves. An increase in the total leukocytic count with an increased level of lymphocytes and decreased number of neutrophils in the G2 (after treatment with Marbofloxacin) was detected. This was are in accordance with Asati *et al*. (2008) and El-sayed *et al*. (2019). It might be referred to the fluoroquinolone's capacity to concentrate in neutrophils and macrophages. Moreover, due to the body's natural response against the infection, body defense mechanism exhibited dehydration, hemoconcentration, high leukocyte percentages (Kumar *et al*., 2018).

Also, an improvement of the counts of monocyte and eosinophil was yielded in G2 after the experimental administration of the drug. This finding was similarly with results of Chauhan *et al*. (2017) who recorded significant declines in eosinophil and monocyte in the post treated animal group with Marbofloxacin with a dose 5 mg/kg of body weight for 5 days.

In addition, the significant elevation in levels of serum AST, ALT, urea and creatinine in the present study could be resulted by the direct harmful effect of *Salmonella* toxins on the hepatic and renal cells. This result was previously supported with many studies (Manaa *et al*., 1993; Aly *et al*., 1996; Nabih and Arafa, 2012). Also, chronic inflammations and pathological affections of gastrointestinal tract might lead to reported elevations of serum ALT and AST in the diarrheic calves (Berg, 1981).

For serum urea and creatinine estimation in this study, a significant rise in their values was reported in G1 (without any drug treatment). The rise of urea and creatinine levels could be attributed to deficiency in the renal blood perfusion (glomerular

filtration rate) which consequently, reduce the urine formation alternating the renal functions (Jain, 1993; Singh *et al*., 2014). These results were in accordance with Patel *et al*. (2014) who reported a significant reduction of the values of alanine aminotransferase in the affected sheep however, these values remained within the normal range after a single intravenous administration of Marbofloxacin (2 mg/kg) in the same study.

The serum biochemical analysis of diarrheic calves during our experimental investigation cleared that the mean values of total proteins and albumin were significantly decreased ($p<0.05$) when compared with healthy group. This could be due to anorexia in the affected calves. Also, reduced levels of total protein and albumin in diseased calves might be related to the profound fibrinopurulent necrotizing enteritis where huge amounts of protein-rich effusion could pass into the intestinal lumen causing severe intestinal protein loss. A nonselective loss of protein could be substantiation with the concurrent decrease in albumin and total protein concentrations (Gharieb *et al*., 2015).

Furthermore, a significant elevation in serum globulin of G1 was in agreement with Pekcan *et al*. (2012) since it was attributed to an acute inflammation due to intestinal bacterial pathogens. Although, other authors documented a rise in the total serum protein and albumin but with a decreased level of the serum globulin (Guzelbektes *et al*., 2007; Seifi *et al*., 2006). On the contrary, our findings were disagreed with Mahmood (2013) who discovered a non-significant change in total protein, albumin and globulin following daily intramuscular administration of Marbofloxacin (2 mg/kg) and trovafloxacin (3 mg/kg) in sheep for five days.

In addition, hypoglycemia was recorded in G1 during our experimental study that might be due to glycogenesis and increased aerobic glycolysis and congruent with diarrhea (Tennant *et al*., 1986). Significant hypoglycemia in several cases of calf diarrhea was reported. Some authors stated that it might result from poor or complete absence of normal suckling, lack of the intestinal epithelial transport and endotoxic-septic shock (Naylor, 2002). It is well noted that hyperproteinemia as well as hypoglycemia was manifested due to the action of *Salmonella* enterotoxins since these toxins activated the adenyl cyclase enzyme leading to the production of cyclic adenosine monophosphate (cAMP). This cAMP was found instantly increased the intestinal fluid secretion from the systemic circulation resulting in varying degrees of dehydration, electrolyte imbalance and acidosis. These results were supported with many studies ((Blood *et al*., 1983; Manaa *et al*., 1993; Kaneko, 1997).

Similar mineral serum profile of (decreased levels of Na, Cl, Ca, Zn, Cu, Mg, P and increased level of K) in the diarrheic calves (G1) was in accordance with Nabih and Arafa (2012). Hyponatremia could be generally due to massive loss of sodium which was in relatively equal or in greater proportion to water loss (Roussel 1992). The amount of sodium loss in faeces in the diarrheic calves had been estimated as 27.2 times more than its values in the normal calves (Radostits *et al*., 2000). Also, sever mineral alterations might be caused by increased vascular permeability during the inflammation process. It could be accompanied with the loss of intestinal epithelial integrity, mucosal necrosis and loss of discernible villi or crypt structures (Hassan, 2015).

Moreover, G1 (the *Salmonella* infected group without treatment) showed decreased calcium (Ca) and phosphorus, magnesium serum levels. Hypocalcemia was occur during the persistent diarrhea and dehydration (Ghanem *et al*., 2012b) and low level of phosphorus (P) was resulted by loss of a huge amount of electrolytes than water loss (EL-dessouky and El-Masry, 2005). Low absorption and diarrhea could decrease the serum (Mg) level. Also, other secondary nutritional and metabolic disorders during excessive faecal losses, malabsorption due to various types of bowel diseases including *Salmonella* infections contribute to low Ca and Mg levels (Nabih and Arafa, 2012). Same low serum levels of Ca, P, and Mg during salmonellosis by Santos *et al*. (2002) and Tsolis *et al*. (2000) affirmed our finding.

Hyperkalemia is the most common characteristic finding of severely diarrheic neonatal calves. The same finding of a rise in the potassium serum level in the diseased calves without Marbofloxacin in this study was detected also by Dratwa *et al*. (2012) and Nasir *et al*. (2013). The normal healthy renal tissue function might increase the renal tubular potassium reabsorption in response to acidosis. However, if it had been malfunctioned (that was indicated by high serum urea and creatinine values), the potassium ions would shift from intracellular to extracellular fluid (K+ - H+ exchange) in a response to the acidosis and the hyperkalemia would develop (Naylor, 2002; Radostits *et al*., 2007; Seifi *et al*., 2006; Singh *et al*., 2014). Also, a decline in the levels of Cu and Zn in this study could be due to low gut absorption of nutrients and fecal loss (Khan *et al*., 2009). The increased demand of the immune system for utilization and sequestration of Zn at the tissue level for synthesis of antioxidant enzymes as a compensatory mechanism to counter excessive free radical production (Ranjan *et al*., 2006). Moreover, the estimated hypomagnesemia was related to malabsorption during diarrhea process (Ghanem *et al*., 2012a)

Overall, the current investigation emphasized an improvement of all hemato-biochemical parameters after an experimental study with repeated I/M injection of Marbox®. According to the results, Marbox® drug mitigated the harmful effect of *Salmonella* toxins in tested diarrheic calf cases. This finding was in accordance with El-sayed *et al*. (2019) in which they confirmed that Marbox® was safe and positively impacted on the hematological and biochemical activities of the diarrheic calves concluding that it could act as a cornerstone in the treatment and control of diarrhea caused by bacterial infection.

CONCLUSION

This study emphasized a high proportion of MDR *Salmonella* species as the primary cause in calf diarrhea alongside with other bacterial pathogens that could of a great alert concern for animal welfare and production. Proper animal farm hygiene, internal and external biosecurity measures, hygiene management of litter material, daily usage tools and adsorbents should be constructed. Also, new members of antibiotics should be synthesized to overcome antibiotic failure problems and limit the spread of the genetic resistance elements among human and animal species. Marbofloxacin medication recorded notably good results as an antibacterial drug in this experimental study and positively impacted on haematobiochemical parameters of *Salmonella* infected animal. It might inhibit the probability of *Salmonella* infections and aid in the treatment of the diseased cases in animal farms.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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