ISOLATION AND GENETIC DETECTION OF MORAXELLA BOVIS FROM BOVINE KERATOCONJUNCTIVITIS IN BASRAH CITY

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ABSTRACT

This study was aimed to determine the most sensitive isolation procedures and evaluate the genetic diversity of *Moraxella bovis* because there are large number of pathogenic bacteria and several other infectious agents such as virus and Mycoplasma have been isolated from the eyes infected with infectious bovine keratoconjunctivitis (IBK). This study included examination of (40) eye swabs, from cows from different ages and regions in Basrah city showed clinical signs of an ocular infection. The isolated bacteria that obtained in pure cultures were non-motile, catalase and oxidase positive, and a clear zone of β-hemolytic colonies were produced on blood agar. According to the growth characteristics, morphology, staining and biochemical tests, the isolated bacteria were identified initially as *Moraxella spp.*, the genetic diversity among *Moraxella spp.* was assessed by 16S rRNA and sequencing as *M. bovis.* The study has also indicated that the isolates of *M. bovis* were resistant to tetracycline, chloramphenicol and erythromycin, However, they were sensitive to penicillin and gentamicin and has an intermediate sensitivity to streptomycin. This study concluded that PCR techniques and sequencing were verified to be most accurate tools to indicate the genetic diversity between *Moraxella spp*. in bovine keratoconjunctivitis.

Key words: Cows, Sequencing, Genetic diversity.

عزل وتشخيص جيني لجراثيم *Moraxella bovis* من التهاب القرنية والملتحمة المعدي في الأبقار في محافظة البصرة

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المستخلص

تهدف هذه الدراسة الى تحديد الطريقة الأكثر دقة في العزل وتقييم التنوع الجيني لجرثومة *Moraxella b.* وذلك لوجود عدد كبير من الجراثيم الممرضة والعوامل المعدية الأخرى مثل الفيروسات والميكوبلازما التي تم عزلها من العين المصابة بألتهاب القرنية والملتحمة المعدي في الابقار. اشتملت هذه الدراسة على فحص(40) مسحة عين من أبقار ذات اعمار مختلفة ومن مناطق مختلفة في محافظة البصرة والتي أظهرت علامات سريرية لعدوى في العين. الجراثيم المعزولة التي تم الحصول عليها من المزارع النقية كانت غير متحركة، موجبة لأنزيم الكتاليز وانزيم الاوكسيديز، وكذلك انتجت منطقة واضحة من التحلل الكلي لخلايا الدم الحمراء على وسط أجار الدم. وفقًا لخصائص النمو, الخصائص الشكلية, التصبيغ والاختبارات الكيميائية الحيوية، تم مبدئيا تشخيص الجراثيم المعزولة على أنها *Moraxella spp.*، اما التنوع الجيني بين جراثيم *Moraxella spp.* فقد تم تحديده بواسطة 16S rRNA والتسلسل الجيني حيث شخصت على انها جرثومة *M. bovis*. كما أشارت الدراسة إلى أن عزلات *M. bovis* كانت مقاومة لمضادات التتراسيكلين والكلورامفينيكول والإريثروميسين،في حين اظهرت حساسية للبنسلين والجنتاميسين وكذلك لديها حساسية متوسطة تجاه الستربتومايسين. خلصت هذه الدراسة إلى أن تقنيات تفاعل البلمرة والتسلسل الجيني أثبتت أنها الطرق الاكثر دقة في كشف التباين الجيني بين جراثيم *Moraxella spp.* الموجودة في التهاب القرنية والملتحمة البقري.

الكلمات المفتاحية: الابقار، التسلسل الجيني، التنوع الجيني

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INTRODUCTION

Infectious bovine keratoconjunctivitis, also known as pink eye is a bacterial eye disease affect cattle distributed all over the world, and can cause considerable economic impact. The main factors that play a role in financial loss are lower weight gain, increase of treatment cost, and market discounts due to eye blindness and disfigurement. The disease is also considered as one of the most common cases affecting beef heifers, and the second most common disease of nursing calves more than three weeks old (12,14). The disease is seen all over the world but mainly in regions with high temperature climates. In addition, this disease in seasonal countries in the summer months are more predominant and it ordinarily appears in younger animals. The numbers of face fly population within the hot months are greater, also dust and intensive sunlight prompt eye infection (10). *Moraxella bovis*, is a Gram negative bacterium, is considered the main causative agent of IBK. Although this type of infection is usually accompanied with inflammation of eye and most corneal ulcers heal without loss of vision, it can cause permanent or temporary blindness in severe cases and chronic untreated cases (2,20). These bacteria produce hemolysin, which plays a role in damaging neutrophils that are accumulated into the infected area and can also discharge collagen hydrolysis enzymes that play a role in liquefying ulcers (5). The other possible virulence factors of this bacterium include phospholipases (7), iron acquisition systems, outer membrane proteins (16) and proteolytic and hydrolytic enzymes (8). This study was designed for isolation and molecular detection of *Moraxella bovis* from bovine keratoconjunctivitis in Basrah city, Iraq.

MATERIALS AND METHODS

1- Clinical diagnosis

In this study, keratoconjunctivitis was defined clinically by acute and rapid appearance of photophobia, lacrimation, conjunctival hyperemia, blepharospasm, opacity in the center of the cornea and chemosis that develop during a day of exposure followed by keratitis with corneal edema. The main complaint from the owner was the observation of eye problems in adults and young animals for a few weeks.

2- Sample collection

A total of 40 eye swabs, from cows from different ages and regions in Basrah city showed clinical signs of an ocular infection. The samples were collected using swab sticks soaked with sterile normal saline. The eyelid was opened and the swab stick was gentle rotated from front and back on the cornea and conjunctiva surfaces. The swab was then aseptically dipped in the sterile test tubes containing 5 ml of sterile nutrient broth. Subsequently, all samples were transferred to the lab of microbiology at the College of Veterinary Medicine/ University of Basrah, Iraq.

3- Bacterial isolation and identification

The tubes were incubated aerobically in the incubator at 37°C for 24 hours. The samples were then cultured on blood agar comprising 5% blood of sheep and incubated aerobically at 37°C for 24 hours (13) and then sub cultured to obtain pure colonies. The morphology and overlapping of colonies were determined after Gram staining (17). The pure colonies were kept in brain heart infusion accompanied with agar 1.5% and incubated for 24 hours at 37ºC for extraction of DNA and for the purpose of conducting biochemical tests, gelatin hydrolysis, catalase, oxidase, and motility test (23).

4- Molecular detection of *Moraxella bovis*

A- Extraction of the DNA

The DNA extraction was performed to all isolates using specific commercial DNA extraction kit, intron biotechnology, cat.no. 17045 following the manufacturer's instruction.

B- Detection of 16S rRNA gene using PCR

Moraxella bovis was detected and diagnosed by amplifying the 16S rRNA fragment using universal primers (18) as shows in Table 1. The reagents of PCR reaction were summarized in Table 2, and PCR condition was described in Table 3.

Table 1. Universal 16S rRNA primers used for the detection of *Moraxella bovis*

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250
Reverse	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1	base pair

Table 2. Reagents of PCR amplification (25 µl) for 16S rRNA gene

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	(1 µl)
Reverse primer	(1 µl)
DNA	1.5µl
Nuclease -free Water	16.5 µl
Final volume	25µl

Table 3. PCR condition for amplifying 16S rRNA gene of *Moraxella bovis*

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min.	1 cycle
2-	Denaturation -2	95°C	45 sec	
3-	Annealing	52°C	1 min	35 cycles
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	7 min.	1 cycle

C- Agarose gel electrophoresis

The amplified PCR product was detected using 1% agarose gel prepared with TBE buffer stained with ethidium bromide by used 1500 bp ladder (Promega/USA). The expected size of the amplicon (1250 bp) was estimated by comparison with the standard DNA ladder.

D- Sequencing and sequence alignment

Sequencing of the amplified DNA fragment was analyzed using national instrumentation center for environmental management (nicem) (http://nicem.snu.ac.kr/main/?en_skin=index.html). Sequence analysis and alignment was carried out using program of Basic Local Alignment Search Tool (BLAST) which is existing at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and Bio Edit program.

5- Antibiotic susceptibility tests

The antibiotic susceptibility test was performed by disk agar diffusion method (4). The isolates were tested for susceptibility to 6 different antimicrobial discs, which included Erythromycin E (15 mg), Gentamicin GN (10mg), chloramphenicol C (30 mg), Penicillin PI (30 mg), Streptomycin ST (10mg) and Tetracyclin TE (30 mg).

RESULTS AND DISCUSSION

Visual and physical examinations

Forty eye swab from cattle were suffering from eye infection in one or both eyes. The affected eye(s) showed copious lacrimation, closure of the eyelids, photophobia and blepharospasm. Some calves showed copious watery discharge from the affected eye and matting the hair on the lateral aspect of the face . There was severe conjunctivitis and edema resulted in lateral deviation of the eyeball with lacrimation , and opacity of the cornea (Figure 1) .

Figure 1. Calf with severe conjunctivitis and edema with corneal opacity

Many calves showed keratitis and yellow opacity of cornea and appearance of nictitating membrane (third eye lid) (Figure 2)

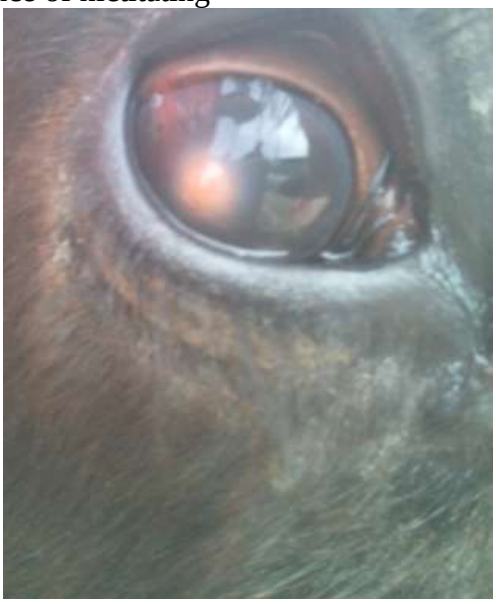

Figure 2. Calf with yellow opacity of the cornea and ulcer in central cornea

Other calves had ocular discharge, edema and white opacity of cornea and matting of the eye lashes with copious lacrimation. In some cases, the cornea became conical in shape surrounded by a hyperemic zone (Figure 3). Most of the animals resented examination of the eyes had depressed appetite because of the ocular discomfort that resulted in inability to locate food, our clinical findings agree with the findings of Kahn et al (15).

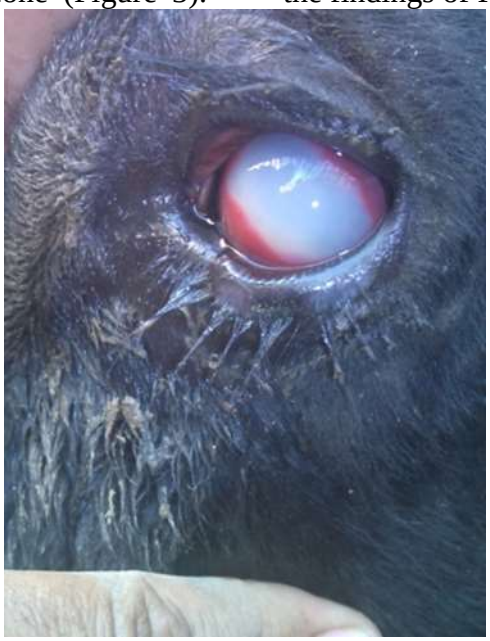

Figure 3. Calf with and opacity of the cornea with hyperemic zone

Isolation and Identification of *Moraxella bovis*

Moraxella bovis was isolated on blood agar and confirmed by determination of the morphology of the characteristics colonies followed by Gram staining. The bacterium isolated from a total of forty eye swabs were non-motile, catalase and oxidase positive (Table 4). According to its growth characteristics, morphology, staining and biochemical tests it was identified initially as *Moraxella bovis* – the etiological agent of infectious bovine keratoconjunctivitis and these results are similar to the results of Mukhtar et al (19). All the isolated bacterium was highly virulent as recognized by the clear zone of hemolysis produced on the blood agar. These findings authenticated the findings of Postma et al (21) who reported that virulent strains of *Moraxella bovis* are formed *β*-

haemolysin toxins which lysed the corneal epithelial cells, and released cytotoxic toxin and pathogenic hyaluronidase, aminopeptidases, fibrinolysin, and phosphatase.

Table 4. Laboratory investigation of *Moraxella bovis*

Morphological and biochemical tests	Result
Gram stain	-ve
Motility	-
Hemolytic activity	+ (β)
Oxidase	+
Catalase	+
gelatin hydrolysis	+

Molecular detection of *Moraxella bovis*

Amplifying of the 16S rRNA gene by PCR technique: The antigenic and genetic difference among *M. bovis* and the assumed incidence of other micro-organisms existence in IBK can be assessed by comparing different sizes of DNA fragments produced from PCR amplification. Universal primer was used, and the amplification products were visualized on agarose gel with a size of approximately 1250bp (Figure 4). The isolated bacteria were genetically identified by PCR targeting 16S rRNA fragment were sequenced and aligned with the sequences available in GenBank (Figure 5), and this result agree with Helena et al (11) who reported PCR-derived tools verified to be most accurate instrument to indicate the presence of genetic variability between *Moraxella* spp., also Faraj et al (1) reported that PCR technique followed by phylogenic tree analysis good methods for detection and identification of genetic variants.

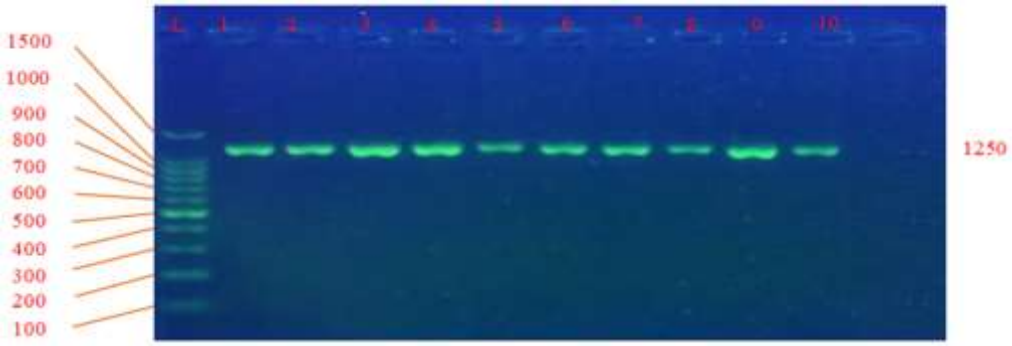

Figure 4. Amplification products of 16S rRNA gene on 1% agarose gel stained with ethidium bromide. The results showed the amplification of 1250 bp of 16S rRNA gene.

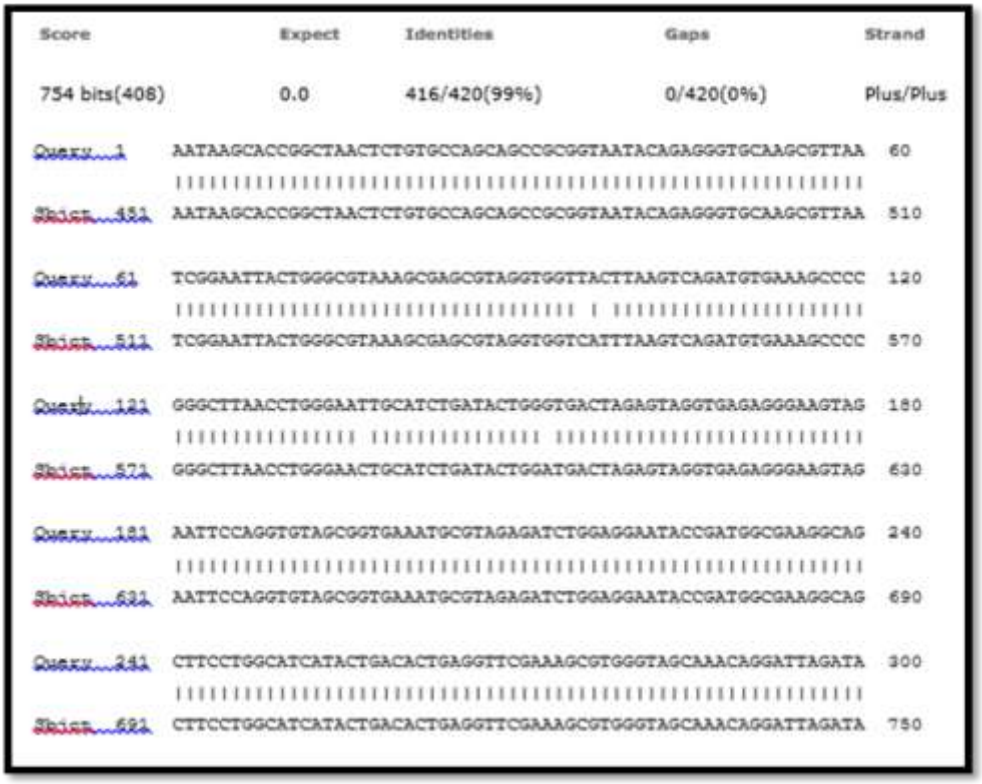

Figure 5. Gene sequencing of *Moraxella bovis* isolated in the present study

Antibiotic susceptibility tests

Antimicrobial susceptibility, as measured by the standard agar disk diffusion procedure, indicated that the isolates of *M. bovis* were resistant to tetracycline, chloramphenicol and erythromycin, but it was sensitive to penicillin and gentamicin and have an intermediate sensitivity to streptomycin (Table 5). These result disagreements with Grazieli et al (9); Shryock et al (22) which reported that *M. bovis* strains showed resistance to penicillin and gentamicin, respectively. While the results of our study is in agreement with Conceição et al (6); Angelos et al (3) they reported that *M. bovis* strains resistant to erythromycin and tetracycline antibiotic classes, respectively.

Table 5. Antibiotic sensitivity test

Antibiotics	Symbol	Disc content (mg)	Diameter of inhibition (mm) (Mean±standard deviation)		
			Sensitive	intermediate	Resistance
Tetracycline	TE	30	-	-	≥8
Penicillin	PI	30	20.5±1.91	-	-
Chloramphenicol	C	30	-	-	≥6
Erythromycin	E	15	-	-	≥3
Gentamicin	GN	10	19.5±1	-	-
Streptomycin	ST	10	-	12.25±0.50	-

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