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Some bacteriological, molecular and biochemical investigation on horses infected with respiratory system bacteria

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Abstract

Respiratory diseases in equines are considered to be a dangerous problem faced equine breeding; these diseases caused severe losses in the diseased equine as well as economic losses from treatment coasts. In this study, 100 nasal swabs were collected from horses with respiratory symptoms and examined. Blood samples were collected from the diseased horses and apparently healthy animals (of both sexes) for biochemical examinations.

Results of the bacteriological examination revealed the isolation of *Staph aureus, Pseudomonase aeroginosa, Strept equi, Klebsiella pneumoniae, E. coli, Pasteurella spp.* and *Coryne spp.* in prevalence rate of 53.3%, 10.7%, 9.3%, 8 %, 6.7%, 6.7% and 5.3% respectivily. Serology of *E.coli* isolates revealed that the 5 tested isolates were belonged to the serogroup O86a, O125, O125, O86a and O125 respectivily. Application of antibiotic sensitivity test of the isolated bacterial species recorded that *Staph. aureus* more sensitive for penicillin G, vancomycine and cefuroxime sodium. *Pseudomonase aeroginosa* more sensitive for kanamycin, ofloxacin. *Strept.equi* more sensitive for penicilline G, cephradine and amoxycillin. *Klebsiella pneumoniae* resist to all used antibiotic discs. *Pasteurella spp*. were more sensitive for erythromycin, cephalexine and doxycycline. *E. coli* more sensitive for ofloxacin, amikacin and amoxycillin. *Coryne.spp.* more sensitive for kanamycin, doxycycline and cephalexine. Virulence genes and antibiotic resistant genes of *Klebsiella pneumoniae*, *Staph aureus* and *Pseudomonase aeroginosa* were detected by PCR showed that the *Staph.*, *aureus* isolates harboured *spa, coa, hlb, hlg* and *hla* virulence genes by amplification at 226bp., 360 bp., 496bp., 937bp. respectively but. *hla* negative. While *Klebsiella pneumoniae* isolates harboured *mag*A virulence genes by amplification at 1282 bp. and *bla*SHV, *bla*TEM antibiotic resistance genes by amplification at at 392 bp., 516 bp. *Pseudomonase aeruginosa* isolates harboured *bla*SHV, *bla*TEM and *bla*CTX antibiotic resistance genes by amplification at 392 bp., 516 bp. and 593 bp. respectively.

Serum biochemical examinations revealed significant increase in the total protein and globulin in diseased horses due to *staph aureus* infection alone and mixed infection in comparison to the apparently healthy animals ($p<0.05$). Also, serum glucose and creatinine showed significant increase ($p<0.05$). On the other hand, no significant changes in ALT and AST values. Significant decrease in iron and zinc in both sex of diseased horses, meanwhile copper showed slightly elevation. The results showed that biochemical parameter alterations may help in choice the proper treatment of the diseased horses.

Keywords: *Respiratory infection, bacterial causes, horses, serum biochemical changes.*

Introduction

The equine respiratory tract is a highly specialized system facilitating the movement of large volumes of air in and out of the lungs each minute. Any structural or functional disorder associated with the lungs can lead to exercise intolerance and poor performance. Respiratory diseases are the result of exposure to dust and other airborne irritants. Besides; bacteria, mold and ammonia levels. Excess nasal discharge, coughing, sneezing and mucus secretion are all signs that a horse may be suffering from respiratory problems (**Sheta and Ashour, 2017).**

Equine infectious upper respiratory disease (IURD) is a problem that affects horses worldwide. Infected horses often develop a fever, cough, and nasal discharge. They also may develop swollen lymph nodes, lethargy, and decreased appetite. Usually, horses recover from IURD without suffering long-term complications **(Vet. Services, 2001).** *Strept. equi* is a common cause of respiratory manifestation in equines **(Neamat-Allah and El-Damaty, 2016)** *Staphylococcus spp.; Pasteurella spp.* and *E coli* are common agents of respiratory diseases in animals suffering from respiratory manifestation (**Abd El-Moez *et al.*, 2013).**

Secondary bacterial respiratory infections are primarily initiated by viral disease, because viral respiratory infections impair and/or destroy respiratory defense mechanisms (ie, influenza destroys the mucociliary apparatus, EHV destroys bronchial-associated lymphoid tissue). The most common organisms associated with pneumonia in horses are opportunistic bacteria originating from the resident microflora of the upper respiratory tract. Clinical evidence of a secondary bacterial infection includes mucopurulent nasal discharge, depression, persistent fever, abnormal lung sounds, hyperfibrinogenemia, and leukocytosis. Secondary bacterial disease may result in mucosal bacterial infections (rhinitis and tracheitis) or may produce more serious invasive disease such as pneumonia and pleuropneumonia. *Streptococcus equi zooepidemicus* is the most common opportunistic pathogen of the equine lung, although *Actinobacillus equuli*, Bordetella bronchiseptica, *Escherichia coli, Pasteurella spp*, and *Pseudomonas aeruginosa* are frequently isolated. *S. equi equi,* the causative agent of strangles which is a primary bacterial pathogen of the upper respiratory tract and is capable of mucosal invasion without predisposing factors. *Rhodococcus equi* is a primary pathogen of the lower respiratory tract of foals and produces pulmonary consolidation and abscessation. *R. equi* pneumonia has been reported in adult horses with a compromised immune system. **(Bonnie, 2016).**

Most respiratory diseases in horses are originated from lesions located in the respiratory airways and lung**.** The major causes of these respiratory diseases are microbial organisms. Foreign objects inhaled during feeding and oral drenching of horses with traditional medicines are predisposing factors for respiratory diseases**.** The upper airway of healthy horses contains many bacterial flora including *streptococcus equi subsp. zooepidemicus, Pasteurella spp., E. coli, Actinomyces spp.* and *streptococcus spp.* **(Debelu *et al.*, 2014)**

Pleuropneumonia is common in horses. Risk factors for development of pleuropneumonia include long-distance transportation, prolonged head elevation, exercise, general anesthesia, and viral respiratory infections that lead to decreased mucociliary clearance and immune suppression. The most common bacterium isolated from horses with pleuropneumonia is *Streptococcus spp.,* other commonly cultured aerobes include *Pasteurella spp., Escherichia coli, Klebsiella pneumoniae* **(Arroyo *et al.*, 2017).**

Respiratory disease is common in racehorses in training, Clinical presentation of inflammatory airway disease (IAD) includes mild signs of respiratory disease such as cough and increased secretions in the trachea and poor performance in racing and training, inflammatory

airway disease has a multifactorial etiology viral, bacterial and environmental components, there is considerable interest in the association between respiratory disease and different bacteria**. (Wood *et al*., 2005).**

Airway infections are an important cause of economic loss for the horse breeding industry. They are responsible for deaths mainly in young foals, and cause hard-to-treat conditions in horses of various ages. Both acute and chronic infections can result in impaired pulmonary function and lead to reduced performance **(Bernard *et al*., 1991).** Respiratory infections in horses are a worldwide phenomenon, and have been the cause of an increasing number of clinical and therapeutic problems.

Metals are importance for both of host and microbe. In bacteria, fungi and humans, maintenance of adequate intracellular concentrations of trace metal ions is essential. Trace elements (Fe, Mn, Cu, Se, Zn) are important cofactors in many enzymes. These metalloproteins are crucial for many cellular functions, including respiration, replication, transcription, translation, signal transduction and cell division, and they are often involved in regulation of bacterial virulence**.** Dietary iron supplementation in areas with a high burden of infectious diseases results in increased morbidity and mortality from infectious diseases, including invasive bacterial infections, gastrointestinal infections and malaria. Zn deficiency can result in a significant increase in the incidence of diarrhea and upper respiratory tract infections, as well as morbidity and mortality from these infections **(Weiss and Carver, 2018)**. Bacteria and yeast are averse to copper and actively export virtually all that is taken up by the cell, as excess levels are toxic (**Solioz, 2016).** Therefore, determination of trace elements profile is important to gain the necessary information concerning animal's health. Moreover, determination of the increase or decrease in these elements will be useful in the diagnosis and treatment **(Shawaf *et al.*, 2017).**

The aim of the present study is the detection of the bacterial causes of respiratory affection in horse, detection of the virulence genes of the isolates as (*Staph aureus* and *Klebsiella pneumoniae.)* and antibiotic resistance genes of the isolates as (*Klebsiella pneumoniae* and *Pseudomonase aeruginosa*) detection of antibiotic sensitivity of the isolates as well as to throw the light on the biochemical alterations associated with this bacterial infection in horses.

Materials and Methods

1) Samples:

A total of 100 nasal swabs were collected aseptically using sterile swab from horse suffering from respiratory affections. Each swab sample was kept in transport media (Amies, Oxoid-CM425). All samples were kept in an ice box and send to the laboratory without delay for bacteriological examination (nasal swab).

The blood samples were drawn from the jugular vein of the diseased and apparently healthy animals (of the same age and sex) into sterilized vacutainer blood collection tubes. Centrifugation at 3000×rpm for 10 min is used to separate the serum, and it was collected in a sterile vial to be preserved at −20°C for serum biochemical examinations.

2) Bacteriological examination:

Each swab sample was inoculated into nutrient broth and incubated aerobically at 37^{o}C for 24 hr. A loopful from each broth was streaked onto the following media: blood agar, macConkey agar, Edwards agar, Eosin methylene blue agar, and mannitol salt agar and incubated aerobically at 37 ^{o}C for 24 - 48 hr. The growing surface colones were picked up, purified and reinoculated into nutrient broth for further identification which based on cultural , morphological and biochemical characteristics according to **(Quinn *et al.*, 2011).** Biochemical identification of suspected *E. coli, Klebsiella and Pseudomonase* colonies were tested using API 20 E (bio – Merieux).

3) Antibiotic sensitivity test:
It was done by disc diffusion method according to **(Cruichshank *et al.*, 1975).** The results were interpreted according to **NCCLS (2004)**.

4) Serotyping of *E. coli* isolates:
Serotyping of *E. coli* isolates. It was done by slide agglutination test (8 polyvalent sera vials & 43 hypermonovalent antisera vials were obtained from DENKA SEIKENCO.LTD. Tokyo. Japan. The procedure outlined of *E. coli* serotyping was carried out according to **Edward and Ewing (1972).**

5) PCR procedure:
Virulence genes of *staph. aureus* (*spa, coa, hlb, hlg*) and *Klebsiella pneumoniae* (*mag*A) were detected using PCR.

Resistance genes was detected for isolate of *Klebsiella pneumoniae* (*bla*TEM, *bla*SHV) and isolate of *Pseudomonase aeroginosa* (*bla*CTX, *bla*TEM, *bla*SHV) using PCR.

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

Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are 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, 4.5 µl of water, and 6 µl of DNA template. The reactions were performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products.
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

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

Target gene	Tested agent	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
coa	*Staphylococcus aureus*	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	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
hla		GAAGTCTGGTGAAAACCCTGA TGAATCCTGTCGCTAATGCC	704 bp	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Fei et al., 2011
hlb		CAATAGTGCCAAAGCCGAAT TCCAGCACCACAACGAGAAT	496 bp	94˚C 5 min.	94˚C 30 sec.	53˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	
spa		TCA ACA AAG AAC AAC AAA ATG C GCT TTC GGT GCT TGA GAT TC	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
hlg		GCCAATCCGTTATTA-GAAAATGC CCATAGACGTAGCAACGGAT	937 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Kumar et al., 2009
*bla*TEM	*Klebsiella pneumonia and* *Pseudomonas aeruginosa*	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom et al., 2003
*bla*SHV		AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
*bla*CTX	*Pseudomonas aeruginosa*	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Archambault et al., 2006
*mag*A	*Klebsiella pneumoniae*	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1282 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 1.2 min.	72˚C 12 min.	Yeh et al., 2007
*rmp*A		ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

6) Biochemical examinations:

a) Detection of biochemical parameters:

Determination of serum total protein and albumin were carried out using the methods described by **Henry (1968)** and **Drupt (1974)**, respectively. Serum globulin was calculated by subtracting the estimated serum albumin value from the determined serum total protein of the same serum sample **Coles (1986)**. Serum albumin/globulin ratio (A/G) was calculated by dividing the value of serum albumin by the value of globulin. Glucose was determined according to the method described by **Trinder (1959)**. Serum alanine amino transferase activity (ALT) and aspartate amino transferase activity (AST) were carried out according to the method of **Reitman and Frankel (1957).** Kinetic determination of serum creatinine was performed according to the method described by **Henry (1974).**

b) Estimation of serum trace elements: Concentrations of serum iron, zinc and copper were estimated by Flame Atomic Absorption Spectrophotome-try (FAAS) using a Sens AA.

Results

The clinical examination of affected cases of horses appeared that, they were affected with pneumonia and characterized by dullness; elevated body temperature; anorexia and respiratory manifestations (cough, dyspnea; nasal – mucopurulent discharge and abnormal lung sounds). Dehydration was shown in some cases.

Resuts of Bacteriological examination:

Table (2) revealed that from 100 nasal swabs samples, 75 (75%) were bacteriologically positive (75 containing bacterial isolates and 20 of them have more than one isolates, mixed isolates). The most prevalent were *Staph. aureus* (53.3%). Followed by *Pseudomonase aeruginosa*, *Strept. equi*, *Klebsiella pneumoniae, E. coli, pasteurella spp.* and *Coryne.spp,* 10.7%, 9.3%, 8%, 6.7%, 6.7% and 5.3%, also mixed infection with (*Staph. aureus), Strept. equi, Pasteurella spp, Klebsiella pneumonia, Coryne. spp* and *Pseudomonase aeruginosa* respectively in an incidence of 35%, 25%, 20%, 10% and 10% respectively as shown in Table (2).

Table (3), showed that *Staph. aureus* more sensitive for penicillin G, vancomycine and cefuroxime sodium. *Pseudomonase aeroginosa* more sensitive for kanamycin, ofloxacin. *Strept. equi* more sensitive for penicilline G, cephradine and amoxycillin. *Klebsiella pneumoniae* resist to all used antibiotic discs. *Pasteurella spp*. more sensitive for erythromycin, cephalexine and doxycycline. *E. coli* more sensitive for ofloxacin, amikacin and amoxycillin. *Coryne. spp.* more sensitive for kanamycin, doxycycline and cephalexine**.** Table (4) showed that 5 strains of isolated *E. coli* were belonged to the serotypes O86a (2) and O125 (3). Application of PCR for detection of virulence and antibiotic resistance genes of random isolates of each of *Staph.*, *aureus*, *Klebsiella pneumoniae* and *Pseudomonase aeruginosa* (table 5) and (fig.) 1, 2, 3 showed that the *Staph.*, *aureus* isolates harboured *spa, coa, hlb, hlg* and *hla* virulence genes by amplification at 226bp., 360 bp., 496bp., 937bp. respectively but. *hla* negative. While *Klebsiella pneumoniae* isolates harboured *mag*A virulence genes by amplification at 1282 bp. and *bla*SHV, *bla*TEM antibiotic resistance genes by amplification at at 392 bp., 516 bp. *Pseudomonase aeruginosa* isolates harboured *bla*SHV, *bla*TEM and *bla*CTX antibiotic resistance genes by amplification at 392 bp., 516 bp. and 593 bp. respectively.

Results of biochemical examinations: protein profile of affected horses with pneumonia in case of single infection with *staph. aureus* and mixed infection revealed significant increase in the total protein and globulin in both male and female diseased horses in comparison to the apparent healthy animals ($P<0.05$). Serum albumin showed significant decrease in female horses with single staph infection (table 6, 7)**.**

Serum glucose and creatinine showed significant increase in both sexes of respiratory diseased horses due to *staph aureus* infection alone and mixed infection in comparison to the apparent healthy animals at P< 0.05. On the other hand, no significant changes in ALT and AST values (table 8, 9). Estimation of serum trace elements revealed significant decrease in iron and zinc in both sexes of respiratory diseased horses. Meanwhile, copper slightly elevated when compared to the apparent healthy animals (table 10, 11).

Table (2). Bacterial species isolated from diseased equine samples:

Total Bacterial isolates		
Single infection 75		
	No.	*** %**
Staph. aureus	40	53.3
Pseudomonase aeruginosa	8	10.7
Strept. equi	7	9.3
Klebsiella pneumoniae	6	8
E.coli	5	6.7
Pasteurella spp.	5	6.7
Coryne.spp.	4	5.3
Mixed infection 20		
Staph. aureus + Strept. equi	7	35
Staph. aureus + Pasteurella spp.	5	25
Staph. aureus+Klebsiella pneumoniae	4	20
Staph. aureus + Coryne. spp.	2	10
Staph. aureus + Pseudomonase aeruginosa	2	10

* % in relation to total number of isolates =75 & mixed isolates= 20
No: number %: percent

Table (3). In- Vitro Antibiotic sensitivity of different bacterial isolates :

Antibiotic discs		Isolates													
		Staph. aureus (40)		*Coryne.spp. (4)*		*Klebsiella pneumo-niae.(6)*		*Pseudomonases aeruginosa (8)*		*E. coli (5)*		*Pasteurella spp. (5)*		*Strept. equi (7)*	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
Kanamycin (K30)	**No.**	12	28	4	-	3	3	8	-	3	2	3	2	4	3
	%	30	70	100	-	50	50	100	-	60	40	60	40	57.1	42.9
Ofloxacin (OFX_5)	**No.**	11	29	1	3	2	4	8	-	5	-	2	3	2	5
	%	27.5	72.5	25	75	33.3	66.7	100	-	100	-	40	60	28.6	71.4
Doxycycline (DO_{30})	**No.**	11	29	4	-	2	4	3	5	2	3	5	-	2	5
	%	27.5	72.5	100	-	33.3	66.7	37.5	62.5	40	60	100	-	28.6	71.4
Colistin sulphate (CT_{10})	**No.**	10	30	1	3	3	3	3	5	3	2	2	3	5	2
	%	25	75	25	75	50	50	37.5	62.5	60	40	40	60	71.4	28.6
Cephalexine (CL_{30})	**No.**	12	28	4	-	2	4	4	4	2	3	5	-	3	4
	%	30	70	100	-	33.3	66.7	50	50	40	60	100	-	42.9	57.1
Cefuroxime sodium (CXM_{30})	**No**	25	15	1	3	1	5	-	8	-	5	3	2	4	3
	%	62.5	37.5	25	75	16.7	83.3	-	100	-	100	60	40	57.1	42.9
Vancomycine (VA_{30})	**No.**	26	14	-	4	2	4	-	8	-	5	2	3	2	5
	%	65	35	-	100	33.3	66.7	-	100	-	100	40	60	28.6	71.4

Table (3). Continued In- Vitro Antibiotic sensitivity of different bacterial isolates:

Antibiotic discs		Isolates													
		Staph. aureus (40)		*Coryne.sp p.(4)*		*Klebsiella pneu-moniae (6)*		*Pseudomonases aeruginosa (8)*		*E. coli (5)*		*Pasteurella spp.(5)*		*Strept. equi(7)*	
		S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
Penicilline G (P_{10})	**No.**	29	11	2	2	2	4	2	6	-	5	1	4	7	-
	%	72.5	27.5	50	50	33.3	66.7	25	75	-	100	20	80	100	-
Erythromycin (E_{15})	**No.**	12	28	1	3	2	4	3	5	-	5	5	-	4	3
	%	30	70	25	75	33.3	66.7	37.5	62.5	-	100	100	-	57.1	42.9
Amikacin (AK_{30})	**No.**	12	28	1	3	3	3	4	4	3	2	2	3	3	4
	%	30	70	25	75	50	50	50	50	60	40	40	60	42.9	57.1
Cephadroxil (CDX_{30})	**No.**	10	30	1	3	2	4	3	5	2	3	3	2	4	3
	%	25	75	25	75	33.3	66.7	37.5	62.5	40	60	60	40	57.1	42.9
Cephradine (CRD_{30})	**No.**	12	28	1	3	2	4	2	6	2	3	1	4	7	-
	%	30	70	25	75	33.3	66.7	25	75	40	60	20	80	100	-
Amoxycillin (AML_{25})	**No.**	14	26	-	4	3	3	2	6	3	2	3	2	7	-
	%	35	65	-	100	50	50	25	75	60	40	60	40	100	-

K_{30}= Kanamycin, OFX_5= Ofloxacin, DO_{30}= Doxycycline, CT_{10}= Colistin sulphate, CL_{30}= Cephalexine, CXM_{30}=Cefuroxime sodium, VA_{30}= Vancomycine, P_{10}= Penicilline G, E_{15}= Erythromycin, AK_{30}= Amikacin, CDX_{30}= Cephadroxil, CRD_{30}= Cephradine, AML_{25}= Amoxycillin
S: sensitive R: resistance

Five *E. coli* isolates were serologically typed using specific antisera and the results are shown in table (4).

Table (4). Serotyping of *E. coli* isolates:

No. of isolate	Polyvalent	Monovalent
1	1	O86a
2	2	O125
3	2	O125
4	1	O86a
5	2	O125

Table (5). Virulence and antibiotic resistance genes of *Staph. aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa* isolates

Bacterial isolates	Results									
	Antibiotic resistance genes			Virulence genes						
	*bla*CTX	*bla*TEM	*bla*SHV	*rmp*A	*mag*A	*spa*	*coa*	*hla*	*hlb*	*hlg*
Klebsiella pneumoniae	-	+	+	-	+	-	-	-	-	-
Pseudomonas aeruginosa	+	+	+	-	-	-	-	-	-	-
Staph.aureus	-	-	-	-	-	+	+	-	+	+

PCR results as shown in table (5) and Fig. (1), (2), (3):

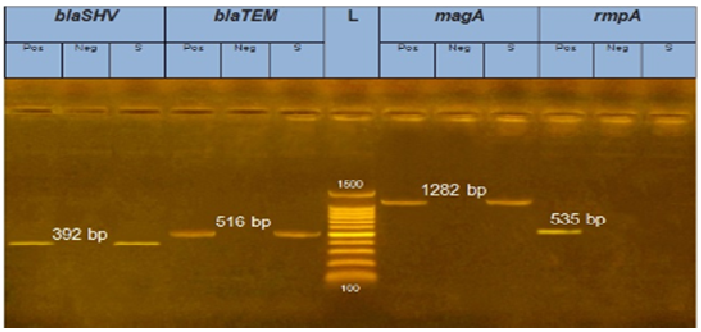

Fig. (1): Gel electrophoresis of viulence and antibiotic resistance genes of *Klebsiella pneumoniae* L: DNA ladder, P: Positive control, N: negative control. Antibiotic resistance genes: Positive amplification for *bla*SHV at 392 bp., positive amplification for *bla*TEM at 516 bp. Virulence genes *mag*A positive amplification at 1282 bp. *rmp*A negative.

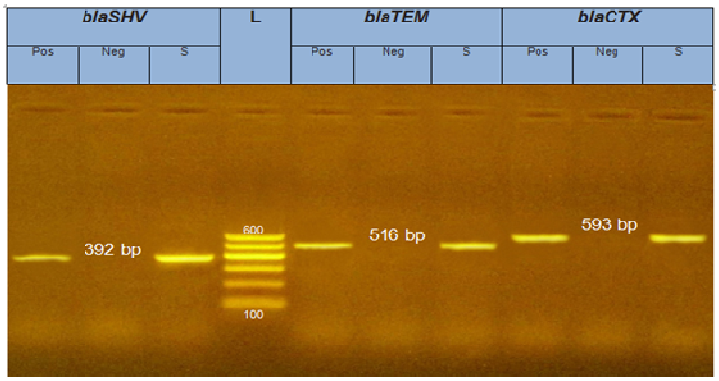

Fig. (2): Gel electrophoresis of antibiotic resistance genes of *Pseudomonas aeruginosa* L: DNA ladder , P :Positive control, N: negative control. Antibiotic resistance genes : Positive amplification for *bla*SHV at 392 bp., positive amplification for *bla*TEM at 516 bp. and positive amplification for *bla*CTX at 593 bp.

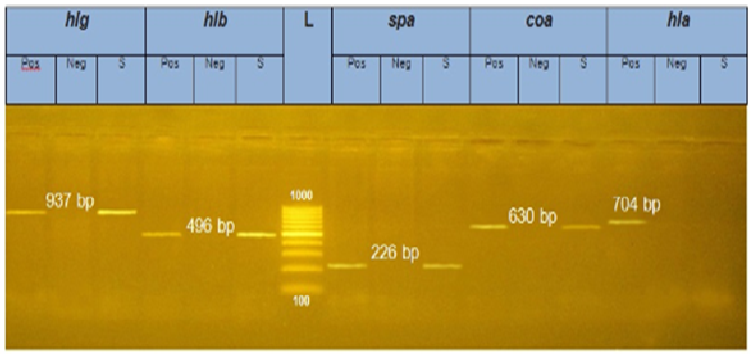

Fig. (3): Gel electrophoresis of virulence genes of *Staph. aureus.* L: DNA ladder , P :Positive control, N: negative control. : viulence genes Positive amplification for *spa, coa, hlb, hlg* at 226bp., 360 bp., 496bp., 937bp. respectively. *hla* : negative.

Table (6). Changes in serum proteins in *Staph aureus* infected horses and apparent healthy animals.

Parameters	Males		Females	
	Apparent Healthy	Diseased	Apparent Healthy	Diseased
Total protein (g/dl)	6.55±0.14	7.01*±0.14	6.59±0.08	6.83*±0.06
Albumin (g/dl)	4.26±0.21	4.07±0.13	4.14±0.18	3.47*±0.09
Globulin (g/dl)	2.29±0.16	2.94*±0.11	2.45±0.16	3.36*±0.07
A/G ratio	1.86±0.14	1.38*±0.08	1.69±0.14	1.03* ±0.04

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t-student test.

Table (7). Changes in serum proteins in case of mixed infection and apparent healthy animals

Parameters	Males		Females	
	Apparent Healthy	Diseased	Apparent Healthy	Diseased
Total protein (g/dl)	6.55±0.14	7.18*±0.13	6.59±0.08	7.02*±0.09
Albumin (g/dl)	4.26±0.21	4.34±0.22	4.14±0.18	3.90±0.26
Globulin (g/dl)	2.29±0.16	2.84*±0.10	2.45±0.16	3.12*±0.19
A/G ratio	1.86±0.14	1.52±0.11	1.69±0.14	1.25±0.16

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t- student test.

Table (8). Serum biochemical parameters in *Staph aureus* infected horses and apparent healthy animals.

Parameters	Males		Females	
	Apparent Healthy	Diseased	Apparent Healthy	Diseased
Glucose (mg/dl)	89.1±1.23	110.96*±1.22	102.18±0.53	113.30*±1.49
ALT (u/ml)	35.46±0.37	34.75±0.49	35.28±0.31	35.83±0.28
AST (u/ml)	102.23±1.96	102.68±0.10	106.62±040	105.87±0.97
Creatinine (mg/dl)	1.20±0.17	2.60*±0.39	1.50±0.29	2.54*±0.35

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t- student test

Table (9). Serum biochemical parameters in case of mixed infection and apparent healthy animals

Parameters	Males		Females	
	Apparent Healthy	Diseased	Apparent Healthy	Diseased
Glucose (mg/dl)	89.1±1.23	112.42*±1.49	102.18±0.53	110.62*±1.39
ALT (u/ml)	35.46±0.37	36.46±0.61	35.28±0.31	35.76±0.36
AST (u/ml)	102.23±1.96	99.78±1.60	106.62±0.40	109.32±1.40
Creatinine (mg/dl)	1.20±0.17	2.80*±0.29	1.50±0.29	2.70*±0.33

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t- student test.

Table (10). Alterations in selected serum mineral profile in *Staph aureus* infected horses compared to the apparent healthy animals

Parameters	Males		Females	
	Apparent Healthy	Diseased	Apparent Healthy	Diseased
Fe (µg/ml)	1.68±0.15	0.84*±0.04	1.66±0.10	0.60*±0.06
Zn (µg/ml)	0.16±0.01	0.10*±0.01	0.19±0.04	0.04*±0.03
Cu (µg/ml)	0.75±0.04	0.79±0.02	0.67±0.04	0.77*±0.02

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t- student test.

Table (11). Alterations in selected serum mineral profile in case of mixed infection compared to the apparent healthy animals

Parameters	Males		Females	
	Apparent healthy	Diseased	Apparent healthy	Diseased
Fe (µg/ml)	1.68±0.15	0.82*±0.03	1.66±0.10	0.56*±0.07
Zn (µg/ml)	0.16±0.01	0.12*±0.01	0.19±0.04	0.03*±0.03
Cu (µg/ml)	0.75±0.04	0.77±0.03	0.67±0.04	0.73±0.03

Values represent means ± standard errors (n=10).
* Significantly at P< 0.05 using t- student test.

Discussion

Respiratory diseases are the result of exposure to dust and other airborne irritants. Bacterial respiratory infections in equines are initiated by viral disease, because viral respiratory infections impair and/or destroy respiratory defense mechanisms leading to activation of many bacterial pathogens.

Our results showed isolation of *Staph. aureus, Pseudomonase aeruginosa, Strept. equi,* and *E. coli* in an incidence of (53.3%), (10.7%), (9.3%) and (6.7%) respectively table (2). **Bzdil *et al*.**, **(2017)** and **Priyanka *et al*., (2017)** reported that *Staph. aureus* was isolated from infected equine with a percentage of 37.2% and 38.71% respectively which nearly similar to our finding. **Van Spijk *et al*.**, **(2016 a)**, **Seung *et al*., (2011)**, **Gutema *et al.,* (2009)** and **Debelu *et al*., (2014)** obtained nearly similar results. Similar results obtained by **Seung *et al*.**, **(2011)** who isolate *Strept.equi*.in an incidence of (10.2%). Also **(Al Seady *et al.,* 2018)** isolated *Strept. equi* during the period from 2015 to April 2017 and concluded that examination of 255 from equine animals showed that 97 were suffered from Upper respiratory tract manifestation so the estimated prevalence was 38%.

The present study showed isolation of *Klebsiella pneumoniae, Pasteurella spp.* and *Corynebacterium spp.* in an incidence of (8 %), (6.7%) and (5.3%) respectively. **Sweeny *et al.,* (1991)** isolated *Klebsiella pneumoniae* at a rate of 13.9% from horses with pneumonia which agreed with our results. **Gutema *et al.,* (2009)** reported that *Klebsiella pneumoniae* was isolated from infected donkey in a percentage of (5.8%) which lower finding to our study. The bacterium was also recovered dominantly from the respiratory tracts of sheep inhabiting the same study area (**Mesele, 2005**). Higher results obtained by **Van Spijk *et al*.**, **(2016 b)**, in incidence of 43%. **Boguta *et al*., (2002**) isolate *Klebsiella pneumoniae* from lung abscess at postmortem. **Gutema *et al.,* (2009)** reported that *Coryne. spp*. was isolated from infected donkeys in a percentage of 15.4% which higher than to our finding**.**

The results illustrated in table (3) showed sensitivity of different isolates recovered from examined clinically diseased cases to 13 different antibiotic discs. *Staph. aureus* more sensitive for penicillin G, vancomycine and cefuroxime sodium. *Pseudomonase aeroginosa* more sensitive for kanamycin, ofloxacin. *Strept. equi* more sensitive for penicilline G, cephradine and amoxycillin. *Klebsiella pneumoniae* resist to all used antibiotic discs. *Pasteurella spp*. more sensitive for erythromycin, cephalexine and doxycycline. *E. coli* more sensitive for ofloxacin, amikacin and amoxycillin. *Coryne.spp.* more sensitive for kanamycin, doxycycline and cephalexine.

Muktha *et al*., (2015) found that *Staph. aureus* isolates were sensitive to ciprofloxacin and gentamycin, on other hand *E. coli* isolates were sensitive to ciprofloxacin, gentamycin and erythromycin, so ciprofloxacin and gentamycin could be used for therapeutic purpose if diseases occur by these organisms in horses. **Seung *et al.,* (2011**) mentioned that antimicrobial susceptibility of bacterial isolates is suggesting that the current initial (trimethoprim/ sulfamethoxazole, oxytetracyclin, penicillin, or amoxicillin/clavulanate), secondary (amikacin and gentamicin nebulization), and tertiary antibiotic (ceftiofur) treatments for respiratory tract infections. **Al Seady *et al.,* (2018**) recorded that *strept. equi* isolates were high sensitive to ceftriaxone, cefquinome, ciprofloxacin and amoxicillin. **Chris *et al*., (2008)** recorded that *strept. equi* isolates were highly sensitive to penicillin and ceftiofur also Procaine penicillin G and gentamicin still appear efficacious for most equine infections.

Table (4) showed that 5 strains of isolated *E. coli* were belonged to the serotypes O86a (2) and O125(3). The resuts are in accordance with **The center for food security & public health (2016)** who showed that one enteroaggregative

E. coli O86:NM was isolated from a fatal case of hemolytic uremic syndrome (HUS) in Japan.

Application of PCR for detection of virulence and antibiotic resistance genes of random isolates of each of *Staph.*, *aureus*, *Klebsiella pneumoniae* and *Pseudomonase aeruginosa*. The results (table 5) and (fig.) 1, 2, 3 showed that the *Staph.*, *aureus* isolates harboured *spa, coa, hlb, hlg* and *hla* virulence genes by amplification at 226bp., 360 bp., 496bp., 937bp. respectively but. *hla* negative. While *Klebsiella pneumoniae* isolates harboured *mag*A virulence genes by amplification at 1282 bp. and *bla*SHV, *bla*TEM antibiotic resistance genes by amplification at at 392 bp., 516 bp. *Pseudomonase aeruginosa* isolates harboured *bla*SHV, *bla*TEM and *bla*CTX antibiotic resistance genes by amplification at 392 bp., 516 bp. and 593 bp. respectively.

The presence of virulence genes explained the pathogenicity of the organism in induction the diseases also presence of antibiotic resistance genes explained failure of treatment of diseased equine by beta lactam antibiotic. **Coelho *et al.,* (2009)** mentioned that The amplification of region X from one yielded a single amplicon for each isolate with the prevalent amplicon size being of 180bp., the amplification of the *coa*-gene displayed four different size polymorphisms with about 400bp., 600bp., 700bp., and 900bp. **Jiang *et al.,* (2018)** reported that the β‑lactamase signaling pathway has been suggested to be involved in antibiotic resistance against β‑lactams in *Klebsiella pneumoniae* the molecular mechanism of the antibiotic resistance of *Klebsiella pneumoniae* was investigated and the results indicated involvement of the β–arrestin recruitment‑induced β‑lactamase signaling pathway. Consistent with the location of *mag*A within the *cps* operon of K1 isolates, both K1 groups were *mag*A positive, while *mag*A was not detected in any other isolate **(Brisse *et al.,* 2009).**

Presence of virulence genes make the bacteria more invasive and more resistant to phagocytosis. Resistance and virulence are not independent properties and their relationship may be play an important role in the pathogenesis of *Klebsiella pneumoniae* **(Vila *et al.,* 2011).**

Virulence and resistance are similar in that most of the determinants have been transmitted between bacteria by horizontal gene transfer and transfer of DNA is probably the most important mechanism for the dissemination and co- selection of virulence and resistance properties **(Dasilva and Mendone, 2012)**. It is possible that a different mechanism of gene transfer such as horizontal gene transfer between serotypes may cause the spread of resistance genes **(Madhusadana and Sarendran, 2010).**

Concerning to the biochemical alterations, protein profile of diseased horses in case of single infection with *staph. aureus* or mixed infection showed significant increase in the total protein and globulin in both male and female diseased horses. Serum albumin showed significant decrease in female horses with single *staph* infection. The increase in total protein level was due to the elevation in globulin. The increase in the serum globulin level might be due to the increase in the immunoglobulins as a result of bacterial infection which indirectly reflect the serum total protein levels (**Fahmy *et al*., 2010; Ijaz *et al*., 2012**). **Salem (2017)** recorded significant increase in total protein and globulin in foals with upper airway affection, caused by *Streptococcu equi* infection. Also, **Neamat-Allah and El Damaty (2016)** reported significant increase in serum total protein and globulin in Arabian horses in Egypt with *Streptococcus equi* infection. **Georgieva *et al.* (2017)** mentioned that serum globulin level rose significantly in rabbit experimentally infected with *staph. aureus*. Also, in dogs experimentally infected with *staphylococcal* **Zapryanova *et al.* (2013)** found significant increases in total protein, globulin and significant decreases in albumin concentrations.

Serum glucose showed significant elevation in disease horses due to *staph aureus* infection

alone and mixed infection in comparison to apparent healthy animals. Hyperglycemia could be attributed to the superantigens (SAgs) that is produced by *staph aureus*, induces inflammation, lipolysis, and insulin resistance. **Vu *et al.* (2015)** mentioned that prolonged exposure to SAgs through frequent *staph aureus* colonization and infection may result in impaired glucose metabolism, hyperglycemia. This is due to the ability of SAgs to cause insulin resistance through inducing chronic systemic inflammation and lipolysis.

Glucose is an energy source inside the horse's cells. Serum glucose fluctuates widely in normal individuals, in response to recent feeding, pain or stress. Hyperglycemia may be attributed to glycogenolysis and gluconeogenesis as result of the neuroendocrine response to stress **(Marik and Bellomo, 2013)**. Hypothalamic-pituitary-adrenal (HPA) axis, sympathoadrenal system and proinflammatory cytokines (TNF-α, IL-1 and IL-6) act collectively and synergistically to induce stress hyperglycemia **(Marik and Bellomo, 2013)**. There are several mechanisms leading to the development of hyperglycemia during critical illness. Glucagon, epinephrine, cortisol, and growth hormone are released, which may oppose the normal actions of insulin and promote gluconeogenesis via increased lipolysis and proteolysis **(Langouche and Van den Berghe, 2006, Nasraway, 2006)**. In addition, inflammation leads to release of proinflammatory cytokines, which may aggravate the hyperglycemic state. There is also a decrease in insulin-dependent glucose uptake, despite an increase in blood insulin concentrations, reflecting the development of insulin resistance in these critically cases **(Hollis *et al.*, 2007).**

Arroyo *et al.* (2017) recorded hyperglycemia in horses with septic pleuropneumonia. They also, mentioned that the most common isolate were *Streptococcus Streptococcus equi subsp zooepidemicus (84%), Actinobacillus spp. (33%), E. coli (28%), Staphylococcus spp. (15%), Klebsiella pneumoniae (13%),* and *Pseudomonas aeruginosa* (10%).

Creatinine is a nitrogenous waste product derived from the breakdown of creatine and phosphocreatine. Creatine is synthesized in the liver, pancreas, and kidneys from the transamination of the amino acids arginine, glycine, and methionine. Creatine then circulates throughout the body and is converted to phosphocreatine by the process of phosphorylation in the skeletal muscle and brain **(Jose *et al.* 2014)**. The majority of the creatinine is produced in the muscle and excreted via the kidneys. Creatinine levels are an important marker of renal function, with levels controlled by the excretion rate. The daily production and excretion is remarkably constant. Elevated levels of serum creatinine are seen in association with reduced glomerular filtration and may result from prerenal (circulatory disturbances, hypovolemia, dehydration, shock, endotoxemia), renal (insufficiency) or postrenal (obstructive) conditions **(Barrelet and Ricketts 2013).** In this study, elevation of serum creatinine level in diseased horses might be due to dehydration and *Staph. aureus* infection either single or mixed infection.

Arroyo *et al.* (2017) found elevation of serum creatinine in (14%) of the horses with septic pleuropneumonia and *Staphylococcus spp.* (15%), among organisms cultured from tracheal aspirate and from pleural fluid of the horses. They attributed the elevation in serum creatinine in these horses to prerenal case, reduction in glomerular filtration rate as a result of decreased fluid intake and increased fluid loss into the pleural cavity. They also mentioned that increased serum creatinine concentration is a negative prognostic indicator and is likely a reflection of dehydration. Also, **Shukla *et al.* (2011)** recorded an increase in serum creatinine level in *Klebsiella pneumoniae* pulmonary infection and attributed the increment in creatinine to the ability of *K. pneumoniae* to fix nitrogen. Thus the increased nitrogen content in the body may lead to more synthesis of amino acids resulting elevation of creatinine

level. There are also evidences that in patients suffering from *Klebseilla* septicemia with pneumonia, creatinine level in the most important risk factor associated with in-hospital mortality (**Tsai *et al*., 2010).**

Trace elements (Fe, Cu, Zn) are found in the body in low concentrations, any increase or the decrease in these element will be hurtful. These metals are essential nutrients to bacterial pathogens. In *Staphylococcus aureus*, metal ions participate in metabolism, DNA synthesis, regulation of virulence factors, and defense against oxidative stress **(Cassat and Skaar 2012)**. Therefore, the clinical concern in determination of trace elements has been increased in the recent years for diagnosis of different diseases **(Shawaf *et al.*, 2017)**.

This study revealed a significant decrease in serum iron and zinc in both sex of horses affected with pneumonia caused by single or mixed infection of staph *aureus*. Meanwhile, copper slightly elevated when compared to apparent healthy animals. The detected decrease (Fe and Zn) could be attributed to an innate immune response to bacterial infection; vertebrate hosts sequester metals in a process termed "nutritional immunity **(Cassat and Skaar 2012)**.

Serum iron refers to ferric ions (Fe 3+) bound to serum transferrin. Concentration of serum iron is highly variable and is affected by dietary iron intake, inflammation, and infection**.** Iron deficiency in infectious or inflammation conditions attributed to inflammatory cytokines that increase the synthesis of hepcidin protein. Hepcidin decreases iron absorption from the gastrointestinal tract and impedes mobilization of iron from macrophages and hepatocytes (**Kelly *et al.* 2017)**. The recorded low serum iron in the present study might be attributed to decrease iron intake, absorption from GIT and /or bacterial infection.

Iron is a necessary element for haemoglobin in red blood cells, myoglobin in muscle, and many metalloproteins and enzymes. It is essential for uptake of oxygen and its delivery to tissues, utilization of oxygen by muscle cells, and mitochondrial energy production (**Kelly *et al.* 2017)**. A sufficient supply of iron is vital for the host to maintain key metabolic processes such as mitochondrial respiration, DNA synthesis, hormone formation or multiple other biochemical processes. Because many of these functions are also essential for microbial proliferation and pathogenicity, they have developed multiple pathways to acquire iron from their environment. Therefore, control over iron availability is a central component deciding the outcome of infections. As a consequence, subtle alterations of iron homeostasis occur in case of an infection or inflammatory disease, often resulting in the development of a mild to moderate anemia, termed 'anemia of chronic disease' or 'anemia of inflammation/infection **(Weiss and Carver, 2018)**. Anemia may lead to lack of exercise which, in turn affects fitness and other body systems (**Asl *et al.,* 2013**).

Zinc is a cofactor in many metalloenzymes and proteins. It plays an important role in formation of SOD and in body antioxidant mechanism by averting radical-induced injury **(Yehia *et al.*, 2016).** Zinc is required for normal development and function of both pathogens and hosts, in which they are important components of cells mediating innate immunity (macrophages, neutrophils, natural killer cells). Zn deficiency can result in an increase in the incidence of upper respiratory tract infections and diarrhoea, as well as morbidity and mortality from these infections (**Weiss and Carver, 2018).**

Limitation of zinc availability may be beneficial to controlling *staph aureus* infection, as several processes that contribute to staphylococcal virulence are zinc dependent, including biofilm formation and superantigen activity**.** So the significant decrease in serum zinc could be due to the sequestration of zinc as innate immune response to bacterial infection; nutritional immunity.

Concerning the slight elevation in serum copper might be due to the increase in ceruloplasmin "acute phase protein" which increased during inflammation and consequently, leads to elevation in Cu level **(Healy and Tipton, 2007).** Copper is a redox-active metal ion, which exerts antimicrobial activities by radical and no radical mediated processes **(Samanovic *et al.*, 2012)**. The copper-induced bactericidal activity has been exploited since the time of the Egyptians and is currently being explored as a means to prevent hospital-acquired infections **(German *et al*, 2016; Besold *et al.*, 2016).** One strategy of host immune defense is to increase copper levels during infection. In particular, there is a well-described copper burst that takes place in the phagosome of macrophages, which serves as an important host defense against infection (**Samanovic *et al.*, 2012; Potrykus *et al.*, 2014).** Copper antibacterial antimicrobial effect is associated with various mechanisms such as damaging the microbial DNA, altering bacterial protein synthesis and altering membrane integrity. The antibacterial effect of copper was already proved for staph *aureus*, and *E. coli* (**Reyes-Jara *et al.*, 2016)**. In addition, **Mahmoodi *et al.* (2018**) reported that copper have antimicrobial activity against a wide range of microorganisms, such as *Staphylococcus aureus*, Salmonella enteric, Campylobacter jejuni, Escherichia coli, and Listeria monocytogenes**.**

Conclusion and Recommendation

Staph aureus was the most frequent isolate of the diseased cases causing severe damage of respiratory system as well as pneumonia and dehydration. Biochemical incidence and trace elements alteration are greatly affected decrease (iron and zinc) or increase. Significant elevation in the levels of serum total protein, globulin, glucose and creatinine were in the infected horses. These changes help in the treatment of diseased animals after the isolation of the organisms. At the choice of advisable antibiotic we have to be away from those having adverse effect in the kidney. We have to check the level of trace element (iron and zinc) in serum after treatment of the infection. The decrease in serum values of iron and zinc, considering the role of these elements in hematopoiesis and immune system, the administration of supplements containing these elements (oral or injection) is suggested after treatment of the infection. Application of good hygienic measures in equine farms to reduce bacterial contamination together with early treatment of infected animals which consequently affected on biochemical parameters and avoidance of their alterations and consequently animal health and activity.

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