Available online www.ijpras.com

International Journal of Pharmaceutical Research & Allied Sciences, 2016, 5(1):230-237

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Sensitivity to Antibiotics and Comparing the Microbiological Culture and PCR in Subclinical Endometritis with Escherichia Coli (E.coli) in Repeat breeder Mares in Yazd

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ABSTRACT

Introduction*: The results of previous studies indicate that E. coli bacteria are the main cause of endometritis in mare. The indiscriminate use of antibiotics can cause bacterial resistance against them. Given the importance of economic and productivity issue of the horse, the prevalence of endometritis with E. coli agent in repeat breeder mares in Yazd province were examined by culture and PCR methods as well as detection test of antibiotic resistance gene.* ***Materials and Methods****: The study included 60 mares with a history of related infertility with endometritis. Escherichia coli antibiotic susceptibility was performed by disc diffusion method and culture in water Peptone, Macconkey and EMB environments. All PCR reactions were conducted by Mastercycle Gradiant, Eppendorf. Statistical analysis was performed by SPSS 16 and sas9.2 programs.* ***Results****: The results of PCR showed that the bacteria were most resistant to ampicillin, tetracycline and streptomycin. In contrast, bacteria are most sensitive to amikacin, Ceftiofur Sodium, Chloramphenicol. Detection of E.coli pathogenic agent in the PCR method was reported less than culture.* ***Conclusion****: The results of PCR tests to identify antibiotic resistance genes indicate that amikacin, Ceftiofur Sodium, Chloramphenicol were recognized as the best effective antibiotics against E.coli bacteria in subclinical endometritis disease in mares, and showed the least effect against ampicillin, tetracycline and streptomycin. The results of culture and PCR were inconsistent in terms of resistance to antibiotics.*

Keywords: *endometritis, Repeat breeder mares, E. coli, virulence gene, PCR*

INTRODUCTION

Since in horse breeding, the economic issues are of great considerable importance, the loss of breeding season incurs a significant economic loss to horse owner. Endometritis is considered as one of the most common diseases of reproduction in mares referring to inflammation of the endometrium in the uterus. The disease leads to various complications in mares which among them, unstable infertility and reproductive are the most important ones [Albihn et al., 2003]. Streptococcus Zooepidemicus and Escherichia coli are the main pathogenic factors for this disorder. The disease may be found with Hemolytic or non-hemolytic E. coli origin [Althouse et al., 1989]. The disease may be classified with Hemolytic or non-hemolytic E. coli origin [Althouse et al., 1989]. Since E. coli bacteria live in the normal flora of the gastrointestinal tract of animals, they are very important.

Albihn et al., (2003), in their study on 239 mares with infertility disorders reported that the most endometritis pathogen agent is related to the strains of E. coli. with virulence gene. In addition, in another study on dairy cows, Bicalho et al (2012), observed that the main cause of endometritis is related to E. coli. The indiscriminate use of antibiotics caused bacterial resistance against them and susceptibility of mare's uterus after antibiotic treatment by fungal endometritis as well as cytotoxic stimulating of many drugs for uterine endometrial layer [Adams, et al., 1987]. Therefore, due to the pathogenic effect of E. coli in connection with endometritis in mares on the one hand, and the bacteria resistant to the antibiotics on the other hand, the prevalence of endometritis with E. coli agent in repeat breeder mares by culture and PCR as well as detection test of antibiotic resistance genes in Yazd were studied.

Materials and methods

The study was carried out in the spring of 2011 in horse stalls of Yazd province and on the Arab mares, Thoroughbred mares , Arab Thoroughbred mares , Arab kord mares , and Darreh shouri mares . A total of 60 repeat breeder

mares aged 9-21 years, with a history of infertility after rectal ultrasonography and confirmed its non-pregnant were selected and entered the study.

Sampling: After covering the mare's tail by rectal gloves and washing as well as disinfecting their vulva using a special catheter,100 ml of normal saline o.9 percent tepid in sterile conditions were injected in the uterus and intrauterine fluids in sterile conditions were immediately collected by siphonage using a gavage syringes. 20 ml of liquid discharged in the test tube was transferred to the laboratory. Test tubes centrifuge was performed with 3500 rpm for 5 minutes and after discarding the supernatant, the deposited liquid was used for isolation and cultivation in different environments.

Preparing slides for cytological smears: In the laboratory, fluids taken from the uterus of mares were directly sampled using a sterile swab and were spread on a slide and exposed to air after drying and staining was performed by Giemsa.

Antibiotic Sensitivity:

The antibiotic susceptibility of Escherichia coli was conducted by disc diffusion method using antibiotic discs (product of medicine antibodies in Iran).

Interpretation of the results was used in accordance with CLSI 2006 (Clinical Laboratory Standards Institute) and the muller-hinton agar medium.

Bacteria sensitive to 13 antibiotics including kanamycin, amikacin, enrofloxacin, gentamicin, streptomycin, tetracycline, trimethoprim-sulfonamide, cefixime, ceftiofur Sodium, erythromycin, chloramphenicol and ampicillin were examined. The diameter of halo around the disc was measured using calipers and by comparing it with standard tables and guidelines for National Committee for

Clinical Laboratory Standards (NCCLS), sensitive, semi-sensitive and resistant specimens were reported.

Culture:

Some of the centrifuged liquids in the Peptone Water environment were inoculated using swaps (Merck, Germany) and were placed for 48 hours at 37 ° C. Colonies for bacteria grown in this environment were transferred using sterile swabs into the MacConkey environment (Merck, Germany) was placed there for 24 hours at 37 ° C. Bacterial colonies grown in this environment were transmitted to MacConkey environment using sterile swabs (Merck, Germany) and were placed for 24 hours at 37 ° C. Specimen formed as pink or purple colonies were reported as suspected specimen of E.coli. Then the cultured specimen in the MacConkey colony environment were selected using sterile swabs and transmitted to EMB environment (Merck, Germany) and were placed for 24 hours at 37 ° C. Finally, specimens formed with metallic green colonies, were considered as E. coli bacteria. Moreover, using indole biochemical tests, TSI (Triple Sugar Iron Agar), H2s production, urease, confirmed citrate samples were studied.

PCR Test steps:

DNA extraction by kit: DNA extraction by kit manufactured by Cinnagen in Iran (DNPTM Kit) was carried out according to the instructions.

Primer sequences: primer sequences used in this study are as follows:

Table 1: primers on virulence genes

Primer name	Seq	Size of product (bp)	Target gene	References
Hly -F Hly -R	GAGCGAGCTAAGCAGCTTG CCTGCTCCAGAATAAACCACA	889	*hlyEHEC*	44
Stx1-F Stx1-R	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG	348	*Stx1*	44
Stx2-F *Stx2-R*	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	584	*Stx2*	44
Eae-F Eae-R	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC	629	*Eae*	44
Cnf1-F Cnf1-R	GGGGGAAGTACAGAAGAATTA TTGCCGTCCACTCTCTCACCAGT	1111	*cnf1*	44
Cnf2-F Cnf2-R	TATCATACGGCAGGAGGAAGCACC GTCACAATAGACAATAATTTTCCG	1240	*cnf2*	44

Table 2: primers on antibiotic resistance genes

Antibiotic	Resistant gene	Sequence	Size (bp)	Annealing temperature (°C)	References
Aminoglycoside	ant(2″)-Ia	(F) TCCAGAACCTTGACCGAAC (R) GCAAGACCTCAACCTTTTCC	700	62	70
	aac(3)-IIa	(F) CGGAAGGCAATAACGGAG (R) TCGAACAGGTAGCACTGAG	780		70
Streptomycin	aadA1	(F) TATCCAGCTAAGCGCGAACT (R) ATTTGCCGACTACCTTGGTC	447	58	104
Tetracycline	tetA	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	57	104
	tetB	(F) CCTCAGCTTCTCAACGCGTG (R) GCACCTTGCTGATGACTCTT	634	56	104
Trimethoprim	dfrA1	(F) GGAGTGCCAAAGGTGAACAGC (R) GAGGCGAAGTCTTGGGTAAAAAC	367	45	88
Fluoroquinolone	qnr	(F) GGGTATGGATATTATTGATAAAG (R) CTAATCCGGCAGCACTATTTA	670	50	88
Gentamicin	aac(3)-IV	(F) CTTCAGGATGGCAAGTTGGT (R) TCATCTCGTTCTCCGCTCAT	286	55	88
Sulfonamide	Sul1	(F) TTCGGCATTCTGAATCTCAC (R) ATGATCTAACCCTCGGTCTC	822	47	111
Cephalothin	blaSHV	(F) TCGCCTGTGTATTATCTCCC (R) CGCAGATAAATCACCACAATG	768	52	111
Ampicillin	CITM	(F) TGGCCAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	462	47	111
Erythromycin	ereA	(F) GCCGGTGCTCATGAACTTGAG (R) CGACTCTATTCGATCAGAGGC	419	52	111
Chloramphenicol	cat1	(F) AGTTGCTCAATGTACCTATAACC (R) TTGTAATTCATTAAGCATTCTGCC	547	55	68
	cmlA	(F) CCGCCACGGTGTTGTTGTTATC (R) CACCTTGCCTGCCCATCATTAG	698	55	68

The final composition of the PCR reaction in volume of 25 ml: PCR reactants in a final volume of 25 ml were mixed together. The mixture contains 5.2 ml of DNA template, 2.0 mM of each primer, 200 mM dNTP Mix, 1/5 mM MgCl2, 2/5 microliter PCR buffer and 2.5 units of Taq DNA Polymerase enzyme. Then 2 to 3 drops of sterile mineral oil were added to PCR reaction mixture to prevent contamination and evaporation, and specimens were immediately placed in the thermocycler (Mastercycler Gradient, Eppendorf, Germany) according to the temperature program. The temperature was adjusted as 95 ° C for 5 min, then 30 cycles of 94 ° C temperature for 1 minute, 57 ° C for 1 min at 72 ° C for 1 min as well as final step as 72 ° C for 5 minutes.

Results of PCR: after electrophoresis and gel transfer by reading device (Gel Ocumentation), results were studied. By binding to DNA, Ethidium bromide makes viewing fluorescent dye easier under UV

light where the DNA bands are. At the end, the result was recorded and interpreted by gel imaging. All PCR reactions were performed by Mastercycle Gradiant, Eppendorf.

Statistical analysis results: analysis of data collected was conducted in two levels of descriptive and inferential statistics. The statistical analysis was performed by SPSS 16 software and one-way analysis of variance. The data obtained in the antibiotic susceptibility were analyzed by sas9.2 statistical software and anova using normal distribution.

Results and discussion

Antibiotic Sensitivity: Uterine bacterial resistance against antibiotic agents is one of the most common problems in the horse breeding industry and it can bring a major economic problem in this area. In order to prevent the development of resistance to antibiotics, the most effective antibiotic susceptibility should be determined through laboratory methods and antibiogram. According to data from previous studies, most pathogens associated with endometritis, are Gram-negative bacteria. Because of the greatest effect on aerobic gram-negative bacteria, including E.coli, K.pneumoniae and P. aeroginosa, gentamicin was selected as an effective drug against bacterial endometritis in the previous studies [Althouse et al., 1989]. In the study by Albihn et al., (2003), the sensitivity of E.coli bacteria to gentamicin was reported 96%, while Strep. B-haemolytic bacteria were resistant to it. In another study, sensitivity to gentamicin by E.coli was reported 86% (Shin Shin., et al., 1979). Antibiotics can be selected according to the sensitivity of the organisms isolated from the uterus. In this study, by evaluating the resistance gene in positive E.coli samples by PCR method, the most resistance of bacteria was reported 69/23 percent to ampicillin and streptomycin. Moreover, in the antibiotic susceptibility in E.coli positive culture samples by fusion disc, the highest resistance to ampicillin was reported 71/41 percent. In contrast, the most sensitivity of bacteria to amikacin was reported about 92 percent (Table 3).

Table 3: Detection of sensitivity for Escherichia coli isolates using 13 antibiotics disk

Resistive percent	semi-sensitive percent	Sensitive percent	Type of Antibiotic effect / antibiotics
23/18	72	76/8	Kanamycin
5/36	2.53	91/84	Amikacin
37/1	2/44	60/55	Gentamicin
51/04	2/13	46/83	Streptomycin

57/3	2/3	40/4	Tetracycline
51/66	3/75	44/59	Trimethoprim
46/02	2/61	51/37	Sulfonamides
37/29	5/9	56/81	Enrofloxacin
8/04	2/3	89/66	Ceftiofur *Sodium*
43/52	2/7	53/78	Erythromycin
21/17	3/05	75/78	Chloramphenicol
71/41	6/8	21/79	Ampicillin
43/04	4/3	52/66	Cefixime

Comparison of culture and PCR results:

LeBlanc (2008), reported that 50 -80 percent of endometritisin in mares is associated with infectious agents, including S. zooepidemicus and E.coli. Shin et al., (1979) in their study on the mares in the United States reported that most of the bacteria isolated from the uterus were Strep. B-haemolytic, E.coli, K.pneumoniae, respectively. In another study in this area, Albihn et al., (2003) on mares with infertility disorders in Sweden, found that(% 67) of endometritis were composed of E.coli bacteria, Strep. B-haemolytic (% 20) and S. zooepidemicus (%14).

According to previous studies in the field of important role of E.coli in induced-endometritis infertility as well as the importance of effective antibiotic in the treatment of mares, this study aimed to help both culture and PCR methods for detecting E.coli in endometritis in mares and determining the most effective antibiotic to treat it. The results showed that 13 heads of 60 mares (21.6 percent) are E.coli bacteria identified with virulence genes. Also with the culture of specimens in water Peptone, MacConkey and EMB, from 60 mares, 29 heads (33/48 percent), E.coli with virulence genes were isolated (Table 4).

Table 4. Detection of the presence of E. coli with virulence genes by both culture and PCR tests

	Culture		PCR test	
Isolated Escherichia coli	N	Percent	N	Percent
Positive E.coli	29	48/33	13	21/66
Negative E.coli	31	51/67	47	78/34
Total	60	100	60	100

In case of symptoms of endometritis, mares are widely treated by antibiotics. However, few studies are available about the type of antibiotics, infusion duration, and effective injection of antibiotics [Bae et al., 1999]. The aim of this study was to evaluate the prevalence of subclinical endometritis with Escherichia coli agent by both culture and PCR methods and determining the most effective antibiotic against it in Repeat breeder mares in Yazd province. In inflammation, neutrophils invade inside the uterine cavity, so endometritis can quickly and accurately be diagnosed by testing cells isolated from the liquid to uterine.

In this study, uterine cytology method was used for grading endometritis so that the number of neutrophils was determined at a magnification 400 times [Georges & Michael 1998]. They were graded into three groups: A) the number of neutrophils from 0 to 2, B) the number of neutrophils between 2 to 5, C) the number of neutrophils more than 5.

66.6 percent of mares were in Group A, 40% of the mares in Group B and 53/33 of mares in Group C. The result of study by Riddle et al., (2007) showed that uterine cytology diagnosis of endometritis was twice compaired with uterine culture. In addition, conception rate per cycle was markedly affected by the number of neutrophils at 400 magnifications. The result of this study showed that conception rate in mares with neutrophil count at 400 times magnification were 0-2, 2-5 and more than 5 were 60, 36 and 23 percent, respectively (Table 5).

PH level in normal uterus of mares in estrus phase is about 6.7 to 7; however, it is to about 6/4 in diesterous. S. zooepidemicus bacteria grows well in PH = 6/4. Therefore,at the time of estrus normal uterus can be used as an unfavorable environment for bacterial growth [Georges & Michael 1998].

Table 5: Number and percentage of E.coli bacteria isolated by culture and PCR methods in uterine fluids in terms of the number of neutrophils with X400 magnification

	Culture		PCR test	
Neutrophil count range	N	Percent	N	Percent
Neutrophil count 0-2	0	0	2	6/89
Neutrophil count 2-5	9	69/23	16	55/18
Neutrophil count More than 5	4	30/76	11	37/93
Total	13	%100	29	%100

Conclusion

Uterine cytology test results showed that about 93 percent of subclinical endometritis had neutrophil count range up to 2 (at magnification 400 times), which 50% of endometritis was associated with E. coli agent. This result indicates that Escherichia coli is the main pathogenic cause for endometritis in mares. The PCR test results also showed that 21/66 % of mares with infertility disorders and infectious agents included E. coli bacteria with virulence gene. Correspondence between the detection of genes of antibiotic resistance and antibiogram test from the species, containing E.coli indicates that the disk diffusion method fusion is reliable for determining the sensitivity of bacteria to treat endometritis in mares and antibiogram can be used as a diagnosis method in the Field. The test results of Antibiogram showed that amikacin with least resistance of 5/36 was recognized as the best effective antibiotics, and the highest resistance to ampicillin was determined 71/41. Also, in the results of PCR test to detect antibiotic resistance genes, it was observed that E. coli with 15/38 percent resistance to chloramphenicol and amikacin was identified as the best effective antibiotics, and by 69/23% showed the highest resistance against ampicillin, tetracycline and streptomycin.

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