A Concordance Study between Polymerase Chain Reaction Assay and Conventional Culture-based Methods for Detection of *Fusobacterium necrophorum*

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Abstract

Foot rot duo to *Fusobacterium necrophorum* is an important clinical economic disease in ovine, in order to inform identification and antibiotics selection, Conventional culture-based method and Polymerase chain reaction assays had been proposed to provide a good inter-rater reliability between these techniques, this study was aimed to differentiate the accuracy of conventional culture-based method and PCR technique for identification of *Fusobacterium necrophorum* in an ovine foot rot.

Method: The current study was conducted on three farms of Kerbala governorate (AL-husseinyia, Al-Hurr and Ain Al-tmor cities) during the period from October 2017 to April 2018, pathological swab samples of the interdigital region of affected (n= 40) sheep and (n=40) goat were enrolled in this study, after the collection of swabs, the samples were divided into two partitions, each swab of the first partition was immediately collected into lighten tube containing 5 mL of thioglycollate broth enriched with 0.5% ground sheep and was transmitted to the laboratory for conventional culture-based method and the another partition of swabs was sealed with other cooled tubes containing 5 mL of thioglycollate broth enriched with 0.5% ground sheep for the laboratory of molecular investigations.

Results: *Fusobacterium necrophorum* was identified in the pathological samples by the two following depended assays: and (46) swabs were positive by conventional culture-based method and (44) swabs were positive with PCR assay, Cohen's kappa coefficient revealed P value for conventional culture-based methods vs. PCR assay of 0.056 and the current study was demonstrated a highly identification rates of *Fusobacterium necrophorum* in conventional culture-based methods rather than PCR assay.

Conclusions: Current study documented conventional culture-based method and PCR assays were facilitate the identification of *Fusobacterium necrophorum* in case of presumed ovine foot rot, it also concluded that molecular assays should be depended in confirmatory detection of *F. necrophorum* in affected animals.

Key words: *Foot Rot, Fusobacterium necroforum, PCR, Sheep, goat*

Introduction

Foot rot is a highly contagious disease of ungulates characterized by exudative inflammation with offensive odor, resulting in acute or sub-acute inflammation and necrosis of the tissues of interdigital space of sheep [3]. It is worldwide distribution but highly incidence rates in cold and wet regions, it may reach to peak occurrence in hot and wet summer in both sheep and cattle [15] .

[13] reported that foot rot is a frequent cause of lameness in sheep with severe economic losses in herds because of decreased weight gain and treatment costs. Also, lame bulls will not breed and occasionally, animals with severe and complicated disease may need to be discarded from the herd. *Fusobacterium necrophorum* is a gram-negative, pleomorphic rod-shaped and anaerobic bacterium, was associated with many necrotic lesions in animals and humans, it is a normal inhabitant of the gastrointestinal tract in animals [28] and humans [27] and this

pathogen is existed within the ruminal fluid of ruminants at concentrations of 10^5 to 10^6 per gram with variations occurring depended upon the diet of the animal [30].

Kumar [14] found that the *Fusobacterium necrophorum* in sheep was the primary etiological agent of liver abscesses, and two subspecies; subsp. *necrophorum* and subsp. *funduliforme*, were different morphologically, biochemically, and by the molecular analysis. The subsp. *Necrophorum* is the more virulent and occurs more frequently in liver abscesses than the subsp. *funduliforme*.

Moreover, [16] recorded many toxins in *F. necrophorum.* such as leukotoxin, endotoxin, hemolysin, hemagglutinin, proteases, and adhesin., have been considered as virulence factors. Leukotoxin is a major virulence factor, it is a secreted protein of high molecular weight, active specifically against leukocytes of ruminants and the complete sequence of nucleotides of the leukotoxin operon of *F. necrophorum* has been detected., the operon consists of three genes (*lktBAC*) of which the second gene (*lktA*) is the leukotoxin structural gene.

Previous study was utilized the *lktA gene* of *F. necrophorum* to determine *F. necrophorum* variants in cattle, sheep and goats which diagnosed with foot rot., It was demonstrated that a particular variant of *F. necrophorum lktA* (designated variant A) tended to be determined in sheep with foot rot and this *lktA* sequence matched the strain of *Fnn* (NCTC 10575) which isolated from bovine with liver abscess [34].

Materials and Method

Animals: eighty ovine (40) sheep and (40) goats were conducted in the present study from un-organized three farms in kerbala city which included (AL-husseinyia, Al-Hurr and Ain Al-tmor cities), all data like age, gender of animals and duration of the study were recorded., the study was extended from October 2017 to April 2018. At all (80) animals were observed on the following properties, the site of lesions of foot rot (unilateral or bilateral) and the claws were cleaned and better trimmed for investigating of bacterial lesions and achieving the pathological swab samples.

Collection of Samples and Bacterial Cultivation

Direct swab smears were collected from the interdigital lesions of the affected animals and directly stained with grams stain. After collection of swab samples, they were divided into two partitions in lighten tubes containing 5 mL of thioglycollate broth enriched with 0.5% ground sheep hoof (Aguiar), the first partition was transmitted anaerobically to the medical microbiology laboratory for bacterial cultivation, then, the swab samples were incubated anaerobically at 6% CO2 and 37Cº for 24 hours, and cultivated on Colombia blood agar plates, incubated anaerobically at 37Cº for 48 hours, then, the cultivated plates were examined morphologically to determine the characteristic colonies of *Fusobacterium necroforum* which appeared on the culture media and biochemical characterization of the colonies of *F. necroforum* according to the procedures of [30,8] were performed.

Biochemical characterization

The following biochemical activities included Catalase test, Oxidase test, Indole test, MR/ VP test, H2S production, Litmus milk, DNase test, Lipase test in addition to fermentation of the following sugars-Glucose, Maltose, Fructose Sucrose, Galactose, Lactose, Mannitol and Mannose as well as Motility test were performed.

Molecular identification:

The second partition of swab samples were used for PCR assay for molecular detection of *Fusobacterium necroforum lktA gene* according to the following procedures.

DNA Extraction:

Preparation of DNA :- each of the contains of 5 ml of enriched broth of swab samples were centrifuged and washed with sterile normal saline and suspended in 2 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 50 mM NaCl. and the suspension which prepared was incubated with shaker incubator at 37°C for 30 min and used for DNA extraction according to the instructions of the commercial kit (the Presto™ Mini gDNA Bacteria Kit/ Korea) and amplification of *Fusobacterium necrophorum lktA gene* was performed according to the following program.

Primer and PCR polymerization:

PCR technique was performed in a final reaction volume of 25 µl master mix in a thermal cycler (**Biometra – Germany)** with 5 µl of extracted DNA and 1 µl of each of specific primers for *leukotoxin A* gene of

F. necrophorum sub sp. *Necrophorum* (*lktA)* (Forward 5-ACAATCGGAGTAGTAGGTTC-3) and (Reverse 5-ATTTGGTAACTGCCACTGC-3) 403 bp, they were designated by [5] based on the published *F. necrophorum* sub sp. *necrophorum* leukotoxin gene sequence (GenBank accession number DQ672338). PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide (5µl /100ml) with DNA ladder at along with 100 bp. and PCR products were visualized as a single orange fluorescent band under UV light.

Statistical Analyses

Chi square and Cohens Kappa coefficient tests were used to investigate the degree of concordance between the two identification assays (conventional culture and PCR), A P value <0.05 was considered as statistically significant by using statistical analysis of social science data software version 22.

Results and Discussion

Obtained results were depended cohens kappa coefficient (K) to measure inter-rater agreement between the two identification assays (conventional culture and PCR), they found the moderate interobsorver agreement (0.645) between the two assays on comparing with the results of routine diagnosis have cohens kappa coefficient 0.645, with no significant association between the two identification assays (P value >0.05), according to the kappa interpretation there was a relatively interobsorver agreement found table 1.

Table (1): The inter-rater agreement between conventional culture-based and PCR assays, according to kappa interpretation results.

Inter-rater agreement -ve		**Conventional culture-based methods**		
		+ve	**Total**	
PCR	-ve	28 (82.4%)	8 (17.4%)	36 (55%)
	+ve	6 (17.6%)	38 (82.6%)	44 (45%)
Total		34 (34.5%)	46 (65.5%)	80

Previous studies revealed that conventional culture-based methods was more robust measurement for detection of *F. necrophorum* than PCR assay, interestingly, the recovery of *F. necrophorum* by culture-based methods was similar to that of PCR and they were consistent with the results of another study demonstrating that PCR was predominantly and useful technique for detecting of low concentrations of *F. necrophorum* [2]. In spite of these different clinical findings, there was a considerable confusion about the causative agents, particularly related to duplication of names for *F. necrophorum* but also due to misattribution of a role of other etiological organism such as *Streptococci* [1], or inadequate anaerobic culture methods. Thus, increasing the likelihood of occurrence false positive.

The clinical examination of (80) infected sheep with foot rot in the current study have been revealed the development of fever in affected animals, the temperature ranged between 40-41Cº according to severity of foot rot lesions, the increase of body temperature reached (41Cº) in (45) animals with marked increase in heart beats to (90 b/m) and respiratory rate to (45 b/m)as demonstrated in another similar study [21] . During the period extended from October 2017 to April 2018 the examination of (80) sheep suffered from foot rot lesions in both gender with different ages in Karbala governorate reveled various degrees of lameness in one or two limbs, These clinical signs were agreed with the study of [6] and [26]. The typical lesion occurs in the skin at the top of the interdigital cleft and takes the form of a fissure with the swollen and protruding edges might extended along the length of the cleft or be confined to the anterior part or

the part between the heel bulbs. Exudates secretions were few but the edges of the fissure were covered with necrotic materials and the lesion has a characteristic offensive odor [23, 21]. Moreover, the lesion was concentrated in hind limbs more than in forelimbs and this feature was agreed with [4 ,10, 22] Table (3).

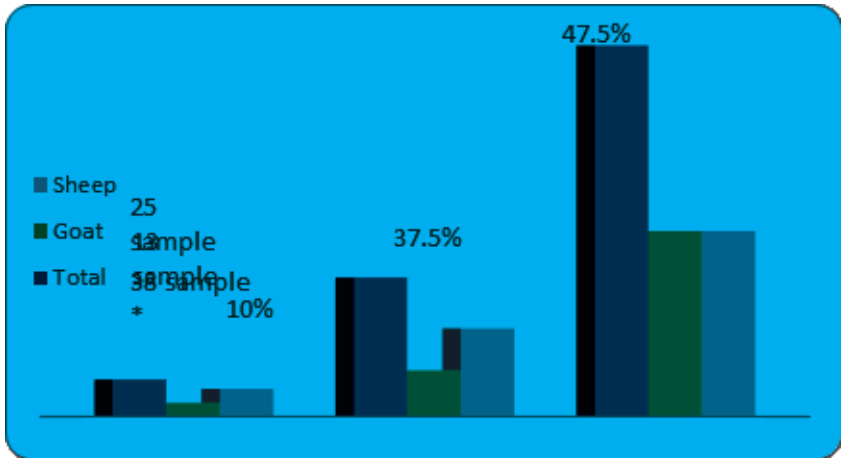


Figure 2: Number and percentage of positive samples and site of lesion on feet in sheep and goat. * matched both tests (+,+). ^{2}X= 0.08 , P>0.05

The highly occurrence of foot rot lesion with the scores (3,4 and 5) were tend to be undetectable lesions in the early stage of infection in animals which led to progress the lesions in deep structures of claws and caused severe inflammation and abscessations; so, there was decreased in the productivity and fertility of animals and this feature was agreed with previous study by [31 , 12].

Table 3: Number and percentage of positive samples location lesion on limbs in sheep and goat.

Type of animal	No.	Fore limbs	Hind limbs	Total +ve
Sheep	40	9	16	25
Goat	40	5	8	13
Total	80	14	24	38*
percentage	%	36.8%	63.2%	47.5%

*** matched both tests** (+,+).^{2}X= 0.022, P>0.05

Table 4: Number and percentage of positive samples of score for sheep and goat.

No. of score	NO. of Sheep	%	NO. of goat	%
Score 1	-	0	-	0
Score 2	-	0	-	0
Score 3	11	44	9	69.2
Score 4	9	36	3	23
Score 5	5	20	1	7.8
Total	25	65.7%	13	34.3%

$^{2}X= 2.307, P>0.05$

Microbiological detection of *F. necrophorum sub necrophorum* in infected animals revealed the presence of this causative agent in 46 out of 80 (65.5%) in affected sheep and goat with foot rot and 34 out of 80 (34.5%) of the other sheep were revealed negative results table.1, this results were disagreement with the results of [20] who found that 4 positive samples with *F. necrophorum* was isolated from 84 sheep have been infected with foot rot.

After 48 hours of anaerobically incubation of Colombia blood agar culture plates at 37C°; the colony morphology of *Fusobacterium necrophorum .sub necrophorum* were appeared as round, grey, and shiny in appearance with diameter about 1-5 µm as demonstrated in (Figure 2 a) while on normal blood agar the colony morphology appeared as tiny round colony have convex surface and some of them were hemolytic but the most of them were non hemolytic colonies as demonstrated in (Figure 2 b).

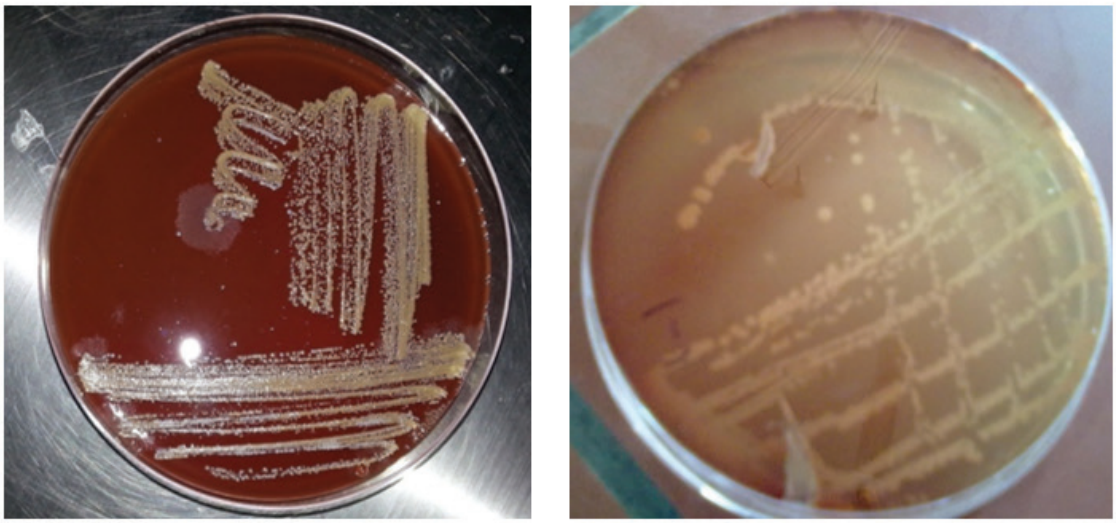
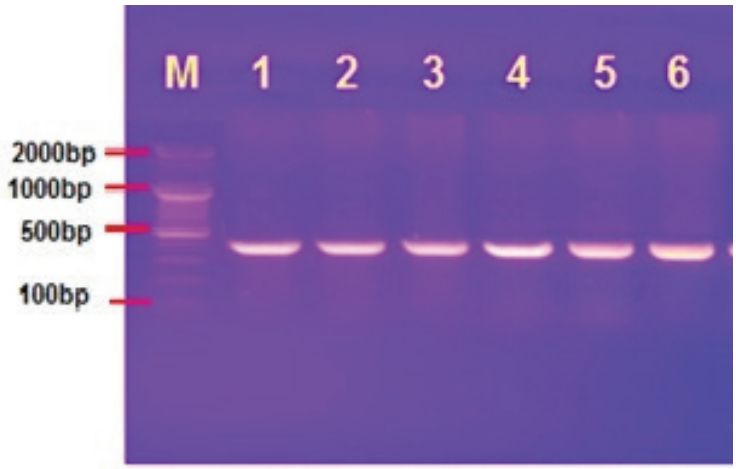


Figure 2: Colony morphology of *Fusobacterium necrophorum* on a- Blood agar and b- Colombia blood agar.

The grams staining of bacterial smears have been showed the characteristic bacterial morphology of *Fusobacterium necrophorum* and they were observed as gram-negative, long, non-branched filamentous pleomorphic bacilli rang about 100µm in diameter with parallel sides and blunt or tapering ends . [4]

The result of PCR technique for detection of *Fusobacterium necrophorum .sub necrophorum*, leukotoxin *gene (lktA),* in (80) foot rot swab samples of the affected sheep) for the extracted DNA from all the isolated bacteria that electrophorised with 1.5 % agarose gel have been showed positive samples with *Fusobacterium necrophorum .sub necrophorum*, leukotoxin *gene (lktA),* in analysis appeared as single band under the U.V light with the length 403 bp. (Figure 2) of amplified DNA of all strains of *Fusobacterium necrophorum .sub necrophorum.*



PCR technique revealed amplified genomic DNA for positive isolates of leukotoxin gene (*lktA*) of *Fusobacterium necrophorum .sub necrophorum* in 1.5 % agarose gel electrophoresis at the wells no. (1,2,3,4,5,6) revealed positive DNA samples with the length of bands 403 bp. and M=100 bp. DNA ladder for the swab samples of sheep with Foot rot.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the Department of Veterinary Medicine/ Veterinary Medicine College/ University of Kerbala, Iraq and all experiments were carried out in accordance with approved guidelines.

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