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Isolation and molecular characterization of *Serratia liquefciense* isolated from pneumonic sheep lungs in Iraq.

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ABSTRACT

The prevalence of *Serratialiquefaciens* to the epidemiology of bacteria associated disease in animals and human in Iraq is not clear. The genus serratia comprises fifteen species and three subspecies but only two are commonly isolated from clinical material, they are S.liquefaciens and S.marcescens.The current work was designed to identify *Serratialiquefaciens* isolated from pneumonic sheep lungs, out of 300 lungs samples , five isolates were distinguished as*S. liquefaciens*in percentage at the rate 1.66% depended on the basis of cultural, morphological and biochemical characteristics . The molecular level confirmation applied with small subunit ribosomal RNA (*16S rRNA*), gene identification ,amplification and sequence. Bacterial genomic DNA was extracted and checked by Nano drop spectrophotometer . Convential polymerase chain reaction (PCR) carried by using specific forward and reverse primers (TGCCTGATGGAGGGGATAA) and (CTCCTTGAGCCCGCCAT) respectively. The PCR products were purified , sequenced and analyzed for nucleotide matching by Blast-NCBI. Our fiveisolates were submitted in GenBank and recorded withaccession numbers KP861223.1, KP861224.1, KP864056.1, KP864057.1 and KP864058.1 Iraqi *Serratialiquefaciens*which have high identity with the reference world strains iDQ123840.1 China isolated from giant panda intestine and AB981192.1 China strain A13 isolated from loach surface. In conclusion, *S. liquefaciens* was isolated from pneumonic sheep lungs samples and detailed molecular characterizations associated with sequence analysis of small subunit (*16SrRNA*)gene provide an excellent method for accurate diagnosis and measurement of evolutionary relationships among species of *Serratialiquefaciens*group.

KEYWORDS: *Serratia*, PCR, Sheep, Phylogenetic Analysis.

INTRODUCTION

The genus *Serratia*a member of the *Enterobacteriaceae*, is comprise of a group of bacteria that are related both phenotypically and genotypically[1]. *Serratialiquefaciens* is an opportunistic pathogen which is capable of colonizing a wide variety of surfaces in water, soil, the digestive tracts of rodents, plants, insects, fish, and humans [2].

*Serratia*species areregarded as significant pathogens to which a variety ofinfections including peritonitis, pneumonia, sepsis andwound infections have been attributed [3],[4] [5],[6],[7],[8]**.** FurthermoreSerratia were one of the etiological agents of hospital acquired bacterial pneumonia [9].

Techniques based on nucleic acids such as DNA hybridization, RFLP analysis and different techniques using PCR and sequencing have been developed to improve identification of bacteria. The nucleic acid sequence particularly is of large potential value since it contains more evolutionary information than the traditionally used phenotypic traits and because it isprecisely defined and relatively simple to determine[10]**.** Two important properties of a nucleotide sequence to be used for bacterial identification are as follows: it must be universal in

its distribution and it must contain sufficient sequence variation. The most commonly used molecule is *16S rRNA* since it is generally accepted that the sequence of *16S rRNA*can be used to distinguish genera and well-resolved species [9],[11],[12],[13].

This work was designed to isolate,identify and phylogenic analysis of locally isolated*Serratialiquefaciens* circulating in Iraq ,and to focalize spot light on the clinical, epidemiological features and molecular characterization of the bacteria.

MATERIALS AND METHODS

Samples collected according to [14], three hundred pneumonic sheep lungs samples which showed congestion, emphysematous lesion and hepatization were collected from sheep in slaughter houses in Al Diwaniyahgovernorateof Iraq . These lungs sections were placed in sterile containers and transported under cooled conditions to laboratory for bacteriological examination by taking a small piece of the lung aseptically after sterilizing the outer surface of the lung by hot spatula. Samples were transferred by sterile forceps in thioglycollate broth tube according to [15].

Identification of bacteria were based on morphological, cultured and biochemical characteristics [15]. The most frequent enrichment medium was blood agar plate containing (5%) whole blood.Thioglycollate broth was also used to promote the growth of bacteria [16],[17].

Bacterial identification by using BioMeriux Vitek2 compact system kit according to [18].

Bacterial isolateswere cultured on sterile brain heart infusion broth(BHI) and glycerol (20%) and incubated at $(37)^0$C for 24hrs, then after turbidity occurred, stored at $(-20)^0$C [15].

Bacterial genomic DNA was extracted from *Serratialiquefaciens* isolates by using (Presto™ Mini gDNA Bacteria Kit. Geneaid. USA). One ml of overnight growing bacteria on(BHI) broth was placed in 1.5ml micro centrifuge tubes and then transferred in centrifuge at 10000 rpm for 1 minute. After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction . After that, the extracted gDNA was checked by Nano drop spectrophotometer, then storedat -20C^o.

PCR assay was carried out by using forward primer (TGCCTGATGGAGGGGGATAA) and Reverse primer (CTCCTTT GAGTTCCCGCCAT) that amplify, (1025bp) PCR product were provided by Genebank of *Serratialiquefaciens* strain *16S ribosomal RNA* gene, complete sequence NCBI Reference Sequence: NR_121703.1[19].

PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer.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 prepared according to kit instructions in 20µl total volume by added 5µlof purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then the PCR premix tube was completed by deionizer PCR water into 20µl and briefly mixed by Exispin vortex (Bioneer. Korea). The reaction was performed in a thermo cycler (MyGene. Korea) by set up the following thermo cycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 55 °C for 30 s, and extension 72 °C for 30 s and then final extension at 72 °C for 7 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

PCR product was purified from agarose gel by using (EZ EZ-10Spin Column DNA Gel Extraction Kit, Biobasic). Specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube. 400 µl Binding Buffer II was added to gel fragment. After that, incubated at 60ºC for 10 minutes and shacked until the agarose gel was completely dissolved.Above mixture was added to the EZ-10 column and let stand for 2 minutes and Centrifuged at 10,000 rpm for 2 minutes and flow-through in the tube was discarded. After that,750 µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Solution was discarded. After that, the step 4 was repeated. Then, centrifuged at 10000 rpm for an additional minute to remove any residual wash Buffer. After that, the column was placed in a clean 1.5ml microcentirfuge tube and 30µl of Elution Buffer was added to the center of the column and was incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and stored at -20ºC.

The PCR products sequenced in both directs by using (ABI prism DNA sequencing kit) and an (ABI sequencer Bio system).

The bacterial *16S rRNA* gene sequences obtained were aligned with selective known *16S rRNA* sequences in GenBank using basic local alignment search tool (Blast and identity score) were generated to identity bacteria.

The nucleotide sequence data in this paper recorded and published in the GenBank, nucleotide sequencedatabases of *Serratialiquefaciens* , partial sequence based on *16S rRNA* gene with the following accession numbers: KP861223.1 ,KP861224.1 , KP864056.1 , KP864057.1 and KP864058.1.

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The alignment provided a list of matching bacteria, ranked for similarity between our isolates sequences and sequences of corresponding bacteria from database bacterial *16S rRNA* gene obtained from GenBank were also aligned by using Clustle W2 program (2007). The evolutionary analyses was inferred using the UPGMA method [20]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) [21]. The evolutionary distances were computed using the p-distance method [22]. The analysis involved 17 amino acid sequences. Evolutionary analyses were conducted in MEGA6 [23].

RESULTS AND DISCUSSION

Bacterial isolation from pneumonic sheep lungs were tentatively identified as*Serratia* by morphological and cultural characters .The results of biochemical characteristics by using vitek2 compact technique, most closely related to *Serratialiquefaciens*.Fivestrains of*S. liquefaciens* isolated in this study at the rate (1.66%).

Isolation of this microorganism from sheep lung may emphasize its role in the pathological changes of lower respiratory tract of animal and may predispose more severe infections by other bacteria. [24] isolated *S.liquefaciens*from a total 17 human patients primarily from respiratory secretion. Furthermore *S.liquefaciens*isolated from urine sample, wound and cerebrospinal fluids, some patients suffered from sepsis[25].

Several different outbreaks of Serratia including *S.liquefaciens* can cause mastitis[26]. Animal source of this bacteria may have medical and public health importance. *Serratialiquefaciens*cause proteolyticspolige of heat treated milk similar as *Psdomonas*[27]. Furthermore [28]stated that serratia species were one of etiological agents of hospital acquired bacterial pneumonia .Some Serratia toxins was hemolytic to sheep RBC and cytotoxic to human respiratory tract and corneal limbial epithelial *in vitro*[29].

Isolation of *S. liquefacien*s from suppurativethrombophilebitic and native valve endocarditis recorded also by[30]

PCR results:

The extracted genomic DNAof *Serratialiquefaciens*were amplified for detection of *16S rRNA*gene.The amplified PCR products were visualizedbyagarose gel electrophoresis, all isolates 16s r RNA gene were found having a molecular weight were at size 1025bp when compared with the DNA marker (Figure 1).

In addition to PCR confirmative diagnosis of the bacteria , the PCR products collected and purified until used in the sequencing technique for analysis of*16SrRNA* gene of predominate strain of*Serratialiquefaciens* isolated from pneumonic sheep lungsin Iraq.

All partially sequenced*16S rRNA* gene of our Iraqi*Serratialiquefaciens*isolates were analysed , recorded and published in GenBank under ACC. Nos. KP861223.1, KP861224.1, KP864056.1, KP864057.1 and KP864058.1.

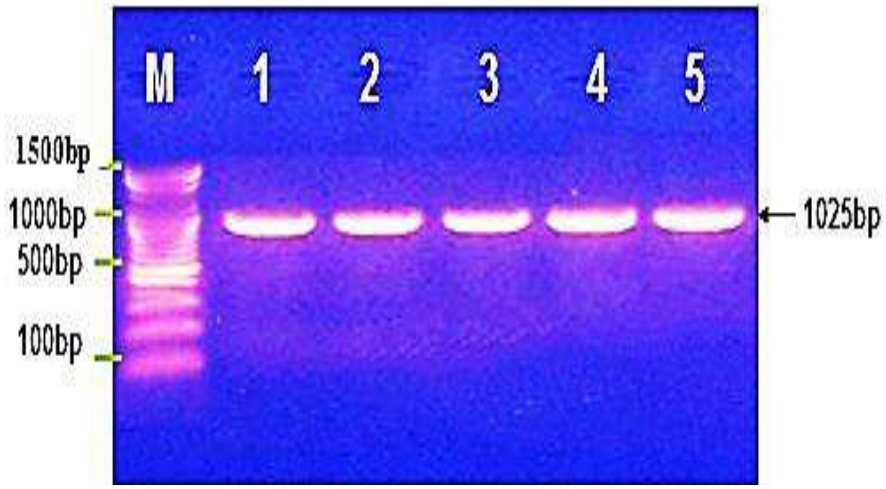
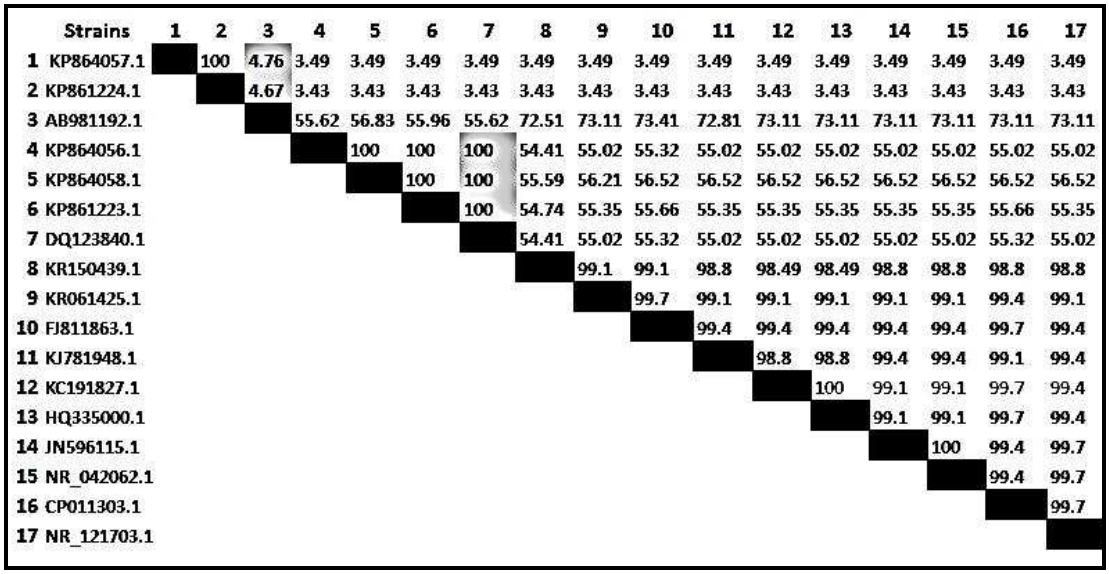


Fig. 1: PCR detection of *16s rRNA* of *Serratialiquefaciens*(M) marker in size was (1500bp), 1 ,2 ,3 ,4 ,5 DNA Samples (1025bp) of the gene.
1: N02 I-D (KP861223.1), 2: N03 I-D (KP861224.1), 3: I.D-Sania (KP864056.1), 4: I.D-Afak(KP864057.1),5: I.D-Hamza(KP864058.1).

Phylogenetic analysis of 16S rRNA:

Results of multiple sequences alignment of the partial*16SrRNA* sequences of five Iraqi*S. liquefaciens* isolates when alimented with others selected world reference *S. liquefaciens* showed high similarity and closed relationships with our isolates.(Figure. 1).



Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 KP864057.1		100	4.76	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49	3.49
2 KP861224.1			4.67	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43	3.43
3 AB981192.1				55.62	56.83	55.96	55.62	72.51	73.11	73.41	72.81	73.11	73.11	73.11	73.11	73.11	73.11
4 KP864056.1					100	100	100	54.41	55.02	55.32	55.02	55.02	55.02	55.02	55.02	55.02	55.02
5 KP864058.1						100	100	55.59	56.21	56.52	56.52	56.52	56.52	56.52	56.52	56.52	56.52
6 KP861223.1							100	54.74	55.35	55.66	55.35	55.35	55.35	55.35	55.35	55.66	55.35
7 DQ123840.1								54.41	55.02	55.32	55.02	55.02	55.02	55.02	55.02	55.32	55.02
8 KR150439.1									99.1	99.1	98.8	98.49	98.49	98.8	98.8	98.8	98.8
9 KR061425.1										99.7	99.1	99.1	99.1	99.1	99.1	99.4	99.1
10 FJ811863.1											99.4	99.4	99.4	99.4	99.4	99.7	99.4
11 KJ781948.1												98.8	98.8	99.4	99.4	99.1	99.4
12 KC191827.1													100	99.1	99.1	99.7	99.4
13 HQ335000.1														99.1	99.1	99.7	99.4
14 JN596115.1															100	99.4	99.7
15 NR_042062.1																99.4	99.7
16 CP011303.1																	99.7
17 NR_121703.1																	

Fig. 2: 16S rRNA gene sequence similarity and divergence of each pair for *S.liquefaciens*Clustal W2

Phylogenetic analysis was performed based on*16SrRNA* gene of our five isolates and twelve world selected reference strains .Phylogenetic tree constructed of our *S. liquefaciens*isolates revealed that the KP861223.1, KP864056.1 and KP864058.1 Iraq isolated from pneumonic sheep lungs in in Al-Diwaniyah\ Iraq were have high similarity and closed relationship together with strain DQ123840.1 China isolated from giant panda intestine[31]Figure 2.Phylogenetic analysis detected 100% similarity among the five Iraqi isolates ,the results showed that the Iraqi strains clustered with strains isolated in china .There was no molecular data documented about *S. liquefaciens* in Iraq or the closest countries ,the phylogenetic relationship may caused by importation of sheep from many sources of the world.

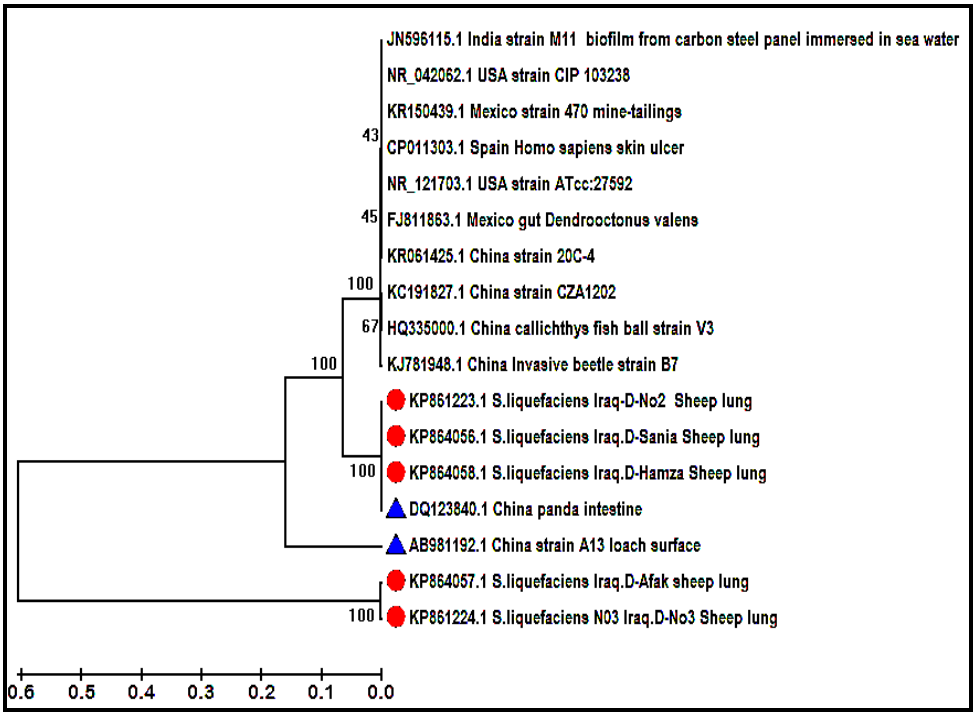


Fig. 3: Phylogenetic tree using the UPGMA method.The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site.The bootstrap test (1000 replicates) are shown next to the branches . Evolutionary analyses were conducted in MEGA6 .

*square red color : *S. liquefaciens*strains isolated from Iraq.

*Triangle blue color: World reference *S. liquefaciens*strains have more similarity with Iraqi isolated strains.

Other strains of this study KP861224.1and KP864057.1 clustered with the AB981192.1 China strain A13 loach surface (Figure 3).

Five strains of *S. liquefaciens* isolated in this study at first time in Iraq from pneumonic sheep lungs at the rate (1.66%),, there was no available data hereto compare our results of infection rate. Molecular technique PCR results were confirmative and rapid [32]and [33]stated thatnucleic acid detectionmethods including PCR are well known sensitive techniques for more rapid detection and specific identification of an organism, therefore, hold promise for sensitive and detection within much shorter time.The basis for PCR

diagnostics applications in microbiology is the detection of infectious causative agents and distinguishing between pathogenic and nonpathogenic strains by virtue of specific gene .Although the small subunit *16S rRNA* gene is usually targeted design of species – specific PCR primers for rapid identification[34].The comparison of ten *Serratia* genomes have identified the universal core genes of*serratia* and unique genes to each strain[35]

Recent developmentin genome sequencing technology cause a major change in the methods of bacterial classification. Sequencing of highly conservedDNA fragment of the bacterial genome, such as the *small subunitrRNA*gene, now create aninternational method of estimating the phylogenetic relationships among all organisms. Such DNA-based phylogenetic analysis induce big recent knowledge about microorganisms that were not used in the past classification. Phylogenetic analysistoday strongly accepted as the prime method of representing taxonomic relationships among bacteria. Many groups based on samephenotypiccharacters may be quite differ from one another based on phylogenetic features.

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