

ISOLATION AND IDENTIFICATION OF THE BACTERIA ASSOCIATED WITH THE RETAINED FETAL MEMBRANES IN COWS BY PCR TECHNIQUE IN BABYLON- IRAQ

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ABSTRACT

The aim of this study was to detect bacterial species in cows suffering from retained fetal membranes (RFM) by PCR technique. For this purpose, seventy two swabs were collected from uterus of cows with RFM. The bacteria was identified by standard microbiological procedures and vitek system. Bacterial DNA was extracted from the predominant bacteria. The results showed that the distribution of bacteria was *Staphylococcus aureus* (13%), *Escherichia coli* (68%), while (18%) of samples showed no growth. Moreover the results showed the 65% of *Escherichia coli* had *hlyA* gene which refer to the virulence of bacteria. In conclusion, it was found that *Escherichia coli* was the most prominent bacteria in RFM in cows in Iraq.

Keywords: Retained fetal membranes, cow, PCR, *Escherichia coli*.

عزل و تحديد البكتيريا المتعلقة مع احتباس الأغشية الجنينية في الأبقار بواسطة تفاعل البوليميراز المتسلسل في محافظة بابل- العراق

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المستخلص

إن الهدف من هذه الدراسة كان للكشف عن أنواع البكتيريا في الأبقار التي تعاني من احتباس الأغشية الجنينية بواسطة تفاعل البوليميراز المتسلسل. لهذا الغرض تم جمع 72 مسحة من رحم أبقار تعاني من احتباس الأغشية الجنينية. تم تحديد البكتيريا بواسطة الإجراءات الميكروبيولوجية القياسية و جهاز الفايترك. تم أستخلاص الحمض النووي DNA للبكتيريا السائدة. أظهرت النتائج أن توزيع البكتيريا كان المكورات العنقودية الذهبية (13%) والإشريكية القولونية (68%)، في حين أن (18%) لم تظهر نمو. وإضافة الى ذلك أظهرت النتائج أن 65% من الإشريكية القولونية تحمل الجين *hlyA* والذي يشير الى ضراوة البكتيريا. نستنتج ان الإشريكية القولونية تمثل البكتيريا الغالبة في احتباس الأغشية الجنينية في الأبقار في العراق.

الكلمات المفتاحية: احتباس الأغشية الجنينية، بقرة، تفاعل البوليميراز المتسلسل، الإشريكية القولونية.

Introduction

Parturition can be divided for three stages: dilatation of birth canal, expulsion fetus and finally fetal membranes should be expelled. Swiefy (1) and Adedeji *et al* (2) defined retained fetal membranes (RFM) as failure of fetal membranes to expel from 6 to 24 hours after expulsion of fetus. Stephen (3) and Shwetha and Chandrashekaramurthy (4) reported the incidence of RFM range from 5-10% and 8.8% respectively. There are many causes of RFM such as 1) managemental system like improper environment (5), 2) Infectious disease like brucellosis and bovine viral diarrhea (6, 7), 3) hormonal in which Michal and Hanna (8) showed that, there was increment in the levels of cortisol and progesterone in cows with RFM, 4) hereditary where Benedictus *et al.* (9) reported that there was a high affinity in class I of histocompatibility complex between dam and fetus that increase risk of RFM, and 5) nutritional like deficiency in vitamins A, E, β carotene and selenium, with defect in calcium/phosphorus ratio rise incidence of RFM (10). Gaafar *et al* (11) studied the effect of RFM on reproductive performance in which they found that RFM increased the interval between first postpartum estrus after parturition, postpartum service interval and service period; also they found that the number of service preconception was higher in cow treated after RFM, although there was longer open days and calving interval. Otherwise, there was reduction in conception rate with decrease in milk yield compared with normal cows. There is many types of bacteria found to be associated with RFM such as *Arcanobacterium pyogenes*, *Bacterioides melaninogenicus*, *Fusobacterium*

necrophorum, *Escherichia coli*, *Streptococcus spp.* and others (12, 13 and 14). Little information is reported about bacteria isolated from cow with RFM; therefore, this study was conducted to determine the types bacteria associated with RFM in cows in Iraq.

Material and methods:

1. Sample Collection:

In this research, 72 swabs samples were collected from uterus of cows with retained fetal membrane (failure to expulse fetal membrane at least after 24 hours from parturition) in Al-Madhatiya province (during a period from December 2013 to May 2015). Each swabs samples were isolated and identified culturing and biochemically using standard microbiological procedures. Subcultures were done on nutrient agar, MacConkey agar, eosin methylene blue (EMB) and triple sugar iron (Difco) and incubated at 37°C for 18 to 24 hr. All the isolates were stored in brain heart infusion broth with 15% glycerol at -20°C until further use.

2. Identification of bacterial isolates

The bacteria isolate were identified their culture, morphology and biochemical characters. For the cultural characteristics, discrete colonies on the agar surface were observed. The shape, size, consistency and color observed. Gram stained slides of the isolate were examined microscopically to study their cellular morphology. The biochemical tests were performed as catalase and oxidase. Individually isolated colonies of the same morphology on appropriate agar plate, the culture media preparation depended to routine methods (15) and then Vitek 2 system was used to detect species of bacteria (Bio Merieux, Inc, Hazelwood, Mo. USA).

Bacterial genomic DNA extraction:

Bacterial genomic DNA was extracted from *Escherichia coli* isolates by using (Presto™ Mini gDNA Bacteria Kit. Geneaid. USA). 1ml of overnight bacterial growth on BHI broth was placed in 1.5ml microcentrifuge tubes and then transferred in centrifuged at 10000 rpm for 1 minute (Eppendorf. Germany). Then, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction procedure and the extraction was done according to company instruction. After that, the extracted gDNA was checked by Nanodrop

spectrophotometer (Thermo Fisher. USA), then store in -20C at refrigerator until perform PCR assay.

Polymerase chain reaction (PCR):

PCR assay was performed for confirmative detection of pathogenic *Escherichia coli* based virulence factor genes hemolysin *hlyA* gene by using specific primer that designed in this study by using NCBI-Primer Blast design. The primers were purchased from (Bioneer Company. South Korea) (Table 1).

Table 1: Showed sequencing of DNA from *Escherichia coli*.

Prime r	Sequence (5'- 3')		Amplico n	GenBank (accession number)
<i>hlyA</i>	F 21bp	TGCAACAGGAGCATCAAAAC C	435bp	AY495950.1
	R 21bp	TTACCGCTTCCTCCTGTCAGA		

The PCR was performed by using AccuPower® PCR PreMix (Bioneer. South Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl₂ 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was performed according to manual instructions in 20µl total volume by adding 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. South

Korea). The reaction was performed in a thermocycler (Mygene Bioneer. South Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 3 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 58.5 °C for 30 s, and extension 72 °C for 1min and then final extension at 72 °C for 10 min. The PCR products were examined by electrophoresis in a 1% agarose gel.

Results and discussion:-

The results, shown in Fig. (1), indicated that from out 72 total specimens

(uterus swabs), *Staphylococcus aureus* in 9 predominant in 49 isolates less than *Escherichia coli* which isolates while rest samples (14 samples) showed no growth or may be anaerobic bacterial isolates.

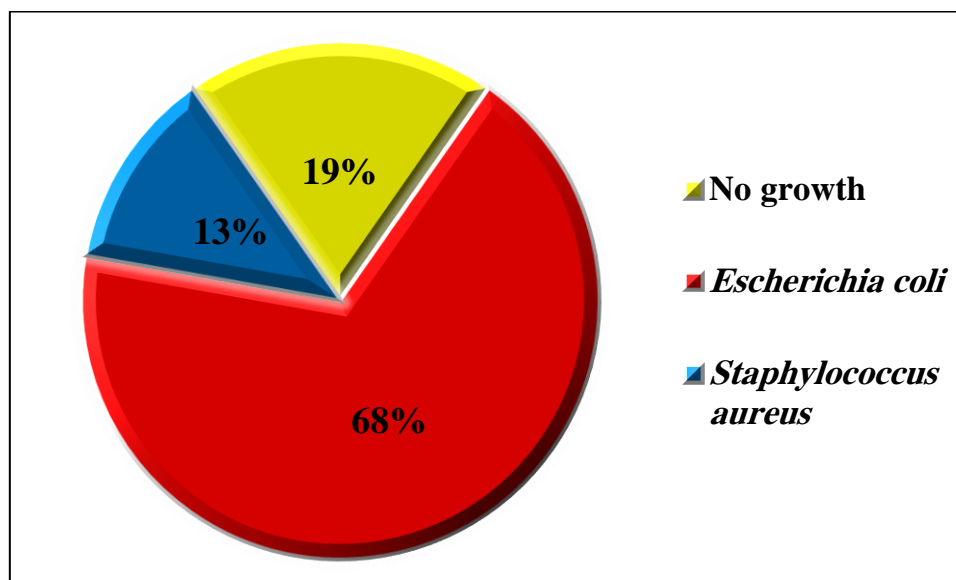


Fig.1: Bacterial isolates associated with RFM.

It is generally considered that bacterial infection of the uterus initiates inflammation of the uterus. This inflammation is a normal adaptive response, however, it may be inadequate or disproportionate response in degree or duration (16). *Escherichia coli* considered as a microflora of female genital system in cow, ewe and she camel (17, 18, 19, 20 and 21). In our study, *Escherichia coli* was the most preponderant bacteria on cow with RFM. This result are in agreement with another studies. Kaczmarowski (14) found that the percentage of *Escherichia coli* and Enterobacteriaceae family was 67.4%, while *Staphylococci* was 12.6% in cows with RFM. Also, Azawi *et al* (22)

found that *Escherichia coli* percentage reach to 26.6% after 24 hr. in relation to RFM. On the other hand, Erin *et al* (23) proved that *Escherichia coli* was the most prominent bacteria in cow with postpartum complications. Deoril and Phookan (24) referred to that *Escherichia coli* considered as normal microflora of gastrointestinal tract which may transferred by contamination of protruded fetal membranes by feces.

Molecular analysis of *E.coli* revealed that from out 20 isolates, 13 isolates (65%) have *hlyA* gene which mean that *E.coli* isolates more virulence and not normal flora or contamination from genital tract during parturition.



Figure 2: Agarose gel electrophoresis image that show the PCR product analysis of *hlyA* gene in *Escherichia coli*. Where M: marker (2000-100bp), lane (1-8) some of positive *Escherichia coli* at (435bp) *hlyA* gene PCR product.

The pore-forming hemolysin (*hlyA*) of *E. coli* represents a unique class of toxins that require a posttranslational modification for activity, specifically the covalent amide linkage of fatty acids to internal lysine residues; produce a catalytic a fragment acting in the eukaryotic target cell (25).

It is widely believed to act mainly by attacking the immune system cells of the host, usually without inducing cell lysis, yet severely impairing their function (26). Stephan and Hoelzle (27) suggest that *E. coli* strains without *hlyA* may possess reduced pathogenicity or may even be nonpathogenic.

Also Wang approved that *E. coli hlyA* may be a more critical virulence factor for disease, which be consider as major

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virulence factors ascribed to the pathogen include a plasmid-encoded enterohemolysin from STEC (enterohemorrhagic *E. coli* [EHEC] *hlyA*) that is often associated with severe clinical disease (28). Among the animals, that gene was only found in cattle (29).

From the above mentioned results, we concluded that *Escherichia coli* considered as a prominent bacteria in genital tract in cows with RFM in Iraq.

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