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Determination of the prevalence of subclinical endometritis and evaluation of molecular characterization of Escherichia coli (E-coli) separated of them in repeat breeder mares in Yazd province

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ABSTRACT

Escherichia coli are known as the most common cause of reproductive tract infection in mare. Due to the progressive process of antibiotics use and increasing prevalence of antibiotic resistance, the aim of this study is evaluate the prevalence of subclinical endometritis and antibiotic resistance genes in Escherichia coli isolated. In this study, 60 mares were used with infertility background. Diagnosis of endometritis was performed using history and ultrasonography. Cytology, culture, Antibiogram were done of samples and PCR test was used to examine the gene virulence and antibiotic resistance. E-coli bacteria was isolated 48/33 % from sample culture. In PCR test 66/21 % of bacteria had virulence gene. It was determined, the lowest resistance to chloramphenicol about 38/15% and greatest resistance into ampicillin, tetracycline and streptomycin with 23/69 percent, respectively. 93% samples cytology had neutrophil more than two and the agent of 50% showed E. coli. The cause of half of subclinical endometritis in infertile maresis E-coli bacteria

Key words: Mares, subclinical endometritis, Escherichia coli, PCR, virulence genes and antibiotic resistance

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INTRODUCTION

The reproduction in domestic animals has economic importance. In the case of mares reveal more importance due to breeding and specific physiological status with the seasonal breeding. This means breeding season will be lost and the birth will be delayed at least a year if the mare does not experience pregnancy in breeding season because of physiological uterine disorders which the endometritis is one of their important. Since in the horse breeding, the economic issues have considerable importance, the loss of the breeding season causes horse owner considerable economic loss. Therefore, one of the most serious duties of veterinarians working in the horse stalls is identify and overcome the reproduction disorder in mares.

Endometritis in the mare is one of the major causes of reduced fertility. Endometritis is inflammation of the uterus endometrial layer. The inflammation is emerged by a series of external factors. The ignorance of endometritis in the mare can eventually lead to infertility or major problems for mare. Endometritis causes pregnancy loss providing a hostile environment for the fetus or premature lysis of the corpus luteum .Endometritis treatment has focused on the antimicrobial agents to the removal of infectious agents. Therapies such as antibiotic therapy is more practical nowadays and the indiscriminate use of antibiotics leads to antimicrobial resistance against chemical agents and specially, mare inclined uterus after antibiotic therapy into fungus as well as stimulating cytotoxic and pointed out the many drugs for uterine endometrial are mentioned. Fungal endometritisis caused in result of the indiscriminate

use of antibiotics in utero and provide the rich environment for the growth and proliferation of various fungi such as Candida species [1].

Fungal Endometritis in mares leads to infertility and treatment is often ineffective and expensive, and recurrence, it is not unusual post-treatment [2].

In fact, endometritis is one of the most common diseases of reproduction in mares that refers to inflammation of the uterine endometrial The disease leads to various complications in mare such as infertility or Non-sustainable fertility[3].

Based on the endometritis etiology and pathology, it is divided into three groups: (1) mating endometritis, 2. chronic infectious endometritis 3. Post- mating endometritis [4].The chronic infectious endometritis is most common which the type of endometritis is influenced of the immunity defense mechanisms defect of uterus to remove the pathogens and thus the pathogens transmitted by the fecal pollution or uterus natural flora are proliferated. This form of endometritis is divided into two categories of fungal and bacterial. The most common pathogenic bacteria that are the most important include Streptococcus Zooepidemicus and Escherichia coli. endometritis induced by Streptococcus Zooepidemicus is seen more as purulentive and caused by Escherichia coli is more as a non-suppurative. Infected by Escherichia may be hemolytic or non-hemolytic but non-hemolytic are common [5].

E. coli is property concerns due to the presence in the intestinal flora of livestock. Escherichia coli is gram-negative bacilli, motile and facultative non- anaerobic and non-spores of the Enterobacteriaceae family which are the normal flora of the intestine of warm-blooded animals. Some bacterial strains with the virulence in livestock involved bacterial diseases include diarrhea, pneumonia and reproductive diseases such as endometritis.

On the other hand, using broad-spectrum antibiotics has caused some bacteria to resist into antibiotics.

About 25 to 60 percent of infertility in infertile mares is due to bacterial endometritis [6] that in cause of irritation and inflammation of the uterus tissue and the presence of bacteria colonies creates an inappropriate environment for fetus.

Also PGF2a is released during the process of inflammation that prohibits the formation of the corpus luteal and prevents of pregnancy. Finally, the aim of endometritis treatment is eliminating bacterial agents and uterus endometrial repair. For successful treatment, the best dissolution is bacteria isolation and Antibiogram culture [7].

The results of study in 1991 at Americashowed86% antibiotic resistance in swab samples taken of mares [6]. Research in 2003 on the 239 mares with reproductive problems, the most isolated bacteria was E-coli. Among 10 Antibiotic were performed in the study using Antibiogram method on isolated samples, there was no any resistance only against Enrofloxacin. Another study was conducted on hospitalized horses, showed that these horses are exposed in infection of E-coli with antibiotic resistance.

at similar study, over 80 colonies of E. Coli has been isolated of swine, only 2/5 % of them had virulence and the most antibiotic resistance was related to tetracycline. The result of study was conducted on dairy cows, shows that most pollution in endometritisis related to of E. coli.

With regard to the mentioned context, the aim of present study is evaluation of prevalence of virulence genes and antibiotic-resistant E. coli isolated from repeat breedermaresendometritis in Yazd province by using molecular methods and comparison the results with Antibiogram test method.

MATERIALS AND METHODS

In the project among 60 Repeat breeder mares aged 9-21 years with fertility and infertility problems were evaluated which have repeated mating background with not pregnancy history in different estrus period in the current reproductive season (spring 1392) and the before reproductive years and in some with non-transparent discharge and were tested using ultrasonography apparatus and were entered into tested statistics. To do this, mare in estrus phase are kept in permeability or completely had bound with shackles of mating at outdoor. During the research a sterile plastic tube (catheter balloons coated) was used to obtain a sample. First, mare's tail is conducted inside a rectal

gloveto avoid entering horse tail hair and its infections into the vagina. Mare's vulva had been washed with warm water, after scrubbing by non irritant antiseptic liquid around the vulva and anus it is cleaned thoroughly and washed. Balloon catheter is kept in gel lubricant of your hand; hand is inoculated with vaginal lubricant gel to insert into the vagina and cervix easily. Catheter is entered into cervical entry and is drive into the uterus from there. As the catheter at the uterus inlet, 100 mltepid normal saline 0/9% is injected into the uterus of mares and was drained in sterile conditions fluid immediately using lavage syringe. In order to better washing, injected liquidintra uterus is shaking by our hand inside rectum and then gently is massage uterus to drain fluid. Due to negative pressure by lowering catheter, it administered fluids to exit their latest. 20 ml of the liquid drained into a test tube was collected in sterile conditions and then was transported to the laboratory. By using swab, the sample was taken from the liquid to Prepare slides for cytology directly. A test tube has been laid off into centrifuges with3,500 rpm for 5 minutes and then is removed. The supernatant fluid is discarded in such a way that at least 2 ml of liquid with sediments to remain that liquid was cultured to separate the bacteria in Peptone Water culture media then MacConkey and finally EMB environment.

To Preparing slides for cytology, the fluids taken from the mares' uterus in test tube that were sent to a laboratory directly picked up by the swab and is played on a slide and after drying smear, Giemsa staining was performed in air exposing.

To microbial culture, the liquid obtained from the centrifuge is inoculated with Merck Germany made peptone water using the swaps and was incubated for 48 hours at 37 ° C. Then, using sterile swabs of grown bacteria in this medium, colonies are transferred to the Merck Germany made MacConkey environment and was incubated at 37 ° C for 24 hours.The samples creating pink or purple colonies on MacCankey environment were reported as samples suspected of bacterial E-Coli. Then one colony was selected of the samples cultured in the MacConkey environment using sterile inoculation loop is transferred to Germany Merck EMB agar environment and was incubated for 24 hours at 37 ° C. samples creating metallic green with metallic luster colonies were considered as E. coli bacteria and was evaluated using biochemical tests indole, TSI or Triple Sugar Iron Agar, H2s production, urease, citrate, examined. The extraction was performed using DNA extraction kit manufactured Cinagen Company of Iran and kit instruction from the typical colonies and presence of bacteria was confirmed using gene-specific primers of 16Sr RNA from bacteria. Antibiotic discs (pad tan tebIran Company) were used to determine susceptibility pattern for E. coli isolates through Disk diffusion antibiotic and Interpretation of results was performed accordance with the standards of CLSI 2006 (Clinical Laboratory Standards Institute). Muller-Hinton agar medium was used for this purpose. The equivalent concentration with 0/5 McFarland of each bacterium was prepared and transferred to Mueller-Hinton medium. The paper discs contain certain concentrations of different antibiotics were used at this method. Disc was on the medium surface and disc antibiotic diffused in the surrounding of agar and, if susceptible, prevents bacteria growth at zone around the disk and make corona. The samples were reported as of titles, sensitive, intermediate or resistant by measuring the diameter of the caliper by Collis and comparison to a standard table and guidance of the National Committee for Clinical Laboratory Standards (NCCLS).

The data obtained from this study were evaluated in two descriptive statistics levels include the average, standard deviation and inferential statistics involve pollution comparison between different seasons of year using SPSS 16 software and ANOVA test

The results:

In samples culturing, the Escherichia coli bacteria was isolated in 29 samples among 60 samples (48/33 %) and the E-coli bacteria with virulence gene was reported by PCR test in 13 samples among 60 samples.

The result of uterus fluid cytology:

Table 1: The number of mares with endometritis in terms of the neutrophil range in uterine fluids X400 magnification

Neutrophils	No 0-2	No 2-5	No 5<	total
Number of mares	4	24	32	60
percent	6/66%	40%	53/33%	100%

In this study to treatment these mares, Amikacin antibiotic with volume of 1/5 grams equally alike of sodium bicarbonate 7/5% were used as intra-uterine injection. In this study, mares were evaluated to treatment passing two

estrous cycles after taking sampling of uterine fluid. The 27 mares had concepted in the first estrus. 22 mares in second estrus and 11 mares were reported as non-pregnant after two estrous periods.

Table 2: pregnancy rate after two estrous cycles according to the number of neutrophils

neutrophils	No 0-2	No 2-5	No 5<	total
number of mares conception rate	3 (75%)	21 (87.5%)	25 (78/13%)	49
number of mares non- conception rate	1 (25%)	3 (12.5%)	7 (21/8%)	11

Table 3:Number of mares and gestation Percentage due to heat period

Neutroohils in heat period	0-2	2-5	5 <
One heat period	2 (50%)	11 (45/83%)	14 (43/75%)
Two heat period	1 (25%)	10 (41/66%)	11 (43/38%)
Non pregnant	1 (25%)	3 (12/5%)	7 (21/87%)

Table 4: Number and percentage of E-coli bacteria isolated by PCR and Culture in terms of the number of neutrophils in uterine fluids

	Neutrophil number range	*E-coli*	percentage
PCR Method	0-2	0	0
	2-5	9	69/23
	>5	4	30/76
	total	13	100%
Culture Method	0-2	2	6/89
	2-5	16	55/18
	>5	11	37/93
	total	29	100%

Table 5: Percentage of non-pregnant and pregnant mares after two periods of estrus

	E-coli	percentage	number
Pregnant mares in microbial culture samples	+	86/2%	25
	_	77/41%	24
Non pregnant mares in microbial culture samples	+	13/7%	4
	_	22/59%	7
Pregnant mares in PCR test samples	+	84/61%	11
	_	80/85%	38
Non pregnant mares in PCR test samples	+	15/39%	2
	_	19/15%	9

CONCLUSION

The aim of this study was to evaluate the prevalence of Escherichia coli subclinical endometritis. To do this, two methods were used such as PCR for separating bacteria with virulence genes as well as microbial culture method for the isolation of all pathogenic and non-pathogenic strains of E. coli. This study was conducted on 60 mares with subclinical endometritis. Endometritis evaluation was examined primarily by ultrasonography in mares. The mares were examined to investigate the infertility agent, that's why when sampling of uterine fluid, were taken for PCR and culture some of the same samples for preparing slides for cytological smears. In PCR positive samples for E-coli bacteria, the bacterial resistance genes were discussed and The E-coli positive cultured samples were cultured to determinate of antibiotic sensitivity using disk diffusion method in Mueller-Hinton agar and the results were analyzed statistically after observation.

Riddle et al (2007) showed in a broad study including the 2123 pairs of uterus culture and uterine cytology samples that endometritis was diagnosed by uterine cytology twice diagnosed by uterine culture. In addition, the pregnancy rate was affected by the number of neutrophil in any field with a magnification of 400 per cycle greatly. In mares that neutrophil count at 400 times magnification were equal to 0-2, conception rate at 28 days gestation was 60%

and the neutrophil count 2-5, 36% and mares with a neutrophil count of more than 5 percent were 23%. The researchers also found that in cytology samples E-coli is less than other bacteria with neutrophils [8,9]. Since endometritis has different grades which uterus fluid cytology was used for grouping to determine the neutrophils number in any field with 400 magnifications, which were divided into 3 groups: Group A (neutrophil count between 0 to 2), Group II (neutrophil count between 2 to 5), Group III (neutrophil count 5 and up). 6/66 percent of mares were placed in Group A, 40% in the group two and 53/33% in groups of three

LeBlanc (2008) announced that cause of 50 -80% of mare's endometritisare because of infected with pathogens Streptococcus Zooepidemicus and E-coli [10]. In another study by Clark et al (2008) at West of Canada, uterus bacteria isolation of 67 mares were earned with the highest bacteria Streptococcus Zooepidemicus, 46/3 percent, E-coli with 17.9 percent and 13.4 percent achieved spp staphylococcus respectively. Testing the sensitivity for bacteria Streptococcus Zooepidemicus, E-coli and spp staphylococcus against Gentamycin was reported 85, 80 and 86 percent respectively [11].

According to the research background in the separation field of endometritis agent and consequently the use of effective antibiotics in the treatment of susceptible maresagainst endometritisit was decided to identify E-coli bacteria with virulence genes in mare endometritisby using PCR method. In this way was reported the isolation of 13 pathogenic E-coli bacteria samples with virulence genes equals 21/66. However, culturing the samples in water peptone medium, MacConkey and EMB were investigated also 29 E-coli were reported positive in cultures equals 48/33 percent of total samples.

Antibiotic choose can be according to the sensitivity range of the organisms isolated from uterus. The therapy rate are equal in mares received the same doses of oxytocin or broad-spectrum antibiotics But the mares that have received both at the same time speed reaches its peak. Oxytocin alone cannot successfully treat all cases of uterine infections. The using of antibiotics, according to the additional costs and risks, leads to success when oxytocin alone fails [12, 13].

Some recommend large volume injection of intrauterine antibiotics is the equivalent of 30 to 60 ml of the drug to prevent rejection [14]. Some prefer injection of more volume (250 ml) to ensure the filling of uterus total endometrial [15]. The 250 ml volume is shown to have more of the drug concentration in the endometrium in proportion to the volume of 60 ml especially in the case Ticarcilin [16]. If a lower volume is used, rectal massage of the uterus needs to be done to distribute the drug through [17].

This study was performed on samples of uterine fluid in evaluating the resistance gene in E-coli samples were positive by PCR most resistant to ampicillin and streptomycin was reported by 69/23 percent. To determination of antibiotic sensitivity in samples E-coli positive using fusion disc the most resistant was against ampicillin with 71/41 percent.

According to the uterine cytology tests, it was resulted about 93 percent of subclinical endometritis has 2 to top neutrophil count of among 50% of endometritis were reported by E. coli agent and this indicates the most dominant factor causing endometritis in mares bacteria is Escherichia coli. PCR tests also showed that 21/66 % of samples have Shigatoxigenic E-coli. Correspondence in antibiotic resistance genes and E-coli positive Antibiogram test show that disk diffusion method is reliable to determine bacterial sensitivity for the treatment of endometritis in the mare and Antibiogram is used as a treatment diagnosis method in the field.

The PCR test to detect antibiotic resistance genes it was shown chloramphenicol has most sensitive with 15/38 percent resistance and ampicillin, tetracycline and streptomycin has allocated the most resistance of 69/23 percent.

The least resistance to amikacin with 5/36 percent and the highest resistance to ampicillin with 71/41 percent were determined in Antibiogram test. In this regard, strategy of treatment endometritis in the mare is uterine lavage 6-8 hours after mating to day 3 and injection antibiotics such as amikacin in the uterus during estrus phase. According to results of this study suggest less use of antibiotics and select effective antibiotic using antibiotic sensitivity tests. Sanitary of maresgenital during the breeding season and the birth and reproductive health in the reproductive season must be done. methods of cultivation, cytology and PCR should be used combined together in order to identify bacteria and designing appropriate treatment. Now use of antibiotic amikacin and seftiofur as proper choice leads into the best treatment in mare endometritis to follow. .

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