Global Veterinaria 13 (5): 877-888, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.13.05.86237

Microbiological Investigations of Equine Infections in Relation to Oxidative Stress Markers

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Abstract: This study aimed to investigate genital tract causes of repeat breeding in equine stable, digestive and respiratory infections, stress and oxidative stress. Vaginal, uterine, nasal and rectal swabs were collected from horses. Blood serum and seminal plasma samples were collected from repeat breeding animals before and after receiving treatment. Microbiological studies revealed that *E. coli* (39%), *S. aureus* (14.63%) and *E. faecalis* (9.75%) were isolated from repeat breeding mares. *S.aureus* (30.09%) and *E.coli* (9.52%) were isolated from respiratory disorders. Enteropathogenic gene (*sfa* gene) was detected in four strains (66.67%) of six *E.coli* isolated from vaginal swabs of repeat breeding mares. Our study indicated that *E.coli* strains which hinder *sfa* gene might play an important role in repeat breeding mare. Real time PCR results confirmed positive Pseudomonas isolates and showed that *E. coli* isolates were negative for *vtx1* and *vtx2*.Cortisol increased in mares with reproductive tract and mixed reproductive-respiratory tract infection compared to other healthy animals. Ascorbic acid levels declined significantly in stallions' serum with respiratory tract infection and mares with reproductive-respiratory tract infection. Glutathione levels decreased in diseased animals than control cases. Cortisol and all antioxidant markers improved after treatment with the selected antibiotic. In conclusion, *E.coli* was the most common pathogens recovered from subclinical endometritis. However, as antimicrobial resistance changes over time, susceptibility assays should aid antimicrobial drug selection. It is recommended to supply horses undergo breeding with some antioxidants such as ascorbic acid to improve their antioxidant status.

Key words: Horses · Bacteria · *sfa* gene · Verotoxins · Genital tract · Oxidative stress · Antibiotic sensitivity

INTRODUCTION

Bacterial uterine infections occur in 25-60% of barren mares and inflict major losses on the equine breeding industry [1, 2]. Accumulation of uterine fluid during the ovulatory period is consistently associated with decreased pregnancy rates [3, 4]. The presence of two or more centimeters of intra-uterine fluid during estrus or between 6 and 36h post-breeding is a good indicator that a mare is susceptible to mating-induced endometritis [5, 6]. Endometritis is most commonly associated with aerobic bacterial infection, but may also be caused by anaerobic bacteria, pneumovagina, urine pooling, exposure to semen and intra-uterine infusion with an irritating substance [7]. Infectious endometritis associated with *Pseudomonas aeruginosa* is an important equine disease resulting in reduced fertility, decreased foal crop and economic loss for the thoroughbred industry [8].

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The ability to adhere to host surfaces is by far the most vital step in the successful colonization by microbial pathogens. Colonization begins with the attachment of the bacterium to receptors expressed by cells forming the lining of the mucosa. Long hair like extracellular appendages called fimbriae, produced by most Gram-negative pathogens, mediate specific attachment to the epithelial cell surface. Associated with the fimbriae is a protein called an adhesion [9]. sfaX(II) gene and presumably other members in the 17 kDa gene family, may play a role in the control of virulence related gene expression in pathogenic *E. coli* [10].

Oxidative stress (OS) arises as a consequence of imbalance between reactive oxygen species (ROS) generation and scavenging mechanism [11]. Oxidants play an important role by inactivating and destructing microorganisms through peroxidation and destabilization of their lipid membranes, oxidation and inactivation of their proteins acting as receptors or enzymes and oxidation of their nuclear material [12, 13]. Glutathione peroxidase (GPx) is one of enzymatic mechanisms to protect against toxic effects of oxidants [14] and is distributed throughout the glandular epithelium of the endometrium and is regulated by estrogen and other sex-steroid hormones [15]. Increasing clinical epidemiological and experimental evidence indicates that excess of production of reactive oxygen free radicals (ROS) is involved in the pathogenesis of a number of airway disorders [16]. Horses suffering from respiratory airway obstruction (RAO) have a decreased pulmonary antioxidant capacity, which may render them more susceptible to oxidative challenge [17]. These enzymatic mechanisms were also detected in equine semen [18, 19]. Other elements of semen, such as vitamin C could act as antioxidants [20].

This study aimed to diagnose genital tract infections in both pure bred Arabian and imported mares clinically by ultrasonography and microbiological examination of vaginal swabs and semen to detect causes of repeat breeding in equine stable. Meantime, rectal and nasal swabs were collected for studying digestive and respiratory affections. Serum samples were collected to assess antioxidant activity and lipid peroxidation status of infected cases and to correlate them with the microbiological findings as well as with control normal cyclic mares. Also, quantitative study of seminal and blood plasma reduced Glutathione (GHD), Malondialdehyde (MDA) and ascorbic acid concentrations were carried out as markers of antioxidant activity and lipid peroxidation, respectively and cortisol as a marker of stress.

MATERIALS AND METHODS

Animals and Sampling: Fifty five mares (age, 3-12) and 23 stallions (age 3-8) located on 3 stud farms were included in the study. Bacterial swabs including genital tract represented by vaginal, uterine and perpetual swabs, digestive tract represented by rectal swabs and respiratory tract represented by nasal swabs samples were collected from horses for routine screening or suspected clinical infection meantime (Table 1). Samples were cultured using routine phenotypic laboratory testing procedures and isolates identified underwent disc-diffusion antimicrobial susceptibility testing. Blood samples were collected in plane vacuum tubes from jugular vein. Semen was also collected from stallions. Sera and seminal plasma were harvested and stored until assaying.

Bacteriological Examination: A double guarded uterine swab (Equivet, UK) was inserted through the cervix to contact the endometrium. The swab was then retracted, sterile caps placed on the ends of the swab to prevent exposure of the cotton tip and immediately transported to the laboratory. Uterine lavage was also collected.

Bacterial swabs were collected under possible aseptic conditions, cultivation of samples, isolation and purification of the isolates were carried out using different media which were purchased from (Oxoid); swabs were inoculated into a tube containing 10 ml tryptic soy broth, half freeze broth media. The broth was incubated at 37°C for 24 hrs then streaked from the enriched broth onto Nutrient, Mannitol, Blood, Edward, Palcam and MacConkey agar plates. Identification of isolates included morphological examination by Gram's Method [21]. Biochemical identification was carried out according to Collee *et al.* [22]. Samples were cultured using routine phenotypic laboratory testing procedures and isolates identified underwent disc-diffusion antimicrobial susceptibility testing according to NCCLS [23].

PCR and rt-PCR Assay Was Carried out to Investigate the Presence of Toxic Gene in the Predominant Isolates.
PCR:

DNA Extraction from Culture Samples: Six *E.coli* strains were selected for investigating the presence of *sfa* gene to prove whether the tested strains contained toxic gene or not. Single colonies of isolates were cultured in Luria-Bertani medium and incubated for 16 h at 37°C. An aliquot (4 ml) of overnight culture (10^9 CFU) was pelletted by centrifugation (13000 rpm for 4 min). The bacterial pellet, re-suspended in 100µl of Elution buffer, the extraction was

Table 1: Samples taken from mares and stallions

	Mare						Stallion			
Swabs	Endometritis	Abortion	Infertility	Normal cyclic	Emaciation	Total	Frequent colic	Normal	Emaciation	Total
Vaginal	1	4	10	6	4	25	-	-	-	-
Uterine	1	-	-	2	-	3	-	-	-	-
Rectal	2	2	1	7	2	14	1	-	9	10
Nasal	1	2	4	4	2	13	-	2	4	6
Semen	-	-	-	-	-	-	-	7	-	7
Total	5	8	15	19	8	55	1	9	13	23

Table 2: Dye settings for multiplex reaction (IPC= Internal Positive Control)

	VT1 Probe	IPC
TaqMan® VT1 (stx1) Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® VT1 (stx2) Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® *E. coli* O104 Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® *E. coli* O157 Assay	Reporter = FAM™	Reporter = VIC®

carried out according to the instruction which was given by Bioflux Company using (Biospin Bacteria Genomic DNA Extraction Kit).

PCR Amplification: On the basis of the DNA extraction, the following oligonucleotides were used in PCR amplification for detection of *sfa* gene, including;

Primers *sfa* 1 (5'- CTCCGGAGAACTGGGTG CAT CTTAC-3') and *sfa*2 (5'- CGGAGGAGT AATTACA AACCTGGCA -3'), which amplified a 410-bp fragment of the *sfa* gene [24]. The amplification was carried out using Pyrostart Fast PCR Master Mix (Fermentas Company). After amplification, 15 µl of PCR samples was loaded on a 2% (wt/vol) agarose gel and horizontal electrophoresis was performed in 0.1 M Tris HCl (pH 8.6)-80 mM boric acid-1 mM EDTA containing 0.5 mg of ethidium bromide per ml. Amplified, ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator at 300 nm.

Screening of VTEC: VTEC screening was carried after overnight incubation of the sample in Buffer peptone water at 37°C for 24 hrs, using Prep man ultra for extraction of DNA, TaqMan Environmental Master Mix, Custom TaqMan *VT2 (stx2)* Assay, Custom TaqMan *VT1 (stx1)* Assay (Applied Bio system), Custom TaqMan O157 Assay and 7500 real time PCR (Applied Bio system). The Master Mix Set-up was prepared as follows: 15 µL of 2X EMM 2.0 and 3 µL of 10X Target Assay Mix and 18 µL of total volume master mix per reaction. The Dye settings for multiplex reaction were prepared as shown in Table (2) and The Thermo Cycler Settings were carried out in 2 steps; enzyme activation and a template denaturation step which occurred at 95°C /10 /15 min and the amplification step which was repeated 45 times, including; stage 1 at 95°C /15 min and stage 2 at 60°C /45 min.

Detection of VTEC from Different *E. Coli* Isolates (EU RL Method, 2011). The Procedure Included 3 Main Steps:

Enrichment: Separate colonies of *E. coli* isolates were cultured into buffer peptone water then incubated at 37°C for 24 hrs.

Extraction of DNA : One ml of enrichment was transferred in micro centrifuge tube and then centrifuged at maximum speed (15000rpm/3 min) to spin down the contents, then the supernatant was removed and the pellet was re suspended in 100 µl of PrepMan® Ultra Sample preparation reagent, then the contents were mixed by vortexing. The tube was heated in a heat block at 95-100°C/10 min and then centrifuged at 15000rpm/ 3 min to spin down the contents. Ten µl of the supernatant (sample DNA) was transferred to a new tube containing 90 µl of water, then vortex to mix the contents and then the sample DNA is ready for PCR.

Preparing the Sample for PCR: According to the number of samples the premix solution of master mix and assay was calculated and added in external screw capped tube. Both the samples and negative controls need 15 µl of master mix and 3 µl assays. Premix solution (18µl) was transferred into each well, gently pipetting at the bottom of the well. 12µl of unknown sample was transferred into each well, gently pipette to mix the solution. 12µl of negative control and positive controls were transferred and the tubes were closed.

Preparation of PCR Runs: The samples undergo running on real-time PCR System, plate was loaded into the instrument and then the cycle was adjusted as follow holding stage where the temperature was gradually raised

to 95.0°C/10mins followed by cycling stages which included 40 cycles; 15 min at 95.0°C and 1 hour at 60°C, then the run started.

Data Analysis and Documentation: Data analysis was carried out according to the flow-diagram of the screening procedure of VTEC according to EURL Method [25] for screening and detection of the presence of Verotoxin genes by Real Time PCR. Negative result to *vtx* gene will be reported as absence of VTEC. Positive samples to *vtx* genes will undergo test for O104 and O157.

Antibiotic Sensitivity Test Against Isolates: *In vitro* sensitivity of isolates (144) against six different antibiotics was carried out using agar diffusion antibiotic sensitivity test according to Beaney *et al*. [26]. The diameters of inhibition zones around wells were measured. Interpretation was carried out according to NCCLS [23].

Antibiotic discs were obtained from Oxoid including B-lactams (amoxicillin/clavulinic acid (20/10 µg/ml)), fluoroquinolones; ciprofloxacin (5 µg/ml), cefoperazone (30 µg/ml), tetracycline c(30 µg/ml) and tobramycin (10 µg/ml). The percentages of sensitive, intermediate and resistant bacterial isolates were shown in Table (3).

Antioxidant Markers Assaying: Nitric oxide (NO) was assayed in serum as described by Montgomery and Dymock [27] using commercial available kits (Bio-diagnostic, Cat. number NO2533). Lipid peroxidation product (Malondialdehyde, MDA) was assayed by the measurement of MDA levels on the base of MDA reacted with thiobarbituric acid and resultant colour read with spectrophotometer at532nm. According to Ohkawa *et al*. [28] using commercially supplied kits (Bio-diagnostic, Kit number MD2529). Blood serum glutathione reduced GSH was determined by spectrophotometer using the Bio-diagnostic kit (Cat number GR2511) according to Beutler *et al*. [29]. Ascorbic acid (Vitamin C, AA) was assayed using commercially supplied kits (Bio-diagnostic, Cat number MD2515) according to Harris *et al.* [30]. Zinc was assayed using commercially supplied kits (Bio-diagnostic, Cat number MD2120) according to Jap [31]. Alkaline Phosphatase (ALK) was analyzed using commercial diagnostic kit (El Nasr Pharamcological Chemicals, BIO ADWIC, Egypt, code No. T5-891) according to Bauer [32] depending on the principle p-nitrophenylphosphate+ H2O phosphate + p-nitropheno.

Hormone Assaying: Serum cortisol was assayed using Enzyme immunoassay kit supplied by Medical Biological Service S.R.L. (Milano, Italy). Sensitivity of the assay was 0.4 µg/dl, intra- and inter-assay CV were 2.9 and 3.8%, respectively [33].

Statistical Analysis: Descriptive statistical analyses were carried out with the SPSS [34]. Simple one way ANOVA between different animal groups and independent sample t-test to compare antioxidant parameters of treated animals before and after treatment with the sensitive antibiotics were processed. Results were presented as means and standard error of mean (SEM). Level of significance was set at a P-value 0.05.

RESULTS

Microbiological studies were carried out for screening of repeat breeder problem in equine stable. A total number of 78 samples was collected, including 25 vaginal swabs, 3 uterine swabs and 7 stallion semen samples. 24 rectal swabs; 14 mares and 10 stallions as well as 19 nasal swabs; 13 mares and 6 stallions. The study revealed that the most predominant bacteria were *E. coli* followed by Pseudomonas then *S. aureus*.

Out of 41 vaginal isolates *E.coli* was isolated with an incidence of 39%, followed by Pseudomonas 19.51%, then *S. aureus* 14.63%. Uterine isolates revealed 60% incidence of isolation of Pseudomonas and 20% for *E.coli* and *S. aureus.* In semen isolates the same bacterial isolates were identified with an incidence of 33.33% as shown in Table (3).

Twenty four rectal swabs were studied; 14 mare and 10 stallion. *E. coli* was isolated from mare rectal swabs with an incidence 34.15%, followed by *E. fecalis* (21.95%), Pseudomonas (19.51%) and then micrococcus (12.19%). Stallion rectal swabs showed isolation of *E.coli* with incidence 34.61% then *E. fecalis* (26.92%), Pseudomonas (19.23%) then *B. cereus* (11.53%) as shown in Table (4).

Mare nasal swabs revealed isolation *S. aureus* (61.54%) Pseudomonas (38.46%) then *E. fecalis* (30.77%).On the other hand, Stallion nasal swabs revealed that the highest rate of isolation was micrococcus (66.67%) followed by Pseudomonas with incidence 50% followed by *E.coli, S.aureus* and Pasturella (33.33%) as shown in Table (5).

Six *E.coli* stains isolated from vaginal swabs of repeat breeding mares undergo further investigations using PCR for detection of enteropathogenic gene (*sfa* gene) which was detected in four strains only with an incidence (66.67%). Our study indicated that *E.coli* strains which hinder *sfa* gene might play an important role in repeat breeding mare.

Table 3: Genital tract bacterial isolates

	Mare				Stallion			
	Vaginal(25)		Uterine(3)		Semen(7)		Total (35)	
Samples type								
Bacterial isolates	No	%	No	%	No	%	No	%
E.coli	16	39.00	1	20.00	1	33.33	18	36.73
Pseudomonas	8	19.51	3	60.00	1	33.33	12	24.49
S.aureus	6	14.63	1	20.00	1	33.33	8	16.33
E. fecalis	4	9.75	-	-	-	-	4	8.16
B.cereus	5	12.19	-	-	-	-	5	10.20
Micrococcus	2	4.87	-	-	-	-	2	-
Total	41	100	5	100	3	42.86	49	100
Mycotic isolates								
C. albicans	7	70.00	-	-	-	-	7	70.00
Asp.flavus	3	30.00	-	-	-	-	3	30.00
Total	10	100.00	-	-	-	-	10	100.00

Percent (%) of each isolated organism is calculated according to total No. of isolates

Table 4:Rectal bacterial isolates

	Mare Rectal (0+14=14)		Stallion Rectal (10+0=10)		Total 24	
Bacterial isolates	No	%	No	%	No	%
E.coli	14	34.15	9	34.61	23	34.33
Pseudomonas	8	19.51	5	19.23	13	19.40
S. aureus	3	7.32	2	7.69	5	7.46
E. fecalis	9	21.95	7	26.92	16	23.88
B. cereus	2	4.88	3	11.53	5	7.46
Micrococcus	5	12.19	-	-	5	7.46
Total	41	100	26	100	67	100
Mycotic isolates						
C.albicans	4	57.14	3	42.86	7	100

% of each organism is calculated according to total No. of isolates

Table 5: Nasal bacterial isolates

	Mare Nasal (7+6=13)		Stallion Nasal (4+2=6)		Total 19	
Bacterial isolates	No	%	No	%	No	%
E.coli	2	9.52	2	15.38	4	11.76
Pasturella	-	-	2	15.38	2	5.88
Pseudomonas	5	23.81	3	23.08	8	23.53
S.aureus	8	30.09	2	15.38	10	29.41
E. fecalis	4	19.04	-	-	4	11.76
Micrococcus	2	9.52	4	30.77	6	17.65
Total	21	100	13	100	34	100
Mycotic isolates						
Asp.flavus	3	50.00	3	50.00	6	100

% of each organism is calculated according to total No. of isolates

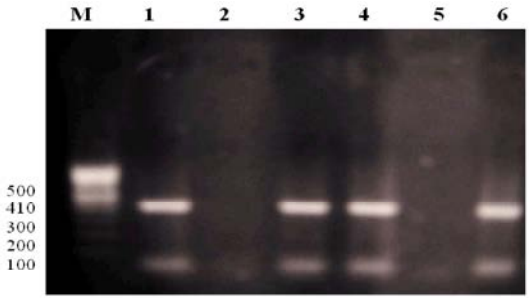
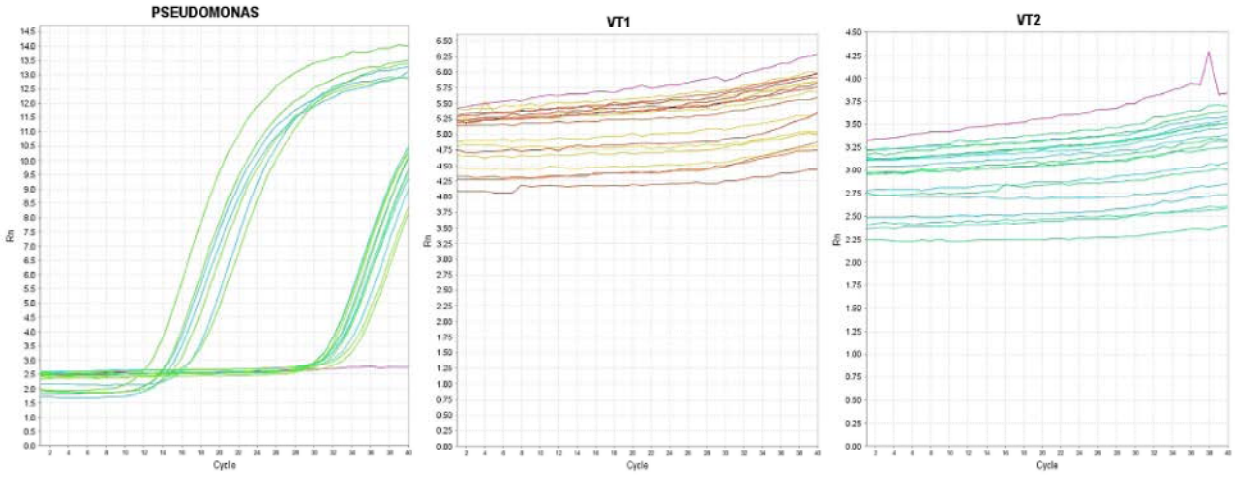


Fig. 1: Gel electrophoresis of DNA fragments showing 410 bp amplified fragment of *sfa* gene among the DNA of examined *E.coli*, isolated from vaginal swabs of repeat breeder (lanes: 1, 2, 5, 6, Mention +ve and -ve isolates?/) and aborted mares (lanes: 3, 4). Lane M represents DNA ladder.

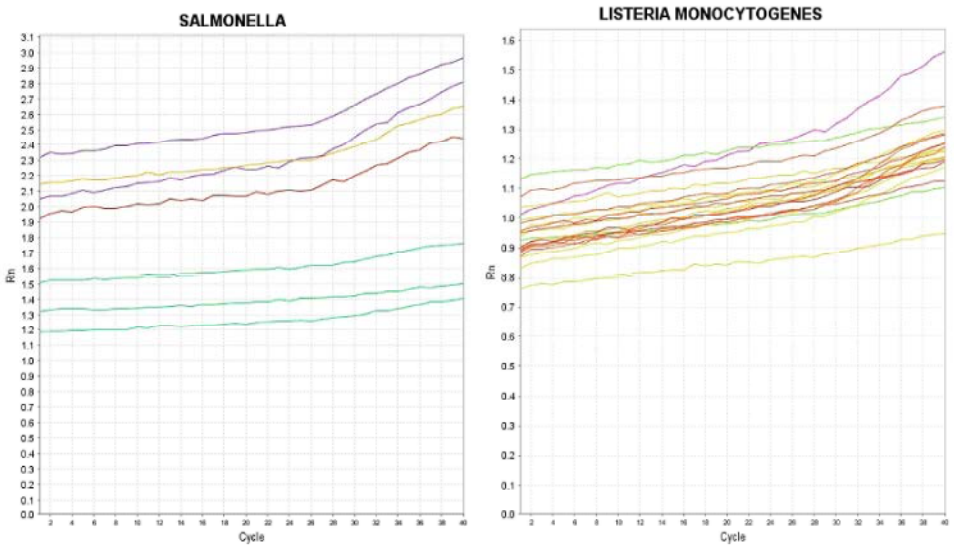
Real time PCR was used for further confirmation of the most predominant isolates ; *E. coli* and Pseudomonas, results confirmed positive Pseudomonas isolates (Figure 2) and all *E. coli* isolates were proved to be free from toxic genes (*vtx1* and *vtx2)* (Figures 3&4). Also, RT-PCR confirmed absence of Salmonella and *L. monocytogenes* infection in all tested samples Figures (5, 6).

Antibiotic sensitivity test was performed for isolated strains (144); *E. coli* (45), Pasturella (2), Pseudomonas (33), *S.aureus* (23), Micrococcus (7), *E. faecalis* (24), *B.cereus* (10). Results revealed that ciprofloxacin was the drug of choice for most cases with an incidence of sensitivity (100.00%) in all tested isolates and 86.96% for *S.aureus*, followed by tobramycin (100.00%) for Pasturella, Micrococcus and *B.cereus* then *E. fecalis* 87.50% and *E. coli* 77.78%. cefoperazone



(2) (3) (4)

Fig. 2: Graph showing positive curve for Pseudomonas samples and the internal positive control.
Fig. 3: Graph showing the negative curves of the VT1 samples.
Fig. 4: Graph showing the negative curve of VT2 samples.



(5) (6)

Fig. 5: Graph showing the negative graph of the salmonella sample.
Fig. 6: Graph showing the negative curve for *L. monocytogenes* in the tested samples.

showed high sensitivity towards *B. cereus* (100.00%), *E. fecalis* (87.50%), *S. aureus* (78.26%) and Micrococcus (71.43%).

Tetracycline was sensitive toward *B.cereus* (100.00%), *E.faecalis* (83.33%) and Micrococcus (71.43%) and finally amoxicillin/clavulinic acid showed sensitivity against *E. faecalis* (70.83%), *S. aureus* (60.87%) and Micrococcus (57.14%). Ciprofloxacin was selected for treatment of infected animals as a result of sensitivity tests performed (Table 6).

Mares with reproductive tract infection and mares with both respiratory-reproductive tract infections had significantly high cortisol levels (Table 7) compared to control mares (cyclic and early pregnant) and control stallions.

In stallions, concentrations of ascorbic acid (AA, Table 8) of blood serum and seminal plasma are significantly low (P=0.037) of stallions with respiratory infection compared to control stallions. Mares with reproductive-respiratory infection had

Table 6: Antibiotic sensitivity test against tested isolates

Antibiotics conc.	Tetracycline (30 µg/ml)		Amoxicillin/ clavulinic acid (20/10 µg/ml)		Ciprofloxacin (5 µg/ml)		Cefoperazone (30 µg/ml)		Tobramycin (10 µg/ml)		Erythromycin (10 µg/ml)	
Isolates (no)	No	%	No	%	No	%	No	%	No	%	No	%
E.coli (45)												
Sensitive	28	62.22	24	53.33	45	100.00	28	62.22	35	77.78	5	11.11
Intermediate	11	24.44	13	28.89	-	-	9	20.00	5	11.11	6	13.33
Resistant	6	13.33	8	17.78	-	-	8	17.78	5	11.11	34	75.56
Pasturella (2)												
Sensitive	1	50.00	1	50.00	2	100.00	1	50.00	2	100.00	1	50.00
Intermediate	1	50.00	1	50.00	-	-	1	50.00	-	-	1	50.00
Resistant	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas (33)												
Sensitive	-	-	-	-	33	100.00	-	-	-	-	-	-
Intermediate	3	9.09	4	12.12	-	-	2	6.06	5	15.15	-	-
Resistant	30	90.91	29	87.88	-	-	31	93.94	28	84.85	33	100.00
S.aureus (23)												
Sensitive	15	65.21	14	60.87	20	86.96	18	78.26	14	60.70	16	69.57
Intermediate	2	8.70	7	30.43	3	13.04	3	13.04	2	8.70	2	8.70
Resistant	6	26.09	2	8.70	-	-	2	8.70	7	30.43	5	21.74
Micrococcus (7)												
Sensitive	5	71.43	4	57.14	7	100.00	5	71.43	7	100.00	4	57.14
Intermediate	2	28.57	3	42.86	-	-	2	28.57	-	-	3	42.86
Resistant	-	-	-	-	-	-	-	-	-	-	-	-
E.faecalis (24)												
Sensitive	20	83.33	17	70.83	24	100.00	21	87.50	21	87.50	16	66.67
Intermediate	4	16.67	7	29.17			3	12.5	3	12.5	8	33.33
Resistant	-	-	-	-	-	-	-	-	-	-	-	-
B.cereus (10)												
Sensitive	10	100.00	5	50.00	10	100.00	10	100.00	5	50.00	5	50.00
Intermediate	-	-	5	50.00	-	-	-	-	5	50.00	5	50.00
Resistant	-	-	-	-	-	-	-	-	-	-	-	-

Table 7: Concentration of cortisol in blood serum of mares with isolates from reproductive tract (RT), both reproductive and upper respiratory (RT+URT) and control cyclic, early pregnant and stallions infected and control groups.

Group	Infected		Control		
Condition	RT	RT+URT	Cyclic	Pregnant	Stallions
N	7	5	22	7	19
Cortisol*	29.46±2.44^{c}	24.80±1.93^{bc}	19.55±1.67^{ab}	17.97±1.99^{ab}	14.04±1.73^{a}

*P=0.0001, means with different superscripts are significantly different at p<0.05

Table 8: Concentrations of ascorbic acid (AA), alkaline phosphatase (ALP) and nitric oxide (NO) in blood serum of mares with isolates from reproductive tract (RT), both reproductive and upper respiratory (RT+URT), stallions with isolates from upper respiratory tract (URT), seminal plasma and control cyclic and early pregnant mares, serum and seminal plasma (S.P.) of control stallions.

Groups	Infected group				Control group			
Gender	Mares		URT Stallions		Mares		Stallions	
N	10	5	3	4	34	6	16	5
Parameter	RT	RT+URT	serum	S.P.	Cyclic	Pregnant	serum	S.P.
AA	40.93±14.31^{ab}	19.15±5.45^{a}	10.15±4.39^{a}	55.21±37.4^{b}	31.35±1.02^{ab}	47.90±30.5^{ab}	23.01±9.68^{ab}	65.41±18.35$_{b}$
ALP*	79.09±29.05^{a}	64.00±58.80^{a}	102.20±23.38^{a}	600.00±00.00^{b}	105.07±23.14^{a}	86.40±19.28^{a}	112.70±56.05^{a}	505.10±94.90^{b}
NO	23.79±2.76	27.69±5.16	22.08±3.89	23.66±7.91	23.15±1.21	26.87±1.49	24.09±2.33	28.63±1.21

*P=0.0001, means with different superscripts are significantly different at p<0.05

Table 9: Concentration of lipid peroxidation product (MDA) and glutathione reduced (GHD) in blood and semen of infected and control groups.

	Infected group				Control group			
Groups	Mares		URT Stallions		Mares		Stallions	
Gender								
N	10	5	3	4	34	6	16	5
Parameter	RT	RT+URT	serum	S.P.	Cyclic	Pregnant	serum	S.P.
MDA	3.67±0.91	5.75±2.54	4.06±1.28	4.26±2.24	5.91±0.71	6.25±1.79	4.31±0.61	8.51±3.01
GHD*	8.78±1.11[a]	9.19±0.99[a]	8.36±0.34[a]	7.32±0.41[a]	10.02±0.77[a]	7.76±0.40[a]	10.06±0.96[a]	26.42±7.15[b]

*P=0.0001, means with different superscripts are significantly different at p<0.05

Table 10: Levels of cortisol, SOD, NO, ALK, AA, GSH, MDA, zinc and copper in treated mares.

Parameter	Before treatment	After treatment
Cortisol**	27.92±2.151	16.38±1.97
SOD*	1312.52±59.63	431.50±39.05
NO	21.51±2.48	10.22±5.16
Alkaline phosphatase	64.00±58.80	122.80±23.87
Ascorbic Acid	21.87±5.39	55.33±.36.77
Glutathione	10.35±1.37	7.78±1.12
Lipid peroxidase (MDA)	7.02±2.17	4.72±2.43
Zinc	0.106±0.01	0.113±0.00
Copper	0.44±0.13	0.48±0.15

* P<0.05, **P<0.01

significantly low ascorbic acid compared to those with reproductive tract infection, control pregnant and cyclic mares.

Semen samples of both control and infected stallions had high alkaline phosphatase enzyme levels (ALP, Table 8) compared to serum of other groups of mares. In addition, a slight insignificant increase of ALP in blood of control stallions compared to infected ones. Similarly, no significant change in ALP was observed in mares but both control cyclic and early pregnant mares had insignificantly high levels compared to infected groups. Nitric oxide (NO) levels are not significantly different (P=0.73) within infected mares and stallions but semen of control stallions and blood sera of mares with respiratory-reproductive tract infection are insignificantly high compared to the other groups(Table 8).

Lipid peroxidation product (Malondialdyhide, MDA) was not significantly different (P=0.41) between groups (Table 9). Seminal plasma of control stallions had high levels of MDA, followed by pregnant, cyclic control and infected mares with both reproductive and respiratory tracts. In contrast to lipid peroxidation, glutathione reduced (GHD) levels differed significantly between groups (Table 9).

Significantly high levels were observed in seminal plasma of control stallion compared to their blood sera. Mares with infected genital tract showed improvement in their oxidative stress markers after receiving treatment with the selected sensitive antibiotic (Table 10).

DISCUSSION

Microbiological studies for screening, repeat breeding problem in equine stable revealed that the most predominant bacteria were *E. coli* followed by *S. aureus* and *E. faecalis* then pasturella and finally diplococcus were isolated were isolated. One case only was infected with *C. albicans* (5.56%). Uterine bacterial isolates in mares showed that *Escherichia coli* (n=729, 29%) and β-hemolytic *Streptococcus equi sub sp zooepidemicus* (733, 28%) being most common [35]. *E.coli* causing pyometra in bitches [36] have been reported. In 55 mares, uterine pathogens were diagnosed in 20.0% of the mares and in five and six of the 55 mares, *Strept. zooepidemicus* 9.1% and *E. coli* 10.9% were detected in monoculture, respectively [37].

Antibiotic sensitivity test for 31 isolated strains revealed that ciprofloxacin is the drug of choice for most cases with sensitivity (96.77%), followed by tobramycin (87.1%), tetracycline and cefoperazone (80.65%) and amoxicillin/clavulinic acid (70.97%) but resistant to Erythromycin (75.56%). *E. coli* isolated from uterine swabs was most resistant to trimethoprim-sulfonamide and ampicillin and least to amikacin and enrofloxacin [35].

On investigating respiratory disorders, nasal swabs revealed high incidence of isolation of *S. aureus* micrococcus and *E.coli* with one case showed mycotic infection with *A. flavus*. Ciprofloxacin was used for treatment of infected cases except one case; stallion showing multiple drug resistant *S. aureus* and Asprigellus who was treated with vancomycin and fluconazole and a mare showing candidacies was given fluconazole as well.

Escherichia coli, the predominant facultative organism of the intestinal flora, can cause severe extra-intestinal infections, including infection of the kidney (pyelonephritis) or bloodstream (bacteremia). When it escapes from its usual habitat, *E. coli* can colonize the genital tract and, as a subsequent step, ascend to the bladder and kidneys [38]. Escherichia coli extraintestinal strains (ExPEC) possess several virulence traits that facilitate colonization, invasion and pathogenesis in

specific body locations [9]. The interaction of ExPEC with host cells is generally mediated by fimbriae, hair-like structures protruding from the bacterial cell surface with an adhesin at the distal end that enable bacterial adhesion to different host receptors. The bacteria also often have the ability to express different fimbriae in an alternative manner and in response to signals from the environment [39]. Proteins needed for production of different types of fimbriae such as S fimbriae is encoded by separate gene clusters as sfa. Analysis of the $SfaX_{II}$ protein sequence suggests that it belongs to the MarR family of transcriptional regulators, a protein family involved in mechanisms such us the control of antibiotic resistance, virulence factor production, as well as the response to oxidative stress [40].

On screening the presence of toxic gene (*sfa*), DNA of six *E.coli* stains isolated from vaginal swabs of repeat breeding mares were tested using PCR, (*sfa* gene) was detected in four strains with an incidence (66.67%), proving that *E.coli* strains hindering *sfa* gene might play an important role in causing cases of repeat breeding mares. Chen *et al.* [41] demonstrated that 17% of *E.coli* isolates from canine pyometra carried two Fimbrial genes (fimH and sfa/foc) and 50% carried three (FimH, sfa/foc and papGIII). The sfa/foc gene is present in 70% of canine pyometra isolates and in 54% of faecal isolates [41]. Many *E. coli* strains isolated from the uteri of infected dogs carry several adhesion genes (fimH, papGIII and sfa) [36].

Cortisol levels were significantly high in mares with reproductive tract infection and mares with both respiratory and reproductive tracts infection compared to control mares and stallions. During this work, repeat breeder mares underwent several gynecological examinations before breeding to access size of mature follicles and after natural breeding to detect early pregnancy using ultrasound and sometimes twitching is used in addition to moving the animal to the examination spot and even detection of infection, all these factors may increase cortisol levels to values higher than those of horses. Although gynaecological examinations in the mare seem to act as stressors and increase cortisol secretion but this does not negatively influence fertility and in animals familiar with that procedure concentrations are not elevated [42]. Concentrations of cortisol in horses increase instantly after exercise [43] and after sexual activity [44] but return again to their basal levels before exercise or sexual excitation. Moreover, long-term exposure as in teaching or research mares results in a customization. But the higher cortisol levels of infected mares are referred to infection. In contrast to cattle and sheep, no effects of cortisol on fertility parameters (oestrus duration, pregnancy rates) could be found in the horse [42]. In agreement with our results, acute abdominal syndrome (AAS), acute diseases and laminitis increased cortisol significantly (6 folds) but castration and chronic diseases increased it nearly 2 folds than controls [46]. In contrast to our findings, chronic inflammation in the horse depressed resting plasma cortisol concentrations [46].

Reactive oxygen species (ROS) are required for normal physiologic processes, but when produced in excess, they can overwhelm endogenous antioxidants, resulting in significant cellular damage and, eventually, cell death. Ischemic events can initiate numerous pathophysiologic mechanisms leading to increased production of ROS, loss of cellular energy production and lipid peroxidation. Although reperfusion is a necessary step in cellular recovery from ischemia, it can be deleterious by leading to the generation of even more ROS and stimulating the accumulation of neutrophils [47].

As well as infection detected during this study resulted in imbalance between oxidants and antioxidants equilibrium in mares and stallions and treatment improved this imbalance, infection with equine infectious anemia virus modified the oxidant/antioxidant equilibrium in the horses, influencing GPx and uric acid levels ($P<0.05$). Time post-seroconversion also contributed to oxidative stress imbalance, exhibiting a significant influence on both SOD and MDA concentrations in the blood [48].

In stallions, concentrations of ascorbic acid in blood were significantly low in stallions with respiratory infection compared to control ones but its levels in seminal plasma of both infected and control stallions were significantly high than blood sera levels. In mares, levels of ascorbic acid were significantly low in mares with both reproductive and respiratory infection compared to those with reproductive tract infection and control pregnant and cyclic mares. In agreement with our results, horses affected with respiratory airway obstruction (RAO) had low levels of Vitamin C in pulmonary epithelial lining fluid which was more pronounced in hoses affected by airway inflammation [49].

Nitric oxide (NO) levels were not significantly different ($P=0.73$) in infected mares and stallions but semen of control stallions and blood sera of mares with both respiratory and reproductive tract infection were insignificantly high compared to the other groups. NO levels were not changed in both plasma and broncho alveolar lavage in horses with summer pasture-associated obstructive pulmonary disease but the intrinsic nitric oxide synthetase (iNOS) increased in bronchial epithelium

[50] and in plasma and synovial fluid in horses with joint disease [51]. Parallel to microbiological infection, infestation with blood parasites leads to oxidant/antioxidant imbalance. A highly significant increase in NO ($P<0.001$), a significant increase in LPO ($P<0.05$) and a significant decrease in GSH, SOD and CAT ($P<0.05$) were found in horses naturally infected with Trypanosoma evansi. The increase in oxidant parameters and decrease in antioxidant enzymes in infected horses indicates the disturbance of oxidant/antioxidant indices [52].

GSH is the major extracellular antioxidant of the lung and the most prevalent cellular thiol, lung can import substantion GSH amounts from plasma [53-55]. Little is known about the relationship between GSH and equine lung disease. Airway inflammation in the horse significantly increased GSH with a strong correlation between severity of the inflammation score and the GSH redox ratio [47]. In contrast to lipid peroxidation, glutathione reduced (GHD) levels differed significantly between groups. Significantly high levels were observed in seminal plasma of control stallion compared to their blood sera. In horses with RAO levels of oxidized GSH increased in pulmonary epithelial lining fluid [56]. Moreover, horses suffering from recurrent airway obstruction (RAO) or heaves are subject to pulmonary and systemic oxidative stress, which has been demonstrated by increased levels of GSH, GSSG, 8-isoprostane, MPO or decreased levels of ascorbic acid in pulmonary epithelial lining fluid (PELF) and increased levels of GSH and GSSG in erythrocytes [49, 56-58]. Hydrogen peroxide (H_2O_2) in exhaled breath condensate has also been assessed and has been correlated with ascorbic acid concentration in PELF [59]. The lowering of ascorbate levels was most pronounced in horses affected by airway inflammation and the number of neutrophils in broncho-alveolar lavage fluid was inversely correlated with the concentration of ascorbate in PELF [49].

CONCLUSION

To prevent the spread of any disease to susceptible horses through breeding, rapid and accurate diagnosis of infected cases and the implementation of appropriate managerial procedures are critical. A stallion carrier of a bacterial disease should not be used for breeding through natural service. Stallions should undergo complete semen evaluation as well as microbiological examination for semen samples as well as pre- and post-ejaculation urethral swabs. A stallion carrier of a bacterial disease should not be used for breeding through natural service.

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