ISSN: 2356-7767

Molecular Studies of Some Bacterial Causes of Incurable Wounds in Equines

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Received in 5/11/2018
Accepted in 11/12/2018

Abstract

Equines are usually subjected to wounds during racing or even breeding from sharp edges. These wounds may be contaminated with various bacterial species resulting in wound infection which mostly become chronic if not treated. A total of 50 wound swab samples were collected from different equine farms and veterinary hospitals at Giza and Cairo Governorates from the period of October 2016 to March 2017 and analysed microbiologically for aerobic and anaerobic bacteria using standard bacteriological techniques and antibiotic susceptibility testing was performed by disc diffusion method according to following CLSI guidelines. Both single and mixed infections were recorded. Among single infection, *S. aureus* was the predominant bacteria, *P. mirabillis, E. coli, K. pneumoniea, C. perfringens* and *P. stuartii* were recorded while mixed infection was found in 8 cases with the isolation of *S. aureus* with *E. coli* and 6 cases *S. aureus* with *C. perfringens* and *P. miabillis*. The isolated strains varied in their sensitivity against different 12 antimicrobial agents. By PCR assay, resistance genes for erythromycin, beta-lactamase, tetracycline, sulphonamides and trimethoprim sulphate were detected in some isolates and the results showed that *C. perfringens* was harboured tetracycline, erythromycin and beta-lactamase resistance genes, while *S. aureus* possessed beta-lactamase gene resistance. On the other hand, *E. coli* and *K. pneumoniea* had resistance gene against trimethoprim sulphate. Field recommendation to avoid wound infection and its complication was discussed.

Keywords: *Equines wounds, gene resistance, antimicrobial sensitivity.*

Introduction

The basic nature of horses seems to put them at risk for traumatic injuries. One of the most common reasons that clients present their horses to the veterinarian is trauma that results in skin and soft tissue wounds.

Lower limb wounds are particularly susceptible to colonization from microorganisms and are notoriously problematic. The risk of infection is heightened because horses are exposed to a vast array of microorganisms that have the ability to colonize open wounds. Maintaining a wound microenvironment that helps to suppress microbial proliferation at problematic anatomical sites, such as dorsal tarsocrural region, is of paramount importance. **(Westgate *et al.,* 2010).**

Both acute and chronic wounds are highly prevalent in horses and represent a significant management challenge to veterinary surgeon **(Carter *et al.,* 2003).** Particular concern exists in equine wounds that are at risk from infection.

The extensive usage of drugs has led to many problems like the emergence of multidrug-resistant bacteria **(Zunita *et al.,* 2008).** The existing approaches for treating animal diseases in developing countries, without being sure what microorganisms exactly are involved in producing the diseases, would build an army of indestructible organisms that will become more resistant to new drug **(Levy, 2002).**

Clark *et al.,* (2008) found that the most commonly isolated bacteria from contaminated horses wounds were Enterobacteriaceae, non-β -hemolytic streptococci and coagulase negative

staphylococci. They added that due to the inclusion of septic foals and iatrogenic infections, there was a high rate of antimicrobial resistance and concluded that the combination of a cephalosporin and amikacin became the standard recommended antimicrobial therapy for all musculoskeletal infections.

Shuaib *et al.,* (2016) threw the light on bacterial infections on skin wounds in equines,
the study revealed 10 different species of aerobic and anaerobic bacteria including *S. aureus, S. epidermidis, S. citreus*, *Streptococcus* species, *Pseudomonas* species, *E. coli*, *Salmonella* species, *Clostridium* species*, Shigella* species and *Klebsiella* species.

With the increase in multiple antibiotic resistances, treating these infected wounds is not as cut-and-dried as it used to be. **(Beckstett, 2016).** Today veterinarians cannot just reach for the nearest antibiotic, they must consider the wound's entire ecology, the patient's immune health, and the pathogens involved.

This study was aimed to identify different bacterial species isolated from incurable chronic wounds in horses and to throw the light upon sensitivity and resistance profile of these isolates against different antibiotics. Moreover, detection of antibiotic resistance genes of some isolates by PCR was carried out.

Materials and Methods

1. Animals: This study was carried out on 50 horses suffering from chronic wound infections at different sites of the body (shoulder, whither, hind quarter, fore and hind limbs) in some equine farms and veterinary hospitals at Cairo and Giza Governorates during period from October 2016 to July 2017.

2. Samples: A total of 50 wound swab samples were collected in duplicate from the infected horses using sterile swabs. The collected swabs were preserved in transport media (Amies, Oxoid-CM425) and kept in an ice box and sent to the laboratory without delay for bacteriological examination (Aerobic and anaerobic). The distribution of the samples according to the location of collection and location of wound are illustrated in table 1.

3.Identification of bacterial isolates: (Koneman *et al.* 1997 and Quinn *et al.*, 2011).

-Aerobic bacteriological examination: Swabs were cultivated into nutrient broth and then plated onto blood agar, manitol salt agar and McConkey agar media and incubated aerobically at 37ºC for 24 hours.

- Anaerobic bacteriological examination:
The collected swabs were inoculated in cooked meat broth and were incubated anaerobically at37ºC for 24 hours then plated onto blood agar and neomycin blood agar (with 200 µg neomycin sulphate) plates and incubated anaerobically at 37º C for 24 hours.

The growing surface on all media colonies were identified by colony characters, microscopical examination, biochemical characters and API test.

Table (1). Distribution of samples according to location of sample collection and wound location

Location of sample collection	No. of samples	Site of wounds	No. of samples
Centre (1)/Giza	8	Shoulder	8
Centre (2)/Giza	10	Whither	3
Centre (3)/Cairo	7	Hind quarter	14
Centre(4)/Cairo	8	Fore limb	10
Vet. Faculty Hospital	17	Hind limb	15
Total	**50**		**50**

4. Antibiogram test (CLSI, 2006):

The isolated bacterial species were tested for their antibiotic sensitivity by culture onto Muller Hinton agar (Bauer-Kirbys disc diffusion method) according to clinical laboratory standard institute (CLSI) guidelines **(CLSI, 2006)** to check the sensitivity of microorganism. Twelve different antibiotics were used to detect the susceptibility and resistance of isolates. Sensitive microorganisms show zone of inhibition around the disc. Resistance gene profiles were examined using PCR to confirm the antibiogram results.

5. Detection of resistance genes of the bacterial isolates 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 (2). PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

6. Analysis of the PCR Products (Sambrook *et al.*, 1989): 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, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen, Germany, GmbH) and a 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 (2). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplif. segment (bp)	Primary Denature.	Amplification (35 cycles)			Final extension	Reference
				Secondary-denature..	Annealing	Extension		
ErmB (C. perfringens)	GAA AAG GTA CTC AAC CAA ATA AGT AAC GGT ACT TAA ATT GTT TAC	638	94˚C 5 min.	94˚C 30 sec.	57˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Soge *et al*., (2009)**
Bla (C. perfringens)	ATGAAA-GAAGTTC AAAAATA TTTAGAG TTAGTGCC AATTGTTC ATGATGG	780	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Catalán *et al*., (2010)**
tetK (C. perfringens)	TTATGGTG GTTGTAG CTAGAAA AAAGGGT TAGAAAC TCTTGAA A	382	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Ghola-miandehkordi *et al*., (2009)**
blaZ (S. aureus)	ACTTCAA CACCTGCT GCTTTC TGAC-CACTTTTA TCAGCAA CC	173	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Duran *et al*., (2012)**
Sul1 (E. coli, K. pneumoniea, C. perfringens, S. aureus and *P. mirabilis)*	CGGCGTG GGCTACC TGAACG GCCGATC GCGTGAA GTTCCG	433	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Ibekwe *et al*., (2011)**
dfrA (E. coli, K. pneumoniea)	TGGTAGC TATATCG AAGAATG GAGT TATGTTA-GAGGCGA AGTCTTG GGTA	425	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Grape *et al*., (2007)**

Results

Prevalence of wounds among animal bodies:

According to the number of samples collected, it had been shown that hind limbs were mostly infected with wound (15 samples as 30%), followed by hind quarter (14 samples as 28%), fore limbs (10 samples as 20%), shoulder (8 samples as 16%) and lastly the less affected part of animal body is the whither (only 3 samples were collected as 6%) as shown in table (3).

Table (3). Prevalence of wounds among animal bodies

Serial	Animal body	Collected wound samples	
		No.	%
1	Hind limb	15	30
2	Hind quarter	14	28
3	Fore limb	10	20
4	Shoulder	8	16
5	Whither	3	6

*% calculated according to the No. of samples examined (50)

Prevalence of bacterial species:

All wound samples collected from infected horses were positive for different bacterial species. Table (4) illustrates the various bacterial species isolated from wound swabs. Mixed infections were recorded in 14 samples out of 50.

Table (4). Bacterial species isolated from wound swabs of examined horses

Bacterial isolates	No. of samples	No. of isolates	
Single infection:		**No.**	**%**
Staphylococcus aureus	9	9	12.9
Echerichea coli	10	10	14.3
Proteus mirabillis	5	5	7.1
Klebseilla pneumoniea	5	5	7.1
Providence stuartii	1	1	1.4
Clostridium perfringens	6	6	8.6
Mixed infection		**No.**	**%**
S. aureus + E. coli	8	16	22,9
C. perfringens + S. aureus + P. miabillis	6	18	25.7
Total	50	70	

*percentage was calculated according to the total number of the isolates

Twenty three *S. aureus* isolates were detected out of total microbial isolates in an incidence of 32.9% while *E. coli* was isolated in an incidence of 25.7%. Moreover, 15.7% of the wound samples were *P. mirabillis*. *K. pneumonia, P. stautti, C. perfringens* were detected in an incidence of 7.1%, 1.4%, 17.1%, respectively.

Table (5). Prevalence of bacterial species isolated from wound swab of examined horses in relation to the total number of samples examined (50)

Total number of samples examined	Organisms	Positive samples	
		No.	**%**
50	*S. aureus*	23	46
	E. coli	18	36
	C. perfringens	12	24
	P. mirabillis	11	22
	K. pneumoniea	5	10
	Providence stuartii	1	2

*Calculated % was according to the No. of samples examined (50).

From presented data in table (5), it had been shown that *S. aureus* was detected in 23 samples out of 50 examined ones (46%), *E. coli* was detected in 18 samples out of 50 examined ones (36%), *C. perfringens* was detected in 12 samples (24%) and lastly, *P. mirabillis* was detected in 11 samples (22%). On the other hand, 5 samples were positive for *K. pneumoniea* and only one sample was positive for *Providence stuartii* (10%, 2%) respectively.

Antibiotic sensitivity of the isolated bacterial species:

Table (6) shows the antimicrobial sensitivity test of the isolates against 12 antibiotics. *S. aureus* was proved to be resistant to Amoxicillin, Kanamycin, cloxacillin and tetracyclin. While *K. pneumoniea* was resistant to manyantibiotics specially trimethoprim sulphate, cephalothin, erythromycin and penicillin. Mean while, *P. mirabilis* was almost resistant to all antibiotics except to epicoflocin. However, *E. coli* was totally resistant to Trimethoprim sulphate and in a large extent to erythromycin and tetracyclin. Finally, *C.perfringens* was completely resistant to streptomycin and amikacin and 75% of their isolates were resistant to kanamycin and oxytetracyclin.

Table (6) Shows the antimicrobial susceptibility test of bacterial isolates against 12 antibiotics using Bauer-Kirbys disc

isolate	*S. aureus* (23)				*K. pneumoniea* (5)				*P. mirabillis* (11)				*P. stautti* (1)				*E. coli* (18)				*C. perfringens* (12)			
	S		R		S		R		S		R		S		R		S		R		S		R	
antibiotic	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	No.
Epi-coflocinO FX5	21	91.3	2	8.7	5	100	0	0	9	81.8	2	18.2	1	100	0	0	16	88.9	2	11.1	10	83.3	2	16.7
Trimethoprim Sulphate SXT_{25}	21	91.3	2	8.7	0	0	5	100	11	100	0	0	1	100	0	0	0	0	18	100	10	83.3	2	16.7
Cloxacil-line CX_{15}	3	13	20	86.9	0	0	5	100	11	100	0	0	1	100	0	0	14	77.8	4	22.2	4	33.3	8	66.7
Oxytetra-cycline T_{30}	20	86.9	3	13	5	100	0	0	0	0	11	100	0	0	1	100	15	83.3	3	16.7	3	25	9	75
Amikacine AK_{30}	1	4.3	22	95.4	5	100	0	0	7	63.6	4	36.4	0	0	1	100	16	88.9	2	11.1	0	0	12	100
Cepha-lothin KF30	22	91.3	0	0	0	0	5	100	4	36.4	7	63.6	0	0	1	100	2	11.1	16	88.9	12	100	0	0
Erythro-mycin E_{15}	23	100	0	0	0	0	5	100	2	18.2	9	81.8	0	0	1	100	3	16.7	15	83.3	10	83.3	2	16.7
Kanamy-cin K_{30}	1	4.2	22	95.4	0	0	5	100	0	0	11	100	1	100	0	0	5	27.8	13	72.2	3	25	9	75
Strepto-mycin S_{10}	23	100	0	0	0	0	5	100	3	27.3	8	72.7	1	100	0	0	18	100	0	0	0	0	12	100
Penicillin p_{10}	11	47.8	12	52.2	0	0	5	100	2	18.2	9	81.8	0	0	1	100	0	0	18	100	10	83.3	2	16.7
Amoxicil-lin AX_{25}	3	13	20	86.9	3	60	2	40	5	45.5	6	54.5	0	0	1	100	4	22.2	14	77.8	12	100	0	0
Tetracy-cline TE_{30}	2	8.7	21	91.3	3	60	2	40	0	0	11	100	0	0	1	100	3	16.7	15	83.3	3	25	9	75

% calculated according to the No. of tested bacterial isolates

Results of PCR:

One random isolate of each of *C.perfringens, S.aureus, E.coli* and *K.pneumoniea* was subjected to PCR test to investigate the presence of resistance genes against some antibiotics that show little or no inhibitory effect on them in sensitivity test. Photo (1) shows resistance pattern of *C.perfringens* against some antibiotics. Resistance genes against tetracyclin, erythromycin and beta-lactamase were detected at 382,638 and 173 bp. While photo (2) illustrates the resistance pattern of *S.aureus* against beta-lactamase at 173 bp. On the other hand, *E.coli* and *K.pneumoniea* had resistance gene against trimethoprim gene (drfA) at 425 bp as shown in photo (3). Meanwhile, resistance gene of sulphonamide was detected in all isolates at 433 bp. (photo 4)

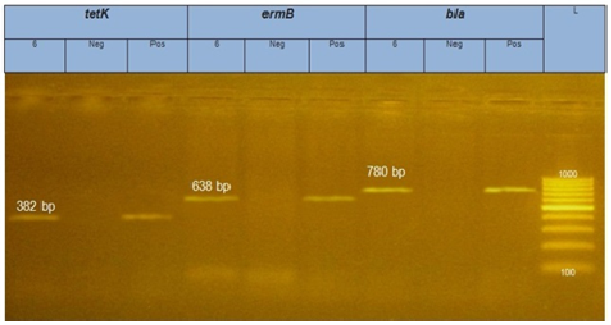

Photo (1): Agarose gel electrophoreses for the amplified product of some antibiotic resistance genes in *C.perfringens* : tetracycline *(tetK)* at 382 bp.:
lane1: the positive tested sample, lane2: negative control, lane3: positive control; erythromycin (*ermB*) at 638 bp: lane1: the positive tested sample, lane2: negative control; lane3: positive control and beta lactamase (*bla*) at 780bp: lane1: the positive tested sample, lane2: negative control; lane3: positive control. L: the ladder (100-1000).

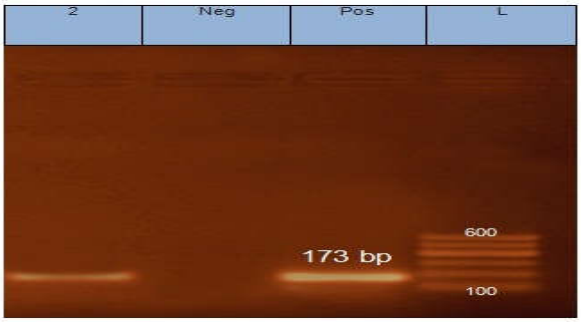

Photo (2): Agarose gel electrophoreses for the amplified product of beta latamase (*blaZ*) in *S.aureus e*at 173 bp.; lane no.2:the positive tested sample ; Neg.: negative control; Pos.: positive control; L: ladder(100-600).

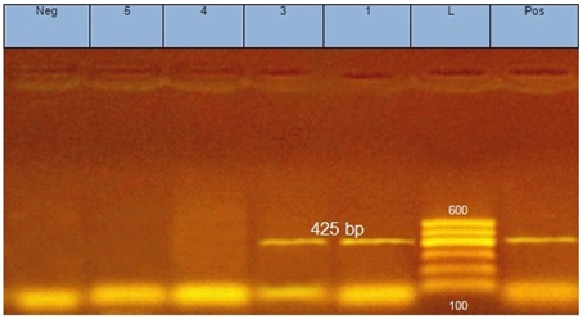

Photo (3): Agarose gel electrophoreses for the amplified product of trimethoprim (*dfrA)* at 425 bp in *E,coli* positive tested sample (lane1) *and K.pneumoniea* positive tested sample (lane3), negative sample of *C.perfringens* (lane4), negative sample of *S.aureus*
(lane5), L:ladder; Pos.: positive control; Neg.: negative control.

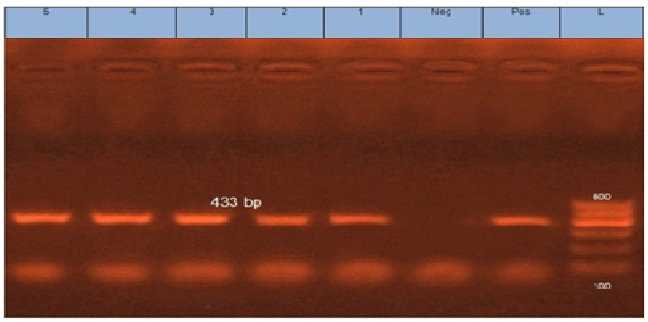

photo (4): Agarose gel electrophoreses for the amplified product of sulphonamide (*sul1*) at 433bp; L:ladder; Pos.: positine control; Neg.: negative control; lane1:*E.coli* positive tested sample; lane2: *K.pneumoniea* positive tested sample*; lane3: S.aureus* positive tested sample.; lane4: *C.perfringens* positive tested sample and lane5:*P.mirabillis*positivetested sample

Discussion

Horse wound have a high risk of becoming infected due to their environment. Infected wounds harbour diverse populations of microorganisms, however in some cases, these microorganisms can be difficult to identify and fail to respond to antibiotic treatment resulting in chronic-healing wounds (**Westgate *et al.,* 2011**). In this study, different samples were collected from wounds at different sites in infected horses. Hind quarters and hind limbs were the most affected sites (28% and 30% respectively). **Westgate *et al.,* (2010)** stated that microorganisms in lower limb wounds are particularly susceptible to colonization and are notoriously problematic. The risk of infection is heightened because horses are exposed to a vast array of microorganisms that have the ability to colonize open wounds. They added that specific anatomical reasons for the failure of distal limb healing that have been proposed, include a relatively poor blood supply and reduced tissue oxygenation compared to the thoracic region. A more meaningful classification of the microbial status of wound infection is the point when the host's immune response is triggered **(Kingsley, 2001).**

In this study, 50 swabs of horse wounds at different sites of the body were collected and examined for the microbial status. Single and mixed infections with Gram +ve and Gram –ve bacteria were detected. Six types of bacteria were detected and included *Staph. aureus* (46%), *E. coli* (36%), *C. perfringens* (24%), *Proteus mirabilis* (22%), *K. pneumonae* (10%) and lastly *Providence stuartii* (2%). The most detected isolates were *S. aureus*, this finding were in agreement with the finding of **Shuaib *et al.,* (2016) and Bzdil *et al.,* (2017**) who stated that *S.aureus* causes supportive wound infection, septicaemia, pyoderma and subcutaneous abscesses and this may be slightly different from that recovered by (**Westgate *et al.,* 2011)** who found that *pseudomonas spp.* was the most predominant species recovered from chronic wound and surgical in horses.

Providence stuartii are G-ve ubiquitous in the environment and are also known to cause nosocomial infection. *Providence stuartii* able to adhere to and include Hela epithelial line, these is important for understanding the molecular mechanism of pathogeneses and control infections caused by *Providence stuartii* **Kurmasheva *et al.,* (2018).**

Equine wounds were polymicrobial and contained pathogens which were significantly more prevalent in chronic wounds than acute wounds (**Westgate *et al*., 2011**). Mixed infections were also recovered in this study as *E. coli* together with *S. aureus* were detected in 22.9% of the isolates while, *S. aureus*, *C. perfringens* and *P. mirabillis* were detected as polymicrobial infection in about 25.7% of the isolates. It is interesting that *Staph. aureus* dominated which correlates with data obtained by **Songer and Post (2005).** The isolation of multiple bacterial species per sample supports the theory that equine in vivo wound enviroments harbour a polymicrobial community of microorganisms **Freeman *et al*., (2009).**

The resistance pattern of isolates from wound infection in horses, was found to be a little but different from those detected by (**Osman *et al*., 2014)** who found that 60.9% of *Klebsiella spp*. were susceptible to sulphamethazole/trimey and 69.6% were susceptible to oxytetracyclin, while none of *Klebsiella spp*. isolated in this study were sensitive to these antibiotics. Moreover, 100% of *Klebsiella spp*. recovered by (**Osman *et al.,* 2014)** were found to be sensitive to kanamycin, while those isolated from wound infection of horses in our study were resistant to it. Variations in the results may be due to strain variations and gene mutations.

Trimethoprim (TMP) affect bacterial folic acid synthesis by the inhibition of dihydrofolate reductase (DHFR), which catalyses the reduction in dihydrofolate **(Huovinen, 2001 and Skold, 2001).** Bacteria may become resistant to TMP by several mechanisms, including the development of permeability barriers, efflux pumps, the existence of naturally insensitive target

DHFR enzymes, mutational and regulation changes in target enzymes and the acquirement of drug-resistance target enzymes **(Huovinen, 2001)**. On the basis of the length of encoded polypeptide, TMP resistant drf genes have been divided into two major types, dfr A and dfr B **(White and Rawlinson, 2001).** Resistance to TMP among clinical *E. coli* isolates varies greatly, ranging from 10-70% in different parts of the word (**Kahlmeter and ECO.SENS, 2003; Lee *et al.,* 2001**) In this study, dfrA gene was detected in *E. co*li and *K. pneumoniae* tested isolates explaining the complete resistance against trimethoprim sulphate in both microorganisms in sensitivity test with incidences of 100%.
Proteus mirabilis which belongs to the *Enterobacteriaceae* family and incriminated in urinary tract infections (UTI), can also cause respiratory, wound infections, bacteremia and other infections **(Mobley and Belas, 1995; Rozalski *et al.,* 1997).**

Bacteria have developed different mechanisms render ineffective the antibiotics used against them. The genes encoding these defence mechanism are located on the bacterial chromosome or on extra chromosomal plasmids **Thomas *et al.,* (2003).** The emergence of bacteria resistant to antibiotics is common in areas where antibiotic are used. The widespread are of antibiotics in medicine and in intensive animal husbandry is indicative of the selection pressure exerted on bacteria **Klare, *et al.,* (1995).**

Conclusion and recommendations
Equine particularly horses are usually subjected to wounds during racing, work or even breeding from adjacent sharp edges in the stable.
Wounds affected horses are usually contaminated with various species of microorganisms, mainly *staphylococcus aureus, Escherichia coli, Proteus mirabillis, Klebsiella pneumoniea, Providence stuatti* and *Clodtridium perfringens*. Some of the bacterial species may be resistant to the antibiotic treatment either local or systemic which resulted in complication of the infected wounds which reflected on the animal health and their activity.

There is a relatively high prevalence of antibiotic resistant *K. pneumoniea* so monitoring of drug resistant isolates are urgently needed in order to better control the emergence and spread.
Strict hygienic measures by using powerful disinfectants should be applied in equine farms to control the distribution of microorganisms.
As possible, avoidance of occurrence of wounds in horses particularly during parturition, application of horse shoes, work ect… and avoid sharp edges in the stable.
Treatment of wounds as soon as possible just occurred by local antibiotic and systemic antibiotic.
Treatment methods that are employed in the management of horse wounds focus on:
- Rapid and efficient wound evaluation.
- Prolonged after care.
-Variation in healing rates.
The use of antibiotics should be regularly reviewed.
Daily care and monitoring of horse wounds can great assit or imped the progress.

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