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MOLECULAR DETECTION OF *PROTEUS VULGARIS* ISOLATED FROM URINE OF COW IN AL-ANBAR PROVINCE OF IRAQ

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Abstract

In this study, 120 urine samples of cows were collected from different area in AL-anbar province. 84 (70%) urine of cow were positive for bacteriological culture. Total of 28 (23.33%) isolates of *Escherichia coli*, 18(15%) isolatesof *staphylococcusaureu*s, 12(10%) isolates of *Proteus miribils*, Mohammed *et al.*, 2016 (8.33%) isolates of *Proteus vulgaris*, 5(4.16%) isolates of *Klebsiella* and 4(3.33%) isolates of *streptococcus*. The identification of these isolates based on microscopic examination, morphological and conducted with biochemical tests. DNA extracted and detection of 16Sr RNA gene of 10 isolated of *P. vulgaris* were done by polymerase chain reaction PCR and it was that found all isolates give positive result for 16Sr RNA 750bp.

Key word: *Proteus vulgaris*, PCR, 16Sr RNA.

Introduction

Proteus spp. is Gram negative, facultative anaerobic bacteria and rod shape. It has swarming motility, non-lactose fermentation, urease activity. It belong to the *Enterobacteriaceae* family, generally characterization of this genus by It is actively motile, non-capsulated non-spore forming, oxidase-negative, but catalase and nitrase positive. The *Proteus* spp, can be identify by using specific tests including phenylalanine deaminase tests and positive urease (Mordi and Momoh, 2009; Brooks *et al.*, 2004). *Proteus* is widely spread in the natural environment. It is found in soil, manure and contaminated water, where it plays an important role in decomposing organic matter of animal origin (Mordi and Momoh, 2009; Ro'zalski *et al.*, 1997). The genus of *proteu*s consists of five species named *(P. mirabilis, P. vulgaris, P. penneri, P. myxofaciens* and *P. hauseri*) and three unnamed genomo-species (Proteus genomo-species; Ro'zalski *et al.*, 1997; O'hara *et al.*, 2000; Harley and Prescott, 2002), *P. vulgaris, P. mirabilis* and *P. penneri* are opportunistic pathogens (Ro'zalski *et al.*, 1997).

P. miribilis is frequently found as free-living organism, water and intestinal tract of animal and human. On (Brooks *et al.*, 2004) indole is considered reliable, because it is positive result for *P. vulgar* (O'hara *et al.*, 2000) *P. miribilis* consider act as the most third common cause of complicated UTI after *E. coli* and *Klebsiella*.

Materials and Methods

Samples collection and their sources

120 urine samples of cow were collected randomly with sterile container from different area in AL-anbar province /Iraq. Through the period from Dcember, 2018 to May, 2019, the samples kept in ice box and transferred to the laboratory for bacteria examination (Harley and Prescott, 2002).

Isolation and Identification of *P. vulgaris*

All samples were streaked on blood agar, MaCcnokey agar and were incubated aerobically for 24hrs at 37°C. The bacteria were identified depending on microscopical feature by using Gram stain to detect their response to stain, arrangement and shape (Quinn *et al.*, 2011). In addition, the morphological features on culture media such as swarming on blood agar, Non lactose ferment on MaCconkey agar also several biochemical tests were used to identify the *Proteus* isolates, such as catalase, indole, citrate utilization tests, methyl red/ Voges-proskauer (MR/VP) tests, urea test, gelatin liquefaction test, motility test and triple sugar iron test (O'hara *et al.*, 2000) finally confirmed with Vitek2 system (Harley and Prescott, 2002).

Table 1: Primers set used for detection of P.vulgaris (Intron/korea) (10).

Target gene	Primer name	(Sequence) (5´-´3)	Product size (bp)	Tm °C	GC %
16 Sr RNA gene	F	´5-CCG AAG GTT AAG CTA CCT AC -3´	750 bp	52.2	50
	R	´5-CCA TGT GTA GCG GTG AAA TG -3´	750 bp	54.1	50

Molecular detection of isolated *P. vulgaris*by PCR

• DNA extraction:

Genomic DNA was extracted from *P. vulgaris*isolates by used G-spin™ Total DNA extraction kit (Intron/ korea). One or two ml of bacterial culture was transferred to 2 ml micro-centrifuge tube then centrifugation for 1 minute at 13.000rpm and discard supernatant by tipping. Add 200 µl Buffer Cl, 20 µl proteinase K and 5 µl RNase A solution and mixed vortexing vigorously. Incubated lysate at 56°C for 10-30 min. After lysis completely add 200 µl of buffer BL into upper sample tube and mix thoroughly. Then incubate the mixture at 70°C for 5min. Centrifuge the sample tube at 13,000rpm for 5 min to remove un-lysed tissue particles. Then carefully transferred 350-400µl of supernatant in to anew 1.5 mml tuber. Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5-6 times or by pipetting. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid. Carefully apply the mixture to the spin Column without wetting the rim, close the cape and centrifuge for 1min at 13,000rpm. Discard the flow-through and reuse the collection tube. Add 700 µl of Buffer WB to spin column without wetting the rim and centrifuge for 1 min at 13,000rpm. Discard the flow-through and place the Column into a 2.0 ml collection tube, Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and collection tube. Place the Spin Column into a new 1.5ml tube and 30-100 µl of Buffer CE directly on to membrane incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000rpm to elute. The purified DNA was checked by 1% agarose gel, 5µl of DNA was mixed with 3 ml of loading dye electrophoresis and viewed using UV illuminator (Sambrook and Russell, 2001).

Amplification genes encoded for 16SrRNAof *P. vulgaris*

A specific primer was used for PCR amplification of 16SrRNA gene: F1:´5-CCG AAG GTT AAG CTA CCT AC-3´ and R1:´5-CCA TGT GTA GCG GTG AAA TG -3´. Used the master mix (Taq polymerase, PCR buffer, DNTPs) and the final concentration of primers was 10 pmol/µl with TBE buffer, PCR was performed under following condition: the Initial Denaturation was 94°C for 5 min followed by denaturation at 94°C for 45 sec., annealing at 54°C for 45 sec for 45 cycle, the amplified sample was directly loaded in a 2% agarose gel and for 1:30h, then visualized by U.V. light (Mokhtar and Bahaa, 2016)

Nucleotide sequence of 16 SrRNA Gene2.3.3

Forward primer of 16Sr RNA was sent to Korea for identifying sequence product PCR. The data of nucleotide sequence of the 16Sr RNA gene of *P. vulgaris* isolates were aligned and compare with similar sequence of the 16Sr RNA gene of the reference strain of *P. vulgaris*in Gen bank searched in the BLAST program of NCBI website. The forward sequence for *P. vulgaris* strains with range of nucleotide from 665 to 1396 has identity with 96% percentage and this strain was designated as

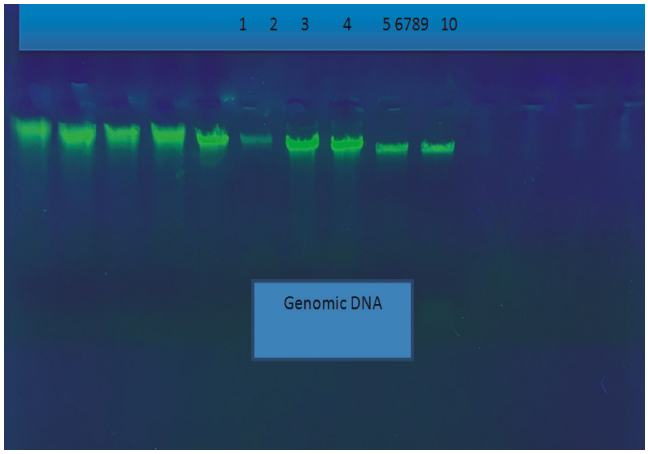

Fig. 1: Gel electrophoresis of genomicDNA extraction from *P. vulgaris*, DNA bands extracted using G-spin Tm Total DNA Extraction Kit with 1% agarose gel, TBE buffer (1X)at 5 vol /cm for 1:15 hour.

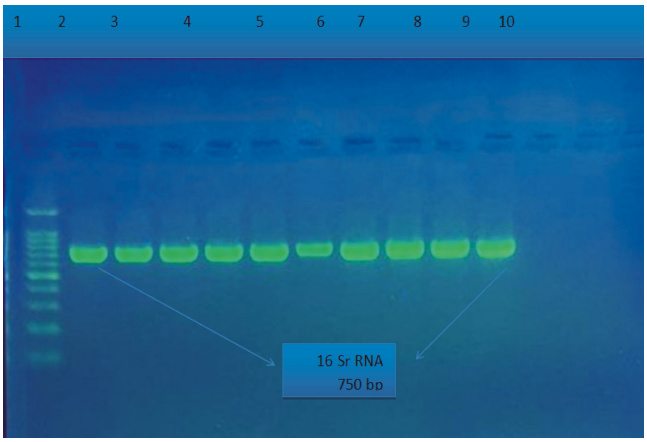

Fig. 2: PCR amplification of 16 SrRNA was electrophoresis on 2% agarose at 5 volt/cm². 1X TBE buffer for 1:30 hours. N: DNA ladder (100), lanes (1-10), visualized under U.V. light, the band size 750 bp.

Table 2: Partial sequence of 16S ribosomal RNA gene for *Proteus vulgaris*.

Source	Identities	Expect	Score	Sequence ID	Range of nucleotide	Nucleotide	Location	Type of subsitiution	No. of sample
Proteus vulgaris	96%	0.0	1199	ID: MH985218.1	665-1396		-		1

Proteus vulgaris strain A7FtNRM1, fig. 3 and table 2.

Result and Discussion

Isolation and Identification of *Proteus vulgaris*

In this study the percentage urine of cow with *P. vulgaris* was 10(8.33%) from 120 collecting urine sample from different area in AL-anbar /Iraq.

On MacConkey s agar plates the colony morphology as small pale colonies, Little convex and circular with smooth edges and were lactose non ferments and turned the media to yellow. *Proteus* strains growing on MacConkey agar because it differentiates it from other Gram negative species and it contains all required nutrients for *Proteus* growth in addition, Proteus culture has special smell (fish smell). Swarming motility its very significant used to differentiated it from other *enterobacteria aceae* family.

Microscopic Examination

Microscopic Examination of bacterial cells isolate are showed negative to gram stain, red color coccobacilli, frequently occurred singly or in short chains, variable in length and non-spore forming.

Biochemical Identification Results for *P. vulgaris*

The 10 isolates of bactria showed catalase, indole, methyl red/ Voges-proskauer (MR/VP) tests, urease test, gelatin liquefaction test, motility test and triple sugar iron test positive, citrate utilization tests and oxidase negative (5).

Vitek2 Systems Results for Diagnosis *P. vulgaris*

The (10) of bacteria isolates that identified as positive with the biochemical tests were subjected to Vitek2 system to verified the diagnosis of biochemical tests. Vitek2 BCL card supply a major advance with highly sensitive method for reliable identification of *P. vulgaris* in comparison with other phenotypic methods (Mohammed *et al.,* 2016), the results of ten isolates of *P. vulgaris* were identified with Vitek2 system as positive isolates and the probability of positive results of biochemical test of Vitek2 system were between 90-95%.

Result of Molecular detection of Proteus vulgaris with PCR

Polymerase Chain Reaction (PCR) has become one of the most important molecular diagnostic method for pathogens detection and is consider to be valuable alternative to culture based detection techniques due to its speed, sensitivity, limited of detection and specificity (Caroline *et al.,* 2003).

All DNA of 10 *P. vulgaris* isolates were successfully extracted and the concentration of DNA was determined by Nanodrop 1000 spectrophotometer at 366nm, the purity of extracted DNA range from 1.5-2. The extracted DNA was visualized under UV after electrophoresis with 1% agarose gel at 70 volt for 30 min. Specific primer were used to determine the 16Sr RNA genes in this study, the optimal condition were identified after several condition it has been found that best volume of DNA template was 5µl and the final volume of reaction mixture was 20µl that transferred to a thermal cycler and the denaturation and annealing temperatures were (94°C, 54°C) respectively, PCR product appeared as a DNA band with about (750)bp. The positive result was confirmed by agarose gel electrophoresis in a 2% agarose stained with red safe stain, electrophoresis in 70 volt for 1:30 hr. and 10 lanes were photographed under UV with (750) bp band size.

Proteus vulgaris strain A7FtNRM1 16S ribosomal RNA gene, partial sequence:

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Query  1     CACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1396  CACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATT  1337
Query  61    CTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGG  120
Sbjct  1336  CTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGG  1277
Query  121   ACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1276  ACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGC  1217
Query  181   CATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC  240
             ||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct  1216  CATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC  1157
Query  241   CTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1156  CTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACA  1097
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Discussion

Out of 120 urine samples of cow, 22 specimens (26.4%) were isolated and identifies *Proteus spp*. These results agree with (Sambrook and Russell, 2001). 12(10%) specimenswere identified as *Proteus miribilis* and 10(8.33%) specimens were identified as *Proteus vulgaris*, these results agree with (Mokhtar and Bahaa, 2016; Mohammed *et al.,* 2016) who mentioned that percentage for *P. mirabilis* isolation was 66.6% and for *P. vulgaris* was 33.3%.

MacConkey agar is widely used for isolation and

enumeration of *P.vulgaris*. In this study the morphological and phenotypic characteristic of local isolates appear was non lactose fermenter, pale, convex, circular and smooth colonies, with special fish-like odour and Swarming phenomenon on blood agar media.

The results agreed with the results recorded (Caroline *et al.*, 2003). On other hand the biochemical and microscopic results obtained by this study concerning the identification of *P. vulgaris* agreed and consistent with what indicated by studied (Lazaro *et al.*, 2007).

The Vitek2 BCL card supply a major advance with highly sensitive method for reliable identification of *P. vulgaris* in comparison with other phenotypic methods. In this study 10 local isolates of *P. vulgaris* were diagnosed by Vitek2 system in the WAHJ ALDNA Laboratory and depending of the results of biochemical tests of BCL Cassette the 10 isolates were positive and identified as *P. vulgaris* with percentage of probability (95%) the result agreed with (Naz and Rasool, 2013) with correct identification percentage (98%).

In this study used Specific primer for determine the 16SrRNA genes; the optimal conditions were identified after several experiments these condition it has been found that the best volume of the DNA template was 3µl and the final volume of reaction mixture was 20µl that transferred to thermal cycler and the denaturation and annealing temperature were (94^{o}C, 54^{o}C) respectively, PCR product appeared as a DNA band with a bout (750) bp. Many studies have already been done to evaluate the occurrence of pathogenic microorganism of *P. vulgaris* that were (Bana *et al.*, 2017; Feglo *et al.*, 2010).

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