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Prevalence of *Staphylococcus aureus* and *Pasteurella multocida* incriminated in calf pneumonia and their treatment

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ABSTRACT

Bovine respiratory disease (BRD) is considered as one of the key health issues and is answerable for serious economic losses all over the world. This study aimed to determine a prevalence and proper treatment of *Staphylococcus aureus* and *Pasteurella multocida* isolated from calves had respiratory manifestations. Bacteriological examination of 45 nasal swab samples collected from respiratory manifested calves, revealed that 19(42.2%) and 13(28.9%) were *S.aureus* and *P. multocida* respectively. *S. aureus* and *P. multocida* displayed good sensitivity for gentamicin, ciprofloxacin and chloramphenicol, according to *in-vitro* antimicrobial susceptibility results.

Twenty calves, (3-5 month) were divided into 2 groups. Gr1: contain five of clinically healthy calves kept as a negative control group. Gr2: fifteen calves had respiratory manifestations and positive for *S. aureus* and *P. multocida* isolation (kept as positive control group), blood samples for hematological and biochemical studies were taken before treatment. Gr2 (diseased calves) sub divided into three equal groups (Gr3, Gr4 and Gr5) (five calves each). Gr3**:** were treated with Gentaprima® 1ml /25kg b.wt. I/M for 3 days. Gr4**:** were treated with Gentaprima® with the same dose+ MENTO-Z® (1 ml/8 litre drinking water for 5 successive days) and Gr5**:** were treated with Gentaprima® with the same dose + Levamizole (single S/C injection at dose of 1 mg/50kg bwt).

From a bacteriological aspect, all swabs collected in 10th day after treatment from groups (Gr3, G4 and Gr5) were negative for *S.aureus* and *P. multocida* isolation.

Hematological study concerning to RBCs count, Hb concentration and PCV% revealed significant decreases in diseased, 3rd and 10th days after

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treatment calves in compared with positive control group. Diseased calves showed significant decrease in PH, PO_2 and SO_2% while there were significant increase in PCO_2, tCO_2 and HCO_3 compared to control animals. At 3rd and 10th days post treatments PH, PO_2 and SO_2% recorded significant increase and PCO_2, tCO_2 and HCO_3 showed significant decrease in comparison with diseased group before treatment. In regard to phagocytic index and percentage showed significant decrease in diseased calves compared with control, meanwhile there were significant increase in phagocytic index and percentage in Gr4 and Gr5 at 3rd and 10th days post treatment in compared with diseased group.

Biochemical finding demonstrated significant increase in serum protein, globulin, glucose, AST and creatinine as well as haptoglobin and fibrinogen in diseased calves compared with control one. Marked improvement in the mentioned parameters in all treated groups at 3 days post treatment which returned to the normal level at 10 days post treatment compared with control one.

INTRODUCTION:

Bovine respiratory disease (BRD) is a multifactorial illness in calves that produces significant mortality and economic losses in cattle farming **(Delabouglise *et al*., 2017).** The pathogenesis of BRD is complex and often involves interactions of infectious agents, environmental and stress factors, and the host immunosuppressive stress that is induced by factors such as poor nutrition, dehydration, early weaning, low or high temperatures, little rest and transportation. Clinical signs of BRD vary from acute to chronic illness and include mostly pyrexia, coughing, nasal and ocular discharge. The occurrence of tachypnoea, anorexia and dyspnoea indicates a more serious in BRD. According to **Duff and Galyean (2007),** in calves with bacterial infections only, there is sporadic morbidity, but higher mortality.

Many bacteria are involved in respiratory syndromes in calves, including; *Staphylococcus aureus, Pasteurella multocida, Escherichia coli, Citrobacter spp., Pseudomonas aeruginosa, Proteus vulgaris, Enterobacter aerogenes, Enterobacter cloacae, Bacillus spp.,* and *Mannheimia haemolytica*. Most of these bacterial species are commensal in the upper respiratory tract of cattle. Stresses or viral infections lower physical and cellular respiratory defenses and increase in susceptibility to infection and pave the way for opportunistic bacteria to colonize the respiratory system, be inhaled into the lungs, and lead to bronchopneumonia **(McMullen *et al.,* 2020).** As a commensal of cattle, *P. multocida* is located in the upper respiratory tract. *P. multocida* is frequently linked to both acute and chronic infections that can cause severe morbidity (manifested as pasteurellosis, pneumonia, atrophic rhinitis, hemorrhagic septicemia and/or cellulitis, abscesses, and meningitis) and mortality, particularly in animals. *S. aureus* are considered potential pathogens involved in calf pneumonia. The upper respiratory tract's mucous membranes are mostly inhabited by *S. aureus*, which uses these tissues as a pathological resource following stress conditions, such as infection by influenza virus and can be become a serious cause of infection in immunosuppressed hosts. The broad range of infections caused by *S. aureus* is related to a number of virulence factors include biofilm, surface proteins, several secreted toxins, and increasing resistance to antibiotics **(Tam and Torres, 2019).**

Polymerase chain reaction (PCR) test allows the detection and identification of different pathogens causing BRD and also has been successfully used for rapid detection of their virulence genes included in pathogenesis **(Enany *et al.* 2018).**

It is likely that a combination of more accurate diagnoses, effective vaccinations, therapeutic interventions, and improved management techniques are needed to effectively control BRD **(Andrés-Lasheras *et al.,* 2021).** So, antibiotic sensitivity testing is a fundamental requirement to distinguish the appropriate antimicrobial agent to be used **(Cid *et al.,* 2019).** Anti-inflammatory medications and the proper antibiotics can be used to treat bovine respiratory disease in order to decrease both of a risk of the illness spreading and economic loss that caused by the cost of treating bovine respiratory disease and its impact on meat quality.**(El-**

zahar *et al.*, 2021).

Blood gases (pH, PCO2, PO2, tCo2 and HCO3) and related parameters are important criteria to make comments on diagnosis, treatment and prognosis of the disease affecting respiratory system and acid base balance **(Radostits *et al.*, 2005)**. Based on evidence showing cattle needing therapy for BRD, the serum haptoglobin (Hp) concentration has been proposed as a tool for management decisions **(Step *et al.*, 2008).** The identification of the inflammatory response associated with pneumonia and the early detection of BRD and illness prediction could be assisted by changes in the results of haematological and biochemical studies **(Kumar *et al.*, 2018).** Stress-induced changes in immune function have been documented in cattle, with alterations to cell-mediated and humoral immunity having a significant impact on immunocompetence which may render an animal more susceptible to infection **(Carroll and Forsberg 2007).**

Therefore, the current work aimed to determine the prevalence of *P.multocida* and *S.aureus* as common bacterial pathogens in calves suffered from respiratory manifestations and to compare between different BRD treatments programmers in calves using antibiotic alone or in combination with immune stimulant drugs.

MATERIALS AND METHODS:

Ethical approval

This study was declared by the Local Committee of the (ARC-IACUC,23,11) committee Institute: Animal Health Research Institute Ethical Committee Approval Number: AHRI/23/11. All methods were done in accordance with the Animal Health Research Institute guidelines according to the OIE standards for use of animals in research and education.

Drugs

Gentamicin: Gentaprima® obtained commercially from Arab company for Primavet, Egypt. Each 100 ml contains Gentamicin sulfate (equivalent to Gentamicin base 10g) by dose 1ml/ 25kg b.wt. I/M for 3 days (recommended dose).

MENTO-Z®: obtained commercially from Biotrade-Italy for Elshinnawy vet-Egypt. Each liter contains menthol (peppermint oil) 80g, cynnamaldelyde (cinnamon oil) 10g, 1.8-cineole (cajeput oil) 5g, alpha pinen (pine oil) 20g, thymol 30g and carvacrol 40 g by dose 1ml/8L drinking water (recommended dose).

Levamizole®: obtained commercially from El Nasr Company, Egypt. Each 100 ml contains Levamisole Hydrochloride 7.5g used as anti-helminthic and immune-stimulant for large animals in farms by dose 1 mg/50kg b.wt. S/C.

Animals

Calves, from 3-5 month old and their weight ranging from 65-70 kg obtained from private farm in Sharkia province during winter season were used in this study.

Samples used for Bacteriological examination

A total of 45 nasal swab samples collected aseptically from 45 pneumonic calves suffered from respiratory manifestations such as cough, fever, nasal discharges, rapid breathing, dyspnea, and anorexia. Samples were transported immediately to the bacteriological laboratory for examination.

Also nasal swabs were collected from calves in each group at 3rd and 10th days post treatment for bacteriological examination.

Experimental design

A total of 20 calves divided into 2 groups before treatment.

Group1 (Gr1): five animals were clinically healthy and negative for *S. aureus* and *P. multocida* isolation kept as a negative control group.

Group2 (Gr2): fifteen calves were positive for *S. aureus* and *P. multocida* isolation and suffering from pneumonia. Gr2 (diseased calves) sub divided into three equal groups (Gr3, Gr4 and Gr5) (five calves each).

Group3 (Gr3)**:** were treated with Gentaprima® 1ml /25kg b.wt. I/M for 3 days.

Group4 (Gr4)**:** were treated with Gentaprima® with the same dose+ MENTO-Z® (1 ml/8

liter drinking water for 5 successive days).

Gr5**:** were treated with Gentaprima® with the same dose + Levamizole (single S/C injection at dose of 1 mg/50 kg bwt).

Bacteriological examination:

Isolation and identification of *P. multocida*:

All examined swabs were inoculated on 5% sheep blood agar and incubated for 24–48 h at 37 °C. *P. multocida* colonies were identified through the colonial morphology, characteristic bipolarity, and biochemical tests **(Glisson *et al.*, 2008).** Isolates were maintained in Brain Hear Infusion (BHI) broth.

Isolation and identification of *Staphylococcus aureus*:

Swabs were streaked onto the surface of mannitol salt agar and paired barker agar. The inoculated plates were incubated at 37C for 24-48h and examined for the bacterial growth Suspected colonies were sub-cultured, purified and preserved in semisolid nutrient agar for further identification, as well as coagulase test was applied according to **Quinn *et al.* (2011).**

Antimicrobial susceptibility testing:

Antimicrobial susceptibility pattern was done by using the disk diffusion method using different antimicrobial disks: Ampicillin, Gentamycin, Streptomycin, Amoxicillin, Erythromycin, Ciprofloxacin, Chloramphinicol, Amikacin and Trimethoprim-Sulfamethoxazole. The diameters of the inhibition zones were measured and interpreted according to **CLSI (2021).**

Polymerase chain reaction (PCR):

According to **Sambrook *et al*. (1989),** molecular identification of (5) *S. aureus* and (5) *P. multocida* isolates and some of their virulence genes by PCR assay. Specific oligonucleotide primers for certain genes *Kmt*1 and *S. aureus 23S rRN*A as pecies-specific for *P.multocida* and *S. aureus,* respectively. Detection of *tox*A (dermonecrotic toxin) and *ptf*A gens (Type 4 fimbriae) (adherence factor) which consider as virulence genes for *P.multocida*, also *spa* **(**protein A) and *Coa* (coagulase) genes for *S. aureus*.

DNA extraction:

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

Oligonucleotide Primer: The primers used were supplied from **Metabion (Germany)** and listed in table (1).

PCR amplification:

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, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:

PCR products were analyzed by electrophoresis and visualized under a UV transilluminator (Spectroline, USA).

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

Target bacteria	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
S. aureus	*S. aureus 23S rRNA*	ACGGAGTTACAA AGGACGAC AGCTCAGCCTTA ACGAGTAC	1250 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 1 min	72˚C 1.2 min.	72˚C 12 min.	**Bhati *et al.* (2016)**
	spa	TCAACAAAGAAC AACAAAATGC GCTTTCGGTGCT TGAGATTC	226 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Wada *et al.* (2010)**
	Coa	ATAGAGATGCTG GTACAGG GCTTCCGATTGT TCGATGC	Four different types of bands may be detected 350 bp 430 bp 570 bp 630 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Iyer and Kumosani (2011)**
P. multocida	*Kmt1*	ATCCGCTATTTA CCCAGTGG GCTGTAAACGAA CTCGCCAC	460 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**OIE (2012)**
	toxA	CTTAGATGAGCG ACAAGG GAATGCCACACC TCTATAG	864 bp	94˚C 5 min.	94˚C 30 sec.	48˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Tang *et al.* (2009)**
	ptfA	TGTGGAATTCAGCA TTTTAGTGTGTC TCATGAATTCTTATGCGCAAAATCCTGCTGG	488 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	

Blood samples:

10 ml of blood were taken from the calves by jugular vein puncture before treatment, 3 and 10 day post treatment. 1st sample was 1 mL of blood transferred into vacuum ethylene diamine tetra acetic acid (EDTA)-coated tubes for hematological examination, 2nd sample was 2 ml of blood collected in tube containing heparin (50 IU/ml) used for blood gases analysis and cellular immunity tests and the remaining amount of blood was taken without anticoagulant and left to clot for serum isolation. Serum samples were harvested by centrifugation at 3000 rpm for ten min and preserved at −20°C till analysis.

1. The hematological studies:

Blood samples with anticoagulant were subjected for detection of cellular blood constituents (CBC) according to **Feldman *et al.* (2000).**

2. Biochemical studies:

All biochemical parameters were carried out using commercial kits, the used protocol for each parameter was done as recommended by the manufacture manual. Total protein was measured according to **Henry (1964).** Electrophoretic analysis was carried out for determination of serum albumin, alpha, beta and gamma globulins according to the technique described by **Davis (1964).** Serum glucose was determined according to the method described by **Lott and Turner (1975).** The aspartate aminotransferase (AST) was estimated according to **Murray (1984)**. Serum creatinine was estimated according to **Henry (1974)**. Plasma fibrinogen concentrations were measured according to **Becker *et al.*, (1984)**. Serum haptoglobin was determined by ELISA as described by **Makimura and Suzuki (1982).**

3. Cellular immune response:

Phagocytic activity and phagocytic index described by **Goddeeris *et al.* (1986)**:

a. Separation of peripheral blood mononuclear cells:

Peripheral blood mononuclear cells (PBMC) were isolated.

b. Phagocytic Assay:

To assess the cell phagocytic activity, we added 0.25 ml of adjusted viable leukocytes suspension to 0.25 ml heat inactivated *C. albicans* in serial plastic tubes. The tubes were incubated at 37°C for 30 minutes in a humidified Co2 incubator. Subsequently, the tubes were centrifuged at 2500 rpm for 5 minutes and the supernatant was removed with Pasteur pipette leaving a drop into which the sediment was resuspended. Smears were prepared from the deposit, dried in air and stained with Leishman´s stain.

c. Evaluation of phagocytic activity:

Under a light microscope using oil immersion lens, a total number of 100 phagocytic cells were counted randomly in about ten microscopic fields. The number of ingested yeast cells in each individual phagocytes were determined to calculate the phagocytic cell activity in each of the tested group. The phagocytic activity is considered as the percentage of phagocytic cells by microscope field. The phagocytic index is the mean number of *C. albicans,* ingested by one phagocytic cell.

4. Analysis of blood gases:

Blood gases were determined in venous blood samples by an automated blood gas analyzer using respiratory cassettes (Gastat fax) according to the manufacturer instructions. Right after obtaining the fresh blood samples in a syringe with heparin, the syringe sample were attached to calibrated cassettes into the analyzer. The overall time of whole process for the analyzing was finished within 60-70 seconds after blood collection.

5. Statistical analysis:

Statistical analysis was performed using the analysis of variance (ANOVA). Duncan's Multiple Range was used to determine differences among treatments mean at significance level of 0.05. All statistics were run on the computer using the SPSS program **(Kinnear and Gray, 2006)**.

RESULTS:

Bacteriological examination

Results of bacteriological examination revealed that, from 45 nasal swab samples examined from respiratory tracts of calves, 26 (57.8%) and 13(28.9%) of staphylococci and *P. multocida* were isolated respectively. Out of

staphylococci isolates 19(42.2%) were identified as *S.aureus*.

Also bacteriological examination of nasal swabs collected in 3rd day after treatment from groups (Gr3, Gr4 and Gr5) were positive for *S.aureus* and *P. multocida* isolation and swabs collected from control group(Gr1) were negative for *S.aureus* and *P. multocida* isolation. On the other hand all swabs collected in 10th day after treatment from groups (Gr1, Gr3, G4 and Gr5) were negative for *S.aureus* and *P. multocida* isolation.

Antimicrobial susceptibility profiles of the bacterial isolates

The antibiotic susceptibility tests of *S.aureus* (n=19) and *P. multocida* (n=13) isolated from nasal swabs showed that most *S.aureus* were found to be sensitive to ciprofloxacin, amikacin, gentamycin and chloramphenicol. However, resistance was noted against amoxicillin, ampicillin and erythromycin. The antimicrobial susceptibility tests conducted for *P. multocida* also indicated that ciprofloxacin, gentamycin and chloramphenicol were the most effective antibiotics; on the other hand it showed high resistance against ampicillin and erythromycin (Figure 1).

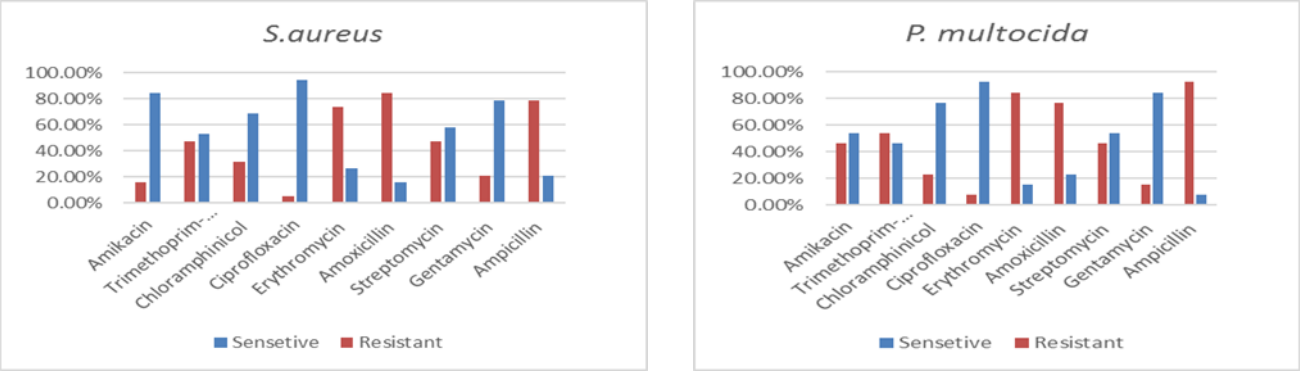

Fig.1: Antimicrobial susceptibility profile of *S.aureus* and *P. multocida* isolates.

PCR characterization of *S.aureus* and *P. multocida* isolates.

All the examined 5 *S.aureus* isolates successfully amplified the *23S rRNA* gene, *spa* gene *and coa* gene with amplicon sizes of 1250bp, 226bp and 570 bp respectively (figure 2-3). On the other hand, from 5 examined *P. multocida* isolates, only 4 isolates were positive for *Kmt*1 gene with amplicon sizes of 460 bp. From these 4 isolates 2 isolates were positive for *tox*A gene with amplicon size 864 bp, while one isolate was positive for *ptf*A gene with amplicon size 488 bp. (figure 4-5).

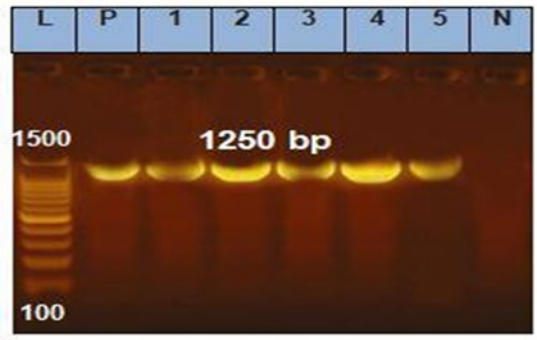

Figure 2: PCR amplicons of *23sRNA* gene of *S.aureus,* Lane L:100-bp ladder; lane N.: negative control; lane P: positive control; lanes 1-5: positive isolates at 1250 bp amplicons.

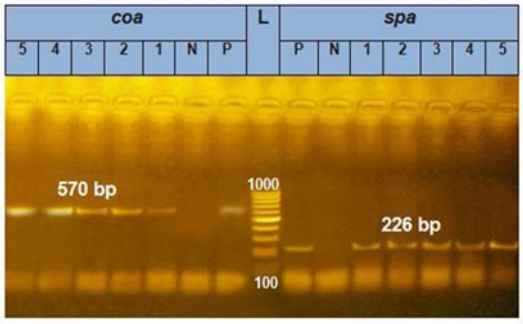

Figure 3: PCR amplicons of *coa* gene and *spa* gene of *S.aureus* , Lane L:100-bp ladder; lane N.: negative control; lane P: positive control; lanes 1-5: positive isolates for *coa* gene at 570 bp amplicons; lanes 1-5: positive isolates for *spa* gene at 226 bp amplicons

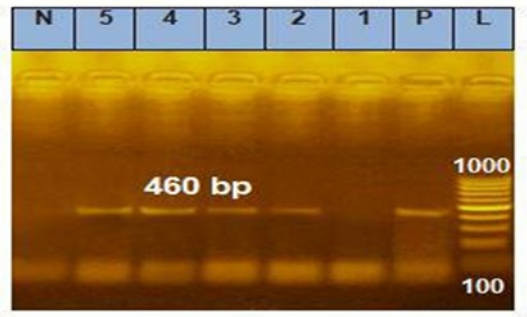

Figure 3: PCR amplicons of *Kmt*1 gene of *P. multocida,* Lane L:100-bp ladder; lane N.: negative control; lane P: positive control; lanes 2-5: positive isolates at 460 bp amplicons

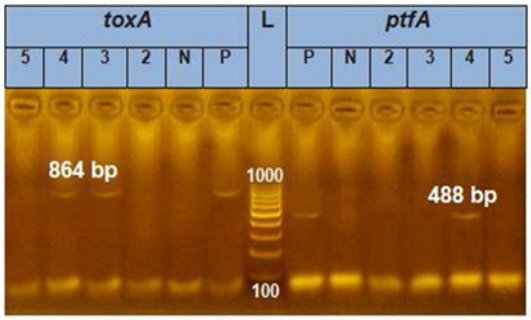

Figure 5: PCR amplicons of *tox*A gene and *ptf*A gene of *P. multocida,* Lane L:100-bp ladder; lane N.: negative control; lane P: positive control; lanes 3 &4: positive isolates for *tox*A gene at 864 bp amplicons; lane 4: positive isolates for *ptf*A gene at 488 bp amplicons.

Hematological study:

Concerning to RBCs count, Hb concentration and PCV% revealed significant decreases in diseased calves in compared with control group, while, 3rd and 10th days after treatment showed significant increase compared with diseased group (G2). Moreover, the leukogram of diseased calves revealed significant increase in TLC, neutrophil, monocyte and eosinophil but lymphocyte count showed a significant decreased compared to control group. TLC, neutrophil monocyte and eosinophil showed significant decrease in all treated groups compared with diseased non treated group, lymphocyte count showed significant increase in Gr4 and Gr5 at 3rd post treatment in comparison with diseased group (Table 2).

Blood gases results:

Diseased calves showed significant decrease in PH, PO_2 and SO_2% while there were significant increase in PCO_2, tCO_2 and HCO_3 compared to control animals. At 3rd and 10th days post treatments PH, PO_2 and SO_2% recorded significant increase and PCO_2, tCO_2 and HCO_3 showed significant decrease in comparison with diseased group before treatment (Table 3).

Immunological and some inflammatory markers results:

In regard to phagocytic index and percentage showed significant decrease in diseased calves compared with control, meanwhile there were significant increase in phagocytic index and percentage in Gr4 and Gr5 at 3rd and 10th days post treatment in compared with diseased group

(Table 4).
Before treatment serum haptoglobin and fibrinogen revealed a significant increase in Gr2 (diseased one) compared with control one (Gr1). At 3 days post treatment there were a significant decrease in serum haptoglobin and fibrinogen in treated groups compared with Gr2. At 10 days post treatment there were non-significant changes in serum haptoglobin and fibrinogen in Gr4 and Gr5 with marked higher value for haptoglobin in Gr3 compared with control one (Table 4).

Protein electrophoresis results:
The results of Protein electrophoresis presented in table (5) before treatment serum protein electrophoretic fractions revealed a significant increase in total protein and total globulin as well as alpha, beta, and gamma globulin compared to control animals. In addition to a significant reduction in serum albumin in Gr2 (diseased one) compared with Gr1 (control group). At 3 days post treatment there was a non-significant increase in total protein and total globulin in treated groups (Gr3, Gr4 and Gr5) compared with Gr2. Concentration of alpha globulin showed non-significant decrease in the same groups compared with Gr2. Serum gamma concentration showed significant decrease in the mentioned groups compared with Gr2. At 10 days post treatment there was a significant decrease in serum total protein and total globulin in Gr3, Gr4 and Gr5 compared with Gr2. On the other hand there was a significant increase in serum albumin and alpha globulin at the same groups compared with Gr2 in addition to significant increase in gamma globulin in Gr5 compared with healthy one.

Biochemical results:
Before treatment serum glucose, AST and creatinine showed a significant increase in Gr2 compared with Gr1. At 3 days post treatment serum glucose showed significant decrease in treated groups (Gr3, Gr4 and Gr5) with non-significant changes in serum AST activity compared with Gr2. Serum creatinine revealed non-significant changes in treated groups compared with Gr1 (control one). At 10 days post treatment serum AST showed significant decrease in treated group compared with Gr2. No significant differences were seen in serum glucose and creatinine in Gr3, Gr4 and Gr5 compared with control one (Table 6).

Table (2): Erythrogram and leukogram of healthy and diseased calves before, 3 and 10 days post treatments.

Groups parameters	Before treatments and at 3 days post treatments					At 10 days post treatments				
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr1	Gr2	Gr3	Gr4	Gr5
RBCsx10^6 /µl	8.97^{a} ±0.14	6.71^{c} ±0.66	7.90^{b} ±0.93	7.98^{b} ±0.80	8.01^{b} ±0.67	9.01^{a} ±0.25	6.71^{c} ±0.66	7.12^{b} ±0.27	7.14^{b} ±0.44	7.31^{b} ±0.36
Hb (gm/dl)	11.89^{a} ±0.21	8.68^{c} ±0.27	9.86^{b} ±0.34	9.88^{b} ±0.34	10.31^{b} ±0.41	12.05^{a} ±0.93	8.68^{d} ±0.27	9.07^{c} ±0.93	9.11^{c} ±0.90	10.03^{b} ±0.84
PCV %	39.98^{a} ±0.25	26.97^{c}± 0.69	32.91^{b} ±0.37	33.65^{b} ±0.94	34.03^{b} ±0.46	40.85^{a} ±1.20	26.97^{d} ±0.69	35.02^{c}± 0.92	35.62^{bc}± 1.42	35.82^{b} ±1.22
TLCx10^3 /µl	8.54^{c} ±0.45	13.32^{a} ±0.29	11.21^{b} ±0.36	11.20^{b} ±0.42	12.30^{b} ±0.31	9.24^{c} ±0.20	13.32^{a} ±0.29	10.53^{b}± 0.23	9.66^{c} ±0.24	9.58^{c} ±0.18
Neutrophilsx10^3 /µl	4.24^{b} ±0.32	7.10^{a} ±0.25	5.00^{b} ±0.33	4.48^{b} ±0.28	5.65^{b} ±0.20	3.63^{b} ±0.15	7.10^{a} ±0.25	4.62^{b} ±0.17	4.12^{b} ±0.20	4.07^{b} ±0.20
Lymphocytesx10^3 /µl	3.69^{c} ±0.15	4.84^{b} ±0.20	5.08^{b} ±0.25	5.67^{a} ±0.19	5.82^{a} ±0.22	4.88 ±0.18	4.84 ±0.20	4.93 ±0.15	4.65 ±0.18	4.48 ±0.15
Monocytesx10^3 /µl	0.36^{c} ±0.02	0.86^{a} ±0.03	0.88^{a} ±0.03	0.65^{b} ±0.02	0.61^{b} ±0.04	0.45^{c} ±0.03	0.86^{a} ±0.03	0.58^{b} ±0.03	0.50^{c} ±0.02	0.55^{b} ±0.04
Eosinophilsx10^3 /µl	0.25^{c} ±0.03	0.52^{a} ±0.02	0.35^{b} ±0.01	0.20^{c} ±0.02	0.22^{c} ±0.01	0.28^{d} ±0.02	0.52^{a} ±0.02	0.40^{c} ±0.01	0.39^{c} ±0.02	0.48^{b} ±0.01

Different letters at the same column means that there was a significant change at $p<0.05$

Table (3): Blood gases of healthy and diseased calves before, 3 and 10 days post treatments:

Groups parame-ters	Before treatments and at 3 days post treatments					At 10 days post treatments				
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr1	Gr2	Gr3	Gr4	Gr5
PH	7.45^{a} ±0.89	7.10^{b} ±0.57	7.29^{a} ±0.47	7.32^{a} ±0.31	7.41^{a} ±0.37	7.65^{a} ±0.86	7.10^{c} ±0.57	7.31^{b} ±0.28	7.45^{b} ±0.35	7.67^{a} ±1.15
PCO_2 Mmhg	40.54^{c} ±0.36	55.17^{a} ±0.59	49.35^{b} ±0.61	49.33^{b} ±0.88	40.76^{c} ±0.32	41.68^{c} ±1.41	55.17^{a} ±0.59	49.85^{b} ±1.70	49.04^{b} ±1.37	41.50^{c} ±1.16
PO_2 Mmhg	32.78^{a} ±0.12	25.99^{c} ±0.31	31.15^{b} ±0.20	31.10^{b} ±0.58	32.70^{a} ±0.27	32.75^{a} ±1.32	25.99^{c} ±0.31	31.05^{b} ±1.57	31.31^{b} ±1.02	33.32^{a} ±1.26
tCO_2 mmhg	27.62^{c} ±0.32	32.42^{a} ±0.31	30.03^{b} ±0.36	30.17^{b} ±0.32	27.39^{c} ±0.40	30.56^{c} ±0.62	32.42^{a} ±0.31	31.38^{b} ±0.91	31.35^{b} ±0.58	30.22^{c} ±0.47
HCO_3 Mmhg	27.45^{c} ±0.40	29.78^{a} ±0.70	28.62^{b} ±0.54	28.36^{b} ±0.55	27.73^{bc} ±0.38	27.55^{b} ±0.98	29.78^{a} ±0.70	28.58^{b} ±0.74	28.46^{b} ±0.82	28.11^{b} ±0.96
SO_2%	66.62^{a} ±0.32	59.62^{c} ±0.31	60.03^{bc} ±0.45	60.85^{b} ±0.60	65.73^{a} ±0.37	67.82^{a} ±1.26	59.62^{c}± 0.31	66.69^{b} ±1.06	66.75^{b} ±1.07	67.56^{ab} ±1.30

Table (4): Cellular immunity and some inflammatory markers of healthy and diseased calves before, 3 and 10 days post treatments:

Groups parameters	Before treatments and at 3 days post treatments					At 10 days post treatments				
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr1	Gr2	Gr3	Gr4	Gr5
Phagocytic index	4.92^{a} ±0.18	3.32^{c} ±0.14	3.45^{c} ±0.17	4.15^{b} ±0.17	4.60^{a} ±0.19	5.00^{a} ±0.18	3.32^{c} ±0.14	3.55^{c} ±0.23	4.52^{b} ±0.35	4.78^{a} ±0.28
Phagocytic %	75.25^{a} ±2.11	60.30^{c} ±1.58	62.40^{c}± 1.90	68.15^{b} ±1.51	70.00^{b} ±2.04	75.00^{a} ±2.15	60.30^{c} ±1.58	63.75^{c} ±1.95	70.50^{a} ±1.80	72.20^{a} ±2.06
Haptoglobin mg/dl	2.00d±0. 19	9.5a ±0.49	6.70b± 1.60	4.80bc ±0.45	3.20cd ±0.38	1.83c ±0.14	9.50a± 0.49	5.40b ±1.3	3.7bc ±0.20	2.9c± 0.48
Fibrinogen mg/dl	345.8b ±42	613.7a ±57	402.4b ±53	311.8b ±11	406.9b ±43	346.33b ±41.2	613.7a ±57	392b± 48.7	306b± 12.4	381b± 34.6

Table (5): Protein electrophoresis of healthy and diseased calves before, 3 and 10 days post treatments:

Groups Parameters	Before and 3 days post treatments					At 10 days post treatments				
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr1	Gr2	Gr3	Gr4	Gr5
Total protein g/dl	6.94^{b} ±0.2	7.91^{a} ±0.1	7.98^{a} ±0.1	7.97^{a} ±0.1	8.00^{a} ±0.2	6.80^{c} ±0.02	7.91^{a} ±0.1	7.2^{b} ±0.1	7.1^{b} ±0.05	7.4^{b} ±0.05
Albumin g/dl	3.75^{a} ±0.1	2.82^{c} ±0.04	3.06^{bc} ±0.08	3.02^{bc} ±0.1	3.14^{b} ±0.02	3.8^{a} ±0.02	2.82^{c} ±0.04	3.50^{a} ±0.01	3.64^{a} ±0.03	3.62^{a} ±0.06
Total globulin g/dl	3.17^{b} ±0.1	5.08^{a} ±0.07	4.92^{a} ±0.07	4.93^{a} ±0.05	4.85^{a} ±0.10	3.18^{d} ±0.02	5.08^{a} ±0.07	3.62^{c} ±0.08	3.55^{c} ±0.01	3.78^{b}± 0.02
α-Globulin g/dl	0.85^{b} ±0.05	0.97^{a}± 0.02	0.92^{ab} ±0.01	0.90^{ab} ±0.02	0.90^{ab} ±0.01	0.93^{b}± 0.01	0.97^{a}± 0.02	1.08^{a}± 0.04	1.07^{a}± 0.03	1.09^{a}± 0.02
β-Globulin g/dl	0.92^{d} ±0.03	1.23^{c} ±0.06	1.37^{b} ±0.02	1.54^{a} ±0.03	1.58^{a}± 0.02	1.04^{b}±0. 05	1.23^{c} ±0.06	1.09^{ab}± 0.04	1.17^{ab}± 0.02	1.19^{a}± 0.03
γ-Globulin g/dl	1.39^{d} ±0.09	2.86^{a}± 0.03	2.62^{b} ±0.04	2.47^{bc} ±0.06	2.38^{c}± 0.09	1.19^{d}±0. 06	2.86^{a}± 0.03	1.44^{bc}± 0.04	1.31^{cd}± 0.04	1.49^{b}± 0.03

Different letters at the same column means that there was a significant change at $p<0.05$

Table (6): Some biochemical parameters of healthy and diseased calves before, 3 and 10 days post treatments.

Groups parameters	Before and 3 days post treatments					At 10 days post treatments				
	Gr1	Gr2	Gr3	Gr4	Gr5	Gr1	Gr2	Gr3	Gr4	Gr5
Glucose mg/dl	78.66^{c} ±10.7	183.6^{a} ±8.5	116^{b} ±9.4	104^{bc} ±3	96.33^{b} ^{c}±3.7	81.6^{b} ±13.7	183.6^{a} ±8.5	105^{b} ±2.9	104.6^{b} ±5.1	97^{b} ±3.5
AST IU/L	31.90^{b} ±5.4	80.16^{a} ±7	67.6^{a}± 8.7	60.8^{a} ±6	69.7^{a} ±6.9	32.86^{c} ±4.5	80.16^{a} ±7	60.6^{b} ±5.2	55.2^{b}± 2.8	61.8^{b} ±5.9
Creatinine mg/dl	0.81^{b} ±.014	3.03^{a}± 0.48	1.86^{b}±. 046	1.30^{b}± 0.2	1.60^{b} ±0.4	0.87^{b} ±0.17	3.03^{a} ±0.48	1.3^{b} ±0.2	1.16^{b} ±0.20	1.2^{b}± 0.35

Different letters at the same column means that there was a significant change at $p<0.05$

DISCUSSION

Respiratory disorders are still serious problem facing animals industry. The importance of respiratory diseases of calves depends on their prevalence, their effect on productivity, the value of the animal and for some diseases, their international spread (**Ali *et al.,* 2009**).

In our study, results of bacteriological examination of nasal swabs collected from 45 calves with respiratory manifestations revealed that 19 (42.2%) and 13(28.9%) of *S.aureus* and *P. multocida* were isolated respectively, which agreed with that obtained by **Algammal *et al.* (2020),** also incidence of *P. multocida* was nearly coincident with results reported by **El-Jakee *et al.* (2016)** (32.7%). Moreover **Hashem *et al.* (2022)** added that *P. multocida*, and *S. aureus* were the most common bacterial pathogens isolated from calves suffering from respiratory manifestations in Menoufiya Governorate. These variation in isolation percentage may be attributed to change in hygienic measure, stress factors, change in management and immune status of infected animals which allow the proliferation of commensal bacteria in the respiratory tract **(Lindsey and Sonia, 2016).**

Concerning to the antimicrobial sensitivity test in-vitro, results were showed that, *P. multocida* isolates were sensitive to Ciprofloxacin and Gentamycin, while showing high resistance against Ampicillin and Erythromycin. Results of Antibiogram of *P. multocia* agreed with results obtained by **Akalu *et al.* (2022)**. Also, **Algammal *et al.* (2020)** illustrated that *P. multocia* isolates were sensitive to gentamicin. With respective to *S.aureus*, isolates were highly sensitive to Ciprofloxacin, Amikacin and Gentamycin, but highly resistance to Amoxacillin, Ampicillin and Erythromycin, similar findings were previously reported by **Ezzeldeen *et al.* (2014)**. Both *P. multocia* and *S.aureus* isolates showed resistance against many antibiotics including Ampicillin, Amoxacillin, Erythromycin and Trimethoprim-Sulfamethoxazole, which is proved by previous studies **(Catry *et al.,* 2016 and Algammal *et al.,* 2020).** This difference in antibiogram pattern might be due to their indiscriminate use of antibiotics in the treatment and prevention of bacterial infections which may result in to acquired drug resistance. Thus, monitoring of antimicrobial susceptibility trends of BRD complex pathogens is an important aid to Veterinarians in selecting the most efficacious and cost-effective therapeutic agents as that reported by **Mohammadi *et al.* (2006).**

According to *in vitro* antimicrobial sensitivity results gentamycin were the drug of choice used in treatment trails in our study. These finding corroborated earlier report that bacterial isolates from nasal swabs of BRD complex affected buffaloes showed high effectiveness of gentamycin **Kumar *et al.* (2015)**.

In our study, we confirmed the diagnosis of isolated bacterial species by PCR, using unique primer *kmt*1 universal gene and both *tox*A and *ptf*A virulence-associated genes in case of *P. multocida* which play important roles in the pathogenesis of BRD. These virulence genes is responsible for the clinical symptoms associated with pneumonia as that was clarified by **Akalu *et al.* (2022)**.

With concerning to *S. aureus*, all 5 investigated isolates were positive for the gene segment encoding a *S. aureus*-specific part of the *23S rRNA*, as well as *coa* gene (coagulase) and *spa* gene (Staphylococcal protein A**).** Similarly *S. aureus* isolates were confirmed genotypically by *23S rRNA* by **Khichar *et al.* (2014).** Previous study of **Bien *et al.* (2011)** explained the central importance of SpA in the pathogenesis of *S. aureus*-induced pneumonia and its absence reduces pneumonia incidents and associated mortality in a mice model of infection.

The primary indicators of bovine health are haematological and biochemical findings, which become more expressive when compared to the history, clinical symptoms, and ultr -asonographic examination of cows with respiratory disorders **(El-zahar *et al.,* 2021)**.

Hematological analysis of BRD infected animals revealed significant reduction in RBCs count, hemoglobin concentration and PCV%. Similar findings were recorded by **Faris and Abd El-Hamied (2007)**. The prevalence of anemia might be attributed to endotoxins secreted from bacteria that resulted in intravascular destruction of erythrocytic cells in the body and lead to hemolysis and Hb breakdown **(Karaivanov, 1984)**.

Treated groups at 3rd and 10th day resulted in improvement of the blood erythrogram compared to diseased calves. MENTO-Z® contains menthol (peppermint oil), cynnamaldelyde (cinnamon oil), 1.8-cineole (cajeput oil), alpha pinen (pine oil), thymol and carvacrol. Studies have shown that peppermint oil can synergize with other agents to produce synergistic antibacterial effects against Gram-positive and Gram-negative microorganisms (**Al-Mariri *et al*., 2012)**. When peppermint oil is used in combination with gentamicin, the dosage of gentamicin required to kill bacterial strains is significantly reduced **(Rosato *et al.,* 2018).**

Leukogram of diseased calves revealed significant increase in TLC, neutrophil, monocyte and eosinophil but lymphocyte count showed a significant decreased compared to control group. The bacterial infection leads to inflammation and suppuration, therefore leukogram response to the inflammatory disease **(Coles, 1986)**. These results agree with **Faris and Abd El-Hamied (2007)** and **Eleiwa *et al.* (2014)**. Neutrophil provide the first line of defense against pathogen may lead to neutrophilia **(Janeway *et al.,* 2005).** TLC, neutrophil, monocyte and eosinophil showed significant decrease in all treated groups compared with diseased non treated group, lymphocyte count showed significant increase in Gr4 and Gr5 at 3rd post treatment in comparison with diseased group. Cinnamaldehyde (CA) is a bioactive compound that has been identified to have antibacterial **(Ali *et al.,* 2005),** anti-inflammatory **(Reddy *et al.,* 2004)**, hypoglycemic **(Khan *et al*., 2003).** Peppermint essential oil **(**PEO) exhibits inhibitory effects on pathogenic bacteria *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* (**Metin *et al.*, 2021)**. It is noted that levamisole has a hormone-like effect due to the increase in the amount of intracellular cGMP and serum factors responsible for leukocyte activation in lymphocytes exposed to levamisole **(Kızıltepe, 2018)**.

Concerning to venous blood gases, diseased calves showed significant decrease in PH, PO_2 and SO_2% while there were significant increase in PCO_2, tCO_2 and HCO_3 compared to control animals this reported by **Cambier *et al.* (2002)** said that, blood oxygen levels binding in hypoxic calves PO2 were investigated they showed that diseased animals exhibited a significant acidosis in arterial and venous blood. Furthermore, in hypoxemic calves, PCO2 higher than healthy animal, at the same time, diseased animals exhibited lower SO2 values than healthy ones. The lung helps regulate pH in the body by exhaling carbon dioxide and in severe acidosis; there is rapid respiration in an effort to exhale more CO2 in an effort to correct the imbalance **(Montgomery, 2009)**. At 3rd and 10th days post treatments PH, PO_2 and SO_2% recorded significant increase and PCO_2, tCO_2 and HCO_3 showed significant decrease in comparison with diseased group before treatment, our results are consistent with **Lekeux (1996)** who reported that acute inflammatory component of pneumonia results in impaired gas exchange and the aim of modulating pulmonary inflammation by the use of NSAIDs are to

block the production and/or the effects of inflammatory mediators and modulators which have a deleterious effect on alveolar exchange of gases. It has become a widely held view that PEO harbors multiple pharmacological effects, such as protection of gastrointestinal, lead to improvement and correlation of PCO_2 and SO_2 **(Zhao *et al.,* 2022)**.

Our study documented significant decreases phagocytic index and percentage showed in diseased calves compared with control. These results are consistent with **Carroll and Forsberg (2007**). The reduction in lymphocyte subsets is most likely attributable to a redistribution of these cells from the peripheral circulation to immune compartments or tissues of greater importance during a stressful event. Trafficking of cells is an important and dynamic factor for effective cell mediated immunity and stress has been shown to influence this process **Viswanthan and Dhabhar (2005)**. In our study there were significant increase in phagocytic index and percentage in Gr4 and Gr5 at 3^{rd} and 10^{th} days post treatment in compared with diseased group. Phagocytes, especially macrophages, are the first-line effector of innate immune system, by eliminating pathogenic microorganisms that invade the host. Activation of macrophages is associated with the recognition of pathogen-associated molecular pattern (PAMP). In a study in vitro, PEO is found to modulate immune activity through phagocytosis **(Lang, *et al.,* 2019)**. Also, PEO can inhibit airway epithelial hyperplasia, collagen deposition and goblet cell activation in asthmatic mice, by decreasing IL-6 level via regulation of phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) **(Kim *et al.,* 2020).** levamisole indirectly stimulates the humoral response by sensitizing phagocytes to antigens and increasing the number of T lymphocytes, levamisole is used to support the immune system in vaccines and infections in humans and animals (**Doğan, 2022)**.

Concerning serum haptoglobin and fibrinogen showed a significant increase in Gr2 (diseased one) compared with control one. Similar result obtained by **Csilla *et al.* (2013**). Also **Faris *et al.* (2010)** recorded a highly significant increase in both serum haptoglobin and fibrinogen in diseased calves before treatment. Haptoglobin is used to guard against the damaging consequences of the inflammatory response on tissue **(Lakritz, 2010).** Additionally, fibrinogen levels rise following BRD pathogen infection **(Ganheim *et al.,* 2003).**

The treated calves showed a significant decrease in serum haptoglobin and fibrinogen in treated groups compared with Gr2. Prior to therapy, serum haptoglobin levels in sick calves were considerably higher than those of healthy calves, and they were significantly lower in all treated calves **(Eleiwa *et al.,* 2014).** In addition to having anti-inflammatory and analgesic properties, peppermint oil suppresses carbachol -induced muscle contraction involving autonomic ganglia, which is particularly beneficial in respiratory diseases **(De Sousa *et al.,* 2010).** Menthol can reduce inflammation and attenuate oxidative stress **(Kim *et al.,* 2021)**. Peppermint oil is now included in animal diets in the agricultural sector to have antimicrobial and anti-inflammatory benefits **(Hejna *et al.,* 2021).**

The proteinogram revealed significant increase in total protein and significant decrease in albumin. Similar results obtained by **Csilla *et al.* (2013**). The protein electrophoresis of diseased calves revealed a significant increase in total globulin as well as alpha, beta, and gamma globulin. A high quantity of total protein in the blood is typically related to inflammatory processes, when the production of immunoglobulins and acute phase proteins increases **(Evans, 2003).** Also added that hyperglobulinemia is usually related to infection and inflammation, because of higher synthesis of acute phase proteins, complement proteins and immunoglobulins. Plasma albumin concentration decreases in case of infection and inflammation **(Eckersall and Bell, 2010)**. Our result agrees with **(Kumar *et al.,* 2018).**

Tothova *et al.* (2012) recorded that when healthy and sick animals' serum protein fractions were compared, the calves with respiratory tract disorders had significantly larger quantities of α1-globulins. The same authors added that higher quantities of α1-globulins may be related to chronic infections as well as acute inflammatory disorders. The most prevalent pattern in animals with inflammatory illnesses is decreased albumin and increased globulin

concentrations, reflecting the compensatory decrease in albumin concentrations to maintain oncotic pressure and viscosity **(Stockholm and Scott, 2008)**.

The treated groups demonstrated a significant decrease in serum total protein and total globulin in Gr3, Gr4 and Gr5 at 10 days post treatment compared with Gr2 in addition to significant increase in gamma globulin in Gr5 compared with healthy one. **Soltan (2009)** investigated that the calves' immune systems were stimulated positively by essential oils. Also levamisole has a reputation for boosting immunological response, especially in immune -compromised states (**Oladele *et al.,* 2012**).

The results showed significant increase in serum glucose, AST and creatinine in diseased calves. Hyperglycemia observed in diseased calves might have been caused by Animals' responses to stress and fasting, which are probably related to factors such as adrenal gland activity, rate of glycogenolysis, rate of lipolysis, or both, quantity and source of nutrients being absorbed from the gastrointestinal tract, and rate of tissue utilization of nutrients **(Kumar *et al.,* 2018)**. Increased activity of AST in diseased calves during illness episodes might result from the increase in the respiration rate and increased movement of the intercostal muscles described by **(El-zahar *et al.,* 2021)**. Meanwhile the increase in serum creatinine is due to kidney dysfunction after infection **(Constable *et al.,* 2017).** The same results for serum AST and creatinine were obtained by **Anwar *et al.* (2019).** Also elevation of AST was observed by **Kumar *et al.* (2018)** in buffaloes suffering from respiratory disease.

In treated groups there were Improvement in the serum glucose and AST at 10 days thus may be due to the role of antibiotic (gentamicin) in controlling infection in diseased calves Gr3. For the treatment of severe respiratory tract infections, such as hemorrhagic septicaemia linked to Mannheimia haemolytica and P. multocida in buffaloes, cattle, goats, pigs, sheep, and rabbits, gentamicin, a broad spectrum aminoglycoside, is frequently employed. It also has the benefit of having a high clinical effectiveness for severe infections **(Sidhu *et al.,* 2014).** In addition to the active ingredient in MENTO-Z® like menthol (peppermint oil), it has cynnamaldelyde (cinnamon oil), and thymol which play role in decreasing serum glucose and AST as in Gr4. Cinnamaldehyde has hypoglycemic effect **(Khan *et al*., 2003).** It has been demonstrated that peppermint oil lowers blood glucose, raises insulin and C-peptide levels, and enhances the structure of pancreatic beta cells **(Abdellatief *et al.,* 2017).** By improving the plasma membrane's integrity and boosting the ability of hepatic cells to repair and regenerate through antioxidative stress, peppermint oil exhibits liver protection **(Bellassoued *et al.,* 2018).** Gr5 administered levamisole and gentamicin revealed significant improvement in the mentioned parameters. Levamisole functions as immune stimulants and immunological potentiators, enhancing the immune response **(Farrah *et al.*, 2017)**.

Conclusion

It could be concluded that, *Staphylococcus aureus* and *Pasteurella multocida* appeared to be the main cause of respiratory manifestations in calves and the use of gentamicin plus MENTO-Z® and gentamicin plus levamizole® in calves which suffered from BRD improved hematological and biochemical parameters in addition to enhance the animal immunity against pathogenic bacteria instead of antibiotic alone.

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