

**ROLE OF VIRULENCE AND MULTIDRUG RESISTANCE OF
STREPTOCOCCUS ZOOEPIDEMICUS IN CAUSING ENDOMETRITIS
AND RESPIRATORY INFECTION IN EQUINE**

By

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ABSTRACT

Streptococcus zooepidemicus is an opportunistic pathogen associated with a wide variety of diseases e.g. pneumonia, septicemia, mastitis, placentitis and endometritis. The pathogenesis of a respiratory infection with *S. zooepidemicus*, factors associated with the bacteria, as well as with the host, appear to play a major role in the clinical appearance and outcome of the disease. *S. zooepidemicus* was isolated from 91 out of 136 samples of foal's disease (38.9%). The highest rate was from nasal swabs of diseased foals (83.5%) followed by lung and lymph nodes of dead foals (66.6%) and the lowest rate (16%) of isolation was from nasal swabs of apparently healthy foals. On the other hand, 17 isolates of *S. zooepidemicus* (42.5%) were isolated from 40 samples collected from mares (vaginal wash and placenta) and 68.3% from aborted feti. Six isolates (3 from respiratory cases and 3 from abortion cases) were subjected to PCR for detection of virulence *sodA* gene, where all isolates were positive. The most effective antibiotic for *S. zooepidemicus* was ceftiofur (75%) and vancomycin. Heat map analysis showed the intensity of resistance of *S. zooepidemicus* isolates were multidrug resistant mainly for β lactams (Cephalosporins), β lactams (Pencillins) and the resistance ranged from 100% to 25%, also the resistance was for tetracyclines (100 to 90%); fluoroquinolones (100 to 50 %), Lincosamides (100 to 75%) and glycopeptides (vancomycin 75 to 25%). Also, heat map analysis showed that *S. zooepidemicus* isolated from aborted feti showed high intensity of resistance to most antibacterial agents used in the study followed by those isolated from mares. These results revealed the isolates causing abortion has different multidrug resistance profiles than that isolated from foal with respiratory symptoms.

INTRODUCTION

S. equi subspp. Zooepidemicus (S. zooepidemicus) appears to be part of the normal bacterial microflora of the upper respiratory tract and caudal reproductive tract of horses, and is also found in healthy carriers of other species such as pigs and monkeys (Salasia *et al.*, 2004).

S. zooepidemicus is an opportunistic pathogen associated with a wide variety of diseases e.g. pneumonia, septicemia, mastitis, placentitis and endometritis (**Timoney et al., 1998**).

The prevailing hypothesis is that isolates of *S. zooepidemicus*, residing in the lower reproductive tract, cause infectious endometritis by an ascending infection in a random manner primarily governed by the uterine defense mechanisms of the mare (**Wittenbrink et al., 2008**). *S. zooepidemicus* is the most frequently isolated pathogen from the uterus of the mare (**Ricketts, 2011**). **Caslick, (1937)** reported that for *S. zooepidemicus* to be able to reach the uterus it has to pass three physio-anatomical barriers; the vulva, the vestibule vaginal sphincter and the cervix. Poor anatomical conformation of the internal and external reproductive organs may weak these barriers and allows bacteria to ascend into the uterus. Contamination of the uterus also takes place during live cover, artificial insemination, (**LeBlanc and McKinnon 2011**).

S. zooepidemicus is considered to be a mucosal commensal of the upper respiratory tract of horses which causes disease as an opportunistic pathogen of the lower respiratory tract (**Erol et al., 2012**). An increased attention has been placed upon antimicrobial resistance in both medical and veterinary field (**Toombs et al., 2015**).

The goal of this study was to detect the role of *S. zooepidemicus* in causing respiratory infection, endometritis and abortion in mares. The antibiotic susceptibility was done to detect the MDR strains of isolates and the virulence encoding gene as *sodA*.

MATERIALS AND METHODS

A total of 236 samples were collected from horses as follows: a) fifty nasal swabs from apparently healthy, b) 26 nasal swabs from diseased foals suffering from pneumonia, c) lung, liver, spleen and lymph nodes of 15 dead foals with pneumonia, d) 30 samples of vaginal wash, e) ten samples of placenta, and f) 60 samples of internal organs and stomach contents of aborted feti (10 of each organ).

All samples were collected aseptically using sterile swabs and bags. Each sample was kept in transport medium (Amies, Oxoid) and kept at 4°C until processing, and internal organs were collected in sterile bags, all samples were kept in an ice box and send to the laboratory without delay for bacteriological examination.

Isolation of *S. zooepidemicus*:

Swabs were placed in 2 ml enrichment broth (Todd hewett) and incubated aerobically at 37°C for 24 h (**Pisoni et al., 2009**).

Each sample of organs and 100 µl of enriched swab were streaked onto the blood agar, staph strept specific media both media contain 5% defibrinated sheep blood and incubated aerobically at 37°C for 24-48 hr. (SMI, 2014).

Colonies were identified as *S. zooepidemicus* based on their colony morphology (small β-hemolysis), microscopic appearance and their ability to ferment lactose, sorbitol and ribose but not trehalose (Schroeder, 2015). The catalase test was negative and oxidase was positive. All identified isolates were confirmed by using the API 20Strep (SMI, 2014 and Quinn *et al.*, 2011).

Antibiotic susceptibility test:

In vitro antibiotic susceptibility test was performed by using disc diffusion method (Bauer *et al.*, 1959) according to the standards of the Clinical Laboratory Standards Institute (CLSI 2007). Isolates were reported as susceptible to an antimicrobial if the diameter of the zone of inhibition was greater than the breakpoint for that drug, according to the CLSI (2007). Penicillin G (10IU), ampicillin (30µg), Levofloxacin (5µg), Tetracycline (30µg), Erythromycin (15µg), Cefotaxime (30µg), Cephalothin (30µg), ceftiofur (30µg), and Vancomycin (30µg) antimicrobial discs (Oxoid Ltd., Basingstoke, UK) were included in the susceptibility tests. Multidrug antimicrobial resistance was also assessed which was described as an isolate being resistant to three or more of β lactams, Fluoroquinolones, Lincosamides, Tetracyclines, Macrolides and Glycopeptides.

Statistical analysis:

Heat map (Microsoft Office Excel, 2013).

Pearson's correlation has been used to test whether there is a linear relationship between MDR against different antibacterial classes and the source of isolates. Calculation of Pearson's correlation coefficient has been achieved through IBM^R SPSS^R software Version 20.

Detection of 16S rRNA and *sodA* encoding genes in *S. zooepidemicus* by PCR.

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

Oligonucleotide Primers used in this study were supplied from Metabion (Germany) as listed in (Table 1). Primers were utilized in a 25µl reaction containing 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20 pmol concentration, 4.5µl of water, and 6µl 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. Generuler 100 bp ladder (Fermentas, Germany) was 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): Target genes, primer sequences cycling conditions and amplicon sizes of PCR performed in DNA of *S. zooepidemicus*.

Target gene	Primer sequences	Primary denaturation	Amplification (35 cycles)				Amplified segment (bp)	References	
			2nd denaturation	Annealing	Extension	Final extension			
<i>16S rRNA</i>	forward	5'-CGG GGG ATA ACT ATT GGA AAC GATA-3'	94c/5 min	94c/30 sec.	55C/40 sec.	72°C/50 sec.	72°C/10 min.	912	Osakabe et al., 2006
	Reverse	5'-ACC TGT CAC CCG ATG TAC CGA AG TA-3'							
<i>SodA</i>	Forward	5'-CAG CAT TCC TGC TGA CAT TCG TCA GG-3'	94°C	94°C	57°C	72°C	72°C	235	Prezioso & Cuteri, 2012
	Reverse	5'-CTG ACC AGC CTT ATT CAC AAC CAG CC-3'	5 min.	30 sec.	30 sec.	30 sec.	7 min.		

RESULTS AND DISCUSSION

Beta hemolytic *Streptococcus* spp., are the most important and widespread agents causing infectious diseases in horses (Bzdil et al., 2017). Of the beta hemolytic streptococci *Streptococcus equi subsp. equi* (*S. equi*), *Streptococcus equi subsp. zooepidemicus*

(*S. zooepidemicus*) and *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*) are three major species, causing severe and economically important diseases in horses (**Erol et al., 2012**). *S. zooepidemicus* is considered to be a mucosal commensal of the upper respiratory tract of horses which causes disease as an opportunistic pathogen of the lower respiratory tract (**Erol et al., 2012**). The agent is also responsible for diseases of reproductive tract especially (**Frontoso et al., 2008**). In the present study, the isolated *S. zooepidemicus* was characterized by β hemolysis on blood agar, Gram positive, catalase negative, Lancefield group C, ferment, lactose and sorbitol but not trehalose and the phenotypic characterization was confirmed by using API 20 Strep. *S. zooepidemicus* was isolated from foals of 91 out of 136 samples (38.9%) with the highest rate from nasal swabs of diseased foals (83.5%) followed by lung and lymph nodes of dead foals (66.6%) and the lowest rate (16%) of isolation was from nasal swabs of apparent healthy foals (Table 2). These result was nearly similar to **Mohamed et al., (2018)** and disagree with **Mete (2018) and Erol (2012)** who isolated *S. zooepidemicus* in a total rate 88%. Despite isolation rate differences between different studies, which might had been due to the geographic and population differences the importance of *S. zooepidemicus* as a cause of upper respiratory disease with its role to be transmitted between horses has been proved (**Paillot et al., 2010a; Newton et al., 1997; Webb et al., 2008**). Seventeen isolates of *S. zooepidemicus* (42.5%) was recovered from 40 samples collected from mares (vaginal wash and placenta) and 68.3% from aborted feti. *Streptococcus equi* subsp. *zooepidemicus* was found responsible for approximately 65% of the cases in a similar study (**Brito and Barth 2003) and Mete, (2018)**.

Table (2): Incidence of *S. zooepidemicus* in different equine samples.

Horses	No of animals	Type of samples	No. of samples	No. of isolated strains	%
Apparent healthy foals	50	Nasal swabs	50	8	16
Diseased foals	26	Nasal swabs	26	10	83.5
Dead foals	15	lung	15	10	66.6
		liver	15	9	60
		spleen	15	6	40
		Lymph nodes	15	10	66.6
Total No. of samples			136	53	38.9
Mares	30	Vaginal wash	30	10	33.3
		placenta	10	7	70
Total No. of samples			40	17	42.5
Aborted feti	10	Liver	10	7	70
		Lung	10	7	70
		spleen	10	7	70
		kidney	10	6	70
		heart	10	7	70
		Stomach content	10	7	70
Total No. of samples			60	41	68.3
Total	131		236	111	47.03

%; calculated according to the Number of samples.

Six isolates (3 from respiratory cases and 3 from abortion cases) were subjected to PCR for detection of virulence *sodA* gene. All isolates were positive for the presence of the gene encoding a manganese-dependent enzyme (manganese-dependent superoxide dismutase (Mn-SOD)). These results were nearly agree with **Javed *et al.* (2016)**.

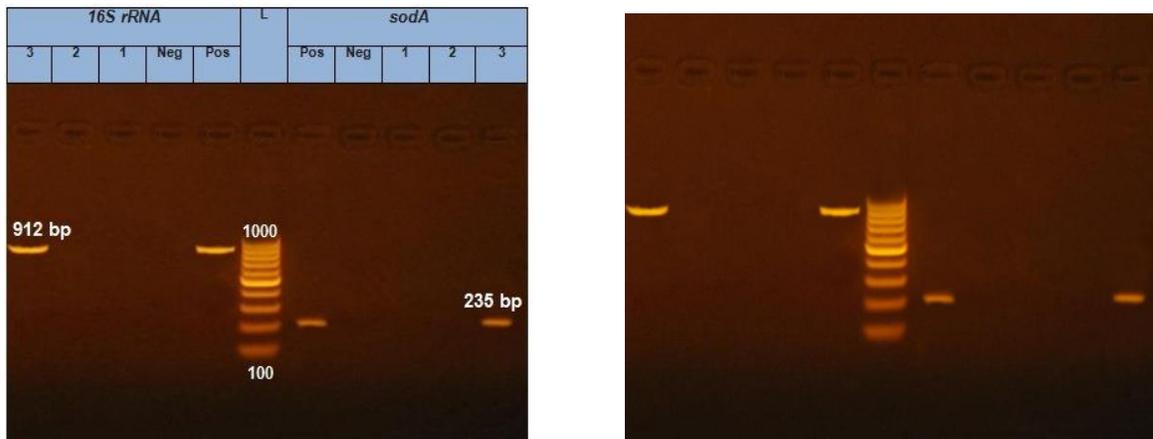


Fig. (1): detection of *sodA* virulence gene in *S. zooepidemicus*: lane 1: control negative, Lane 2: *S. equi* subspp. *Equi* and lane 3 *S. zooepidemicus*.

B-lactam antibiotics are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls, as the peptidoglycan layer is important for cell wall structural wholeness. Tetracyclines is a broad spectrum polyketide antibiotic, it exerts a bacteriostatic by inhibiting the initiation of translation in variety of ways by binding to the 30S ribosomal subunit, which is made up of 16S rRNA and 21 proteins. They inhibit the binding of aminoacyl-tRNA to the mRNA translation complex (Babu *et al.*, 2002). The most effective antibiotic for *S. zooepidemicus* obtained in this study was ceftiofur (75%) the third generation of cephalosporins as well as vancomycin. These results are mostly in agreement with previous studies Bzdil *et al.*, (2017); Erol *et al.*, (2012); Clark *et al.*, (2008) and Toombs-Ruane *et al.*, (2015). There has been also a great concern of multidrug resistant bacteria in veterinary field. Multidrug resistance was defined as resistance to three or more antimicrobial classes in pathogens isolated from animals (Toombs-Ruane *et al.*, 2015).

Heat map analysis in the present study showed that most of tested *S. zooepidemicus* isolated from different diseased horses were multidrug resistant mainly for β lactams (Cephalosporins). β lactams (Pencillins) and the resistance ranged from 100% to 25%. Also the resistance was for tetracyclines (100 to 90%), fluoroquinolones (100 to 50 %), Lincosamides (100 to 75%) and glycopeptides (vancomycin 75 to 25%), Fig. (2). Resistance of most isolates of *S. zooepidemicus* to a number of antibiotics seems to be an outcome of indiscriminate use of those antibiotics in the field.

Also, heat map analysis showed that *S. zooepidemicus* isolated from aborted feti showed high intensity of resistance to most antibacterial agents used in this study followed by those isolated from mares. These results revealed that isolates causing abortion has different MDR profiles than those isolated from foals with respiratory symptoms.

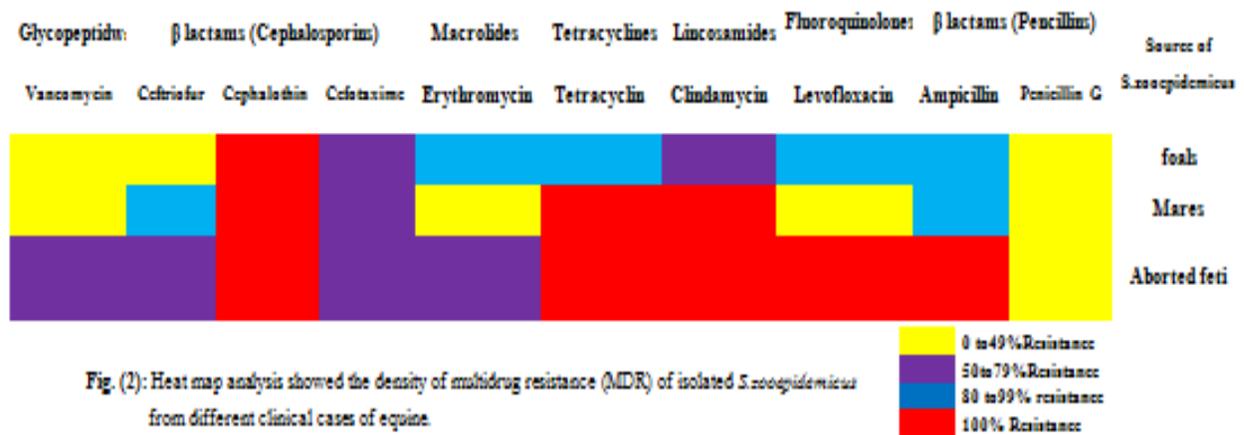


Fig. (2): Heat map analysis showed the density of multidrug resistance (MDR) of isolated *S. zooepidemicus* from different clinical cases of equine.

Table (3): Pearson correlation coefficient (r) of MDR of *S. zooepidemicus*.

Correlation	R coffecient
Foat with mare	0.464643**
Foal with aborted feti	0.705988*
Mares with aborted feti	0.613413*

* Srtong correlation.

** Moderate correlation.

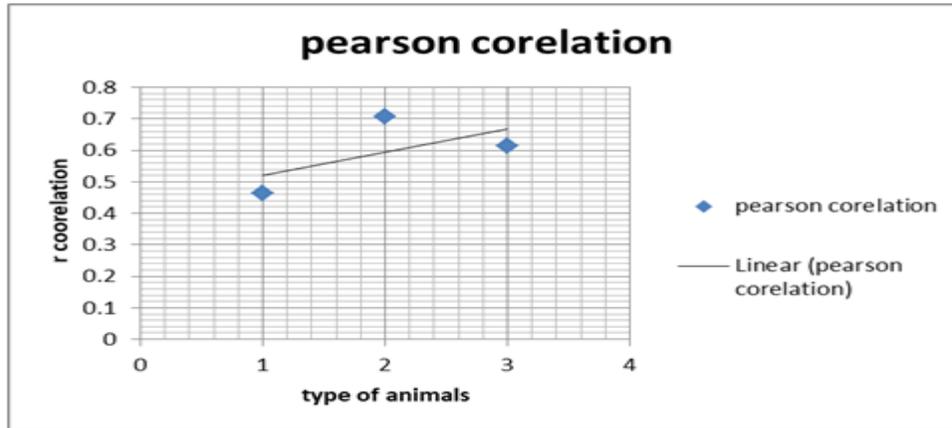


Fig. (3): linear regression analysis of MDR of *S. zooepidemicus* isolated from different diseased cases.

Table (3) shows the Pearson correlation analysis of MDR isolates, where r coefficient was 0.46 in foals with mares, 0.70 between foals and aborted feti and 0.61 between aborted feti with mares Fig. (3) shows no linear regression between MDR *S.zooepidemicus* isolated from iffereent clinical cases. Only strong correlation between MDR isolates obtained from foals and aborted feti and those obtained from mares and aborted feti but it was moderate in isolates obtained from foals and mares. These results revealed that medication against *S. zooepidemicus*. The strong correlation between MDR isolates of foals with aborted feti may indicate that contamination with MDR isolates in stables with bad hygienic sanitation taken after abortion leads to respiratory symptoms in contact foals. Several studies have investigated the relationship between antimicrobial therapy and antibiotic resistance; however, the results of these studies have been inconsistent, possibly due to differences in resistance profiles and antibiotic prescribing practices in different countries. (Metz-Gercek *et al.*, 2009; Meyer *et al.*, 2006; Hsueh *et al.*, 2005; Cook *et al.*, 2004 and Mutnick *et al.*, 2004). These findings support the necessity of monitoring aggregate antibacterial resistance of different sources in order to evaluate the associations between the sources and emerging antimicrobial resistances.

CONCLUSION

The importance of *S. zooepidemicus* as a cause of upper respiratory disease with its role to be transmitted between horses is currently being investigated as well as its responsibility for abortion. There is no difference between isolates from respiratory cases and that from aborted cases in the presence of *sodA* encoding gene. Further studies on different virulence genes are in need. The most susceptible antibiotic for the most susceptible antibiotic for foal obtained in this study was ceftiofur (the third generation of cephalosporins) as well as vancomycin obtained in this study was ceftiofur the third generation of cephalosporins as well as vancomycin. There is a great concern of multidrug resistant bacteria in veterinary field. The most of tested *S. zooepidemicus* isolated from isolates from different diseased horse were multidrug resistance mainly for β lactams (Cephalosporins), β lactams (Pencillins), also the resistance was for tetracyclines; fluoroquinolones, Lincosamides and glycopeptides. *S. zooepidemicus* causing endometritis and abortion has different MDR profile than that isolated from foal with respiratory symptoms. The necessity of monitoring aggregate antibacterial resistance of different sources should be applied in order to evaluate the associations between the sources and emerging antimicrobial resistances.

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دور ضراوة ومقاومة الادوية المتعددة لميكروب الستربتوكوكس زوايبديميكس المسبب للالتهاب الرحمتى
وعدوى الجهاز التنفسى فى الخيول

شيماء رمضان أحمد عبد الموجود - داليا خيرى اسكندر - سمىة السيد أحمد الشافعى

قسم البكتريولوجى . معهد بحوث الصحة الحيوانية - الدقى

الملخص العربى

ستربت زوايبديميكس هو أحد مسببات الأمراض المرتبطة بمجموعة واسعة من الأمراض مثل الالتهاب الرئوى وتسمم الدم والتهاب الضرع والتهاب المشيمة والتهاب بطانة الرحم. وهى من مسببات عدوى الجهاز بـ ستربت زوايبديميكس تم عزلة فى ظهور المظهر السريرى للمرض ونتائج التنفسى ، والعوامل المرتبطة من 91 من أصل 136 عينة (38.9%) من مهرات ، وكانت أعلى نسبة من مسحات أنف المهرات المصابة (83.5%) تليها الغدد الليمفاوية والرئوية للمهور الميتة (66.6%) وأقل معدل (66.6%). 16%) من المعزولة كانت من مسحات أنف مهرات تبدو سليمة. تم اخذ 6 معزولات (3 من حالات الجهاز التنفسى و 3 من حالات الإجهاض) *soda*.. كانت جميع المعزولات موجبة لوجود جين ضراوة. المضادات الحيوية

مضاد حيوى البيتا لاكتام هو مثبت للبكتيريا عن طريق تثبيط تكوين طبقة الببتوجليكان الموجودة فى جدار الغشاء الخلوى . المضادات الحيوية التتراسيكلين هي مثبطات تخليق البروتين وهو أكثر المضادات الحيوية حساسية للبكتريا العنقودية الذهبية التي تم الحصول عليها. فى هذه الدراسة كان السيفتيوفور (75%) الجيل الثالث من السيفالوسبورينات وكذلك الفانكوميسين. أظهرت تحليل الخريطة الحرارية فى هذه الدراسة أن معظم بكتيريا S. المقاومة للتتراسيكلين (100 إلى 90%) ؛ الفلوروكينولونات (100 إلى 50%) ، لينكوساميدات (100 إلى 75%) والبيتيدات السكرية (فانكوميسين 75 إلى 25%) ،

تحليل الخريطة الحرارية أظهرت ان ستربت زوايبديميكس المعزول من الجنين المجهض أظهرت مقاومة عالية للمضادات البكتيرية المستخدمة فى هذه الدراسة تليها تلك المعزولة من الافراس . وكشفت هذه النتائج ان المعزولات المسببة للاجهاض لها خصائص مقاومة مجموعة المضادات الكثرية (MDR) وهى مختلفة عن المعزولات من المهر مع الاعراض التنفسية .