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COMPARATIVE STUDY AND PHYLOGENETIC ANALYSIS OF A BIOFILM *PANTOAE* SP. WITH UNPUBLISHED STRAINS FROM GENE BANK

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Abstract

A total of (64) samples of (blood, urine and milk) presented to the Al-Qasim Green University (Microbiology Lab). Among them 3 of specimens were found to be with *pantoea* sp. bacteriuria. In the present study, it was observed that the number of patients with *Pantoea* spp. were positive by (cultures and the automated system VITEK 2 (bioMerieux, Marcy I'Etoile, France) to achieve final diagnostics and identification of the species level, molecular characterization of *Pantoae* isolates done by PCR technique for the three isolates identification of *pantoea* sp. isolates using specific species primer for *esaI* gene. The analysis of phylogenic established on the *pantoa esaI* gene. Locally MN200754 *Pantoea* sp. isolate fig. 2 (Number 1) illustrated high concern to NCBI-Blast LC388386.1 *Pantoea stewartii* esaI gene were show less genetically different. Whereas other worldwide strain from other countries were being genetically highly distinct from our local Iraqi MN200754 *Pantoea* sp. isolate. Phylogenetic analysis of esaIpgenes by PCR. Sequencing of esaI gene to confirm the results and to determine some variants of this gene with the other unpublished gene bank strains.

Key words: *esaI gene*, *pantoea*, biofilm, Phylogenetic tree analysis.

Introduction

Pantoea sp. are gram-negative bacteria were firstly reported in 1972 as *Enterobacter* and *Erwinia* which belong to enterobacteriaceae family (Bottone and Schneierson, 1972).

Pantoea sp. opportunistic pathogen can cause human diseases by two ways; wound contaminated with plant materials or nosocomial infections (Dutkiewicz *et al*., 2016). Immuno compromised patients were especially infected with these bacteria via contaminated fluid (Shubov *et al*., 2011; Boszczowski *et al*., 2012). Recently increase reported cases with *Pantoea* sp. in 2007 was reported as a causative agents to pediatric infections when isolate from various sites of the body involving urinary tract (Cruz *et al*., 2007). Few studies have been mentioned the possibility of biofilm formation by *Pantoea* sp. (Hasson *et al*., 2018). Normally, biofilm process start with the attachment to a selected surface then colonization as well as grown up to be mature structure of biofilm and ended by detachment

Studies presented in 2010, revealed that cases were decreased at low level to detect the good response to antibiotics treatment (Lee *et al*., 2010). But it return to appear in 2012 as nosocomial outbreak in haemodialysis patients and other cases associated with chronic renal failure (Kazancioglu *et al*., 2014).

Several researches were focused on isolation of *Pantoea* sp. from urine (Cruz *et al*., 2007; Büyükcam *et al*., 2017; Hasson *et al*., 2018) where, *Pantoea* isolated from different specimens of human body and was found 10% and 21.4% isolates from UTI patients respectively.

Materials and methods:

Collection of samples

Different clinical (64) samples of (blood, urine and milk) at 10 ml of each one in sterile test tube and

Table 1: Samples information.

No.	Source	Date of collection
10	Milk/cow	20/5/2018
50	Urine/Human	1/7/2018
4	Blood/Human	23/7/2018

transferred immediately to the Al-Qasim Green University (Microbiology Lab). Routinely, bacterial culture was done to each sample after culturing it on different culture media (Nutrient, MacConkey and blood agar) by streaking method to obtain pure culture then identified and diagnosed by the automated system VITEK 2 (bioMerieux, Marcy I'Etoile, France) to achieve final diagnostics and identification of the species level.

Biofilm testing

The biofilm assay was done by congo red method according to (Freeman *et al*., 1989). The black crystalline colonies were considered as strong biofilm producers while dark colonies without dry crystalline colonies as moderate biofilm producers and dark pink colonies as non-biofilm producers.

Molecular Diagnosis

According to the ViTK2 result for the positive samples. Polymers chain reaction done for the further confirmation, primer was designed by (NCBI) Primer 3 software for *Pantoea* sp. as shown below.

127bp	Provided by	TTTTGCCACCGCGTCAAAAC	F
	Bioneer, South Korea	TGGCGTATCGTTGCTGAATC	R

Genomic DNA extraction done for the samples according to the Extraction Kit from (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA).

Extracted DNA were used for PCR

PCR Master mix preparation regarding Company instructions for polymerase chain reaction (PCR) master mix brought to the total volume of 20 µL as mentioned in the table 2.

Phylogenetic Tree

Phylogentic tree were constructed using our sequence with 5 known sequence upublished in GenBank Clustal W alignment and MegaX software Version 10.0 (Kumar *et al*., 2008; Kumar *et al*., 2018) tree constructed using neighbor joining method with bootstrap 1000 replicate with mega.

Table 2: Extracted DNA were used for PCR.

Volume (µL)	PCR master mix
5	DNA template
1.5	Forward primer (10 pmol)
1.5	Reverse primer (10 pmol)
12	PCR water
20	Total volume

Table 3: Five unpublished Genome from GenBank were used in comparing with the Iraqi Isolate.

Accession No.	Country	Published	Source
CP017581	USA	Unpublished	Pantoea stewartii
L32183	USA	Unpublished	Pantoea stewartii
CP020943	USA	Unpublished	Pantoea ananatis
CP035034.1	China	Unpublished	Pantoea ananatis
AP012032.2	Japan	Unpublished	Pantoea ananatis AJ13355
MN200754	Iraq	Unpublished	*Pantoea* sp.

Results and Discussion

The current study revealed that *Pantoea* sp. isolates which isolated from urine, blood and milk samples were biofilm former (Fig. 1)

(Morohoshi *et al*., 2011, Von Bodman *et al*., 1998), which pointed that *Pantoea* members have been reported to synthesis QS which regulate many phenotypes, such as production of virulence factor, aggregation of cells and biofilm formation (Morohoshi *et al*., 2007).

Pantoea sp. shows QS activity via increase cell density- dependent Exoplysaccharide synthesis (Yunos

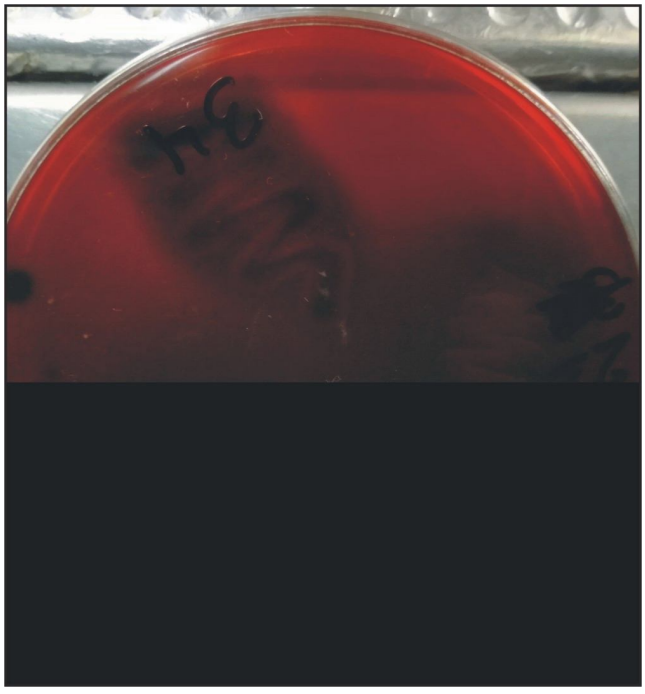


Fig. 1: Black crystalline colonies of *Pantoea* sp. on congo red agar.

et al., 2014; Tan *et al*., 2014). Exoplysaccharide (EPS) is a major component of biofilm bacterial matrix and a powerful virulence factor which protect the bacterial cell from antibiotics action and host immune defense (Minogue *et al*., 2005). *esal* quorum sensing gene was governed synthesis the exopolysaccharide (EPS) in *Pantoea* sp. to appropriate bacterial adhesion and biofilm formation (Koutsoudis *et al*., 2006). *esal* gene consider a typical N-acyl-L homoserine lactones (AHLs) synthase which catalyzes synthesis of N-3-oxo-hexanoyl homoserine lactone (Watson *et al*., 2002).

Some studies were pointed that non biofilm *Pantoea* sp. were uniformly susceptible to most antibiotics tested in their studies (Mardaneh and Dallal, 2013; Kazancioglu *et al*., 2014), but biofilm *Pantoea* sp. were less susceptible to antibiotics at 1000 times than free planktonic bacteria (Donlan, 2000).

In the present study, Pantone sp. detected by PCR. fig. 2, Showed that single amplification product with expected size of (127) bp and noticed in all (3) samples tested (clinical isolates).The exclusive existence of pantoae gene isolates offers a promising tool particularly appropriate for diagnostic. The esaI gene was studied as a diagnostic target for specific identification of pantoae by PCR amplification.

DNA sequencing done for one sample and the result studied to confirm the nucleotide sequences, then seek relationships with others strains in world, by NCBI- Blast-query nucleotide-online. So these results appeared the identities (100%) for Pantoea stewartii esaI gene from genebank strains and results of sequencing suggest that the *esaI* sequences are very preserved, however, that the prevalence of the *esaI* gene was (100%) from all isolation .Through *esaI* gene sequencing appeared partial gene

The phylogenic tree was created by neighbor-joining method in MEGA 1.0 X version. Locally MN200754 Pantoea sp isolate fig. 2 (Number 1) illustrated high concern to NCBI-Blast LC388386.1 Pantoea stewartii esaI gene were show less genetically different. Whereas other worldwide strain from other countries were being genetically highly distinct from our Iraqi locally MN200754 Pantoea sp isolate (White III *et al*., 2018). Pantoea stewartii mentioned in previous research as it belong to the enterobacteriaceae (Walterson and Stavrinides, 2015) family which is associated with human enteric and plant pathogenes like *E. coli* and *salmonella* sp.(Duong *et al*., 2018).

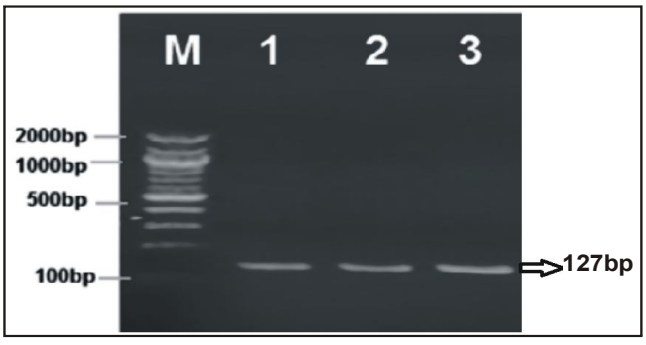
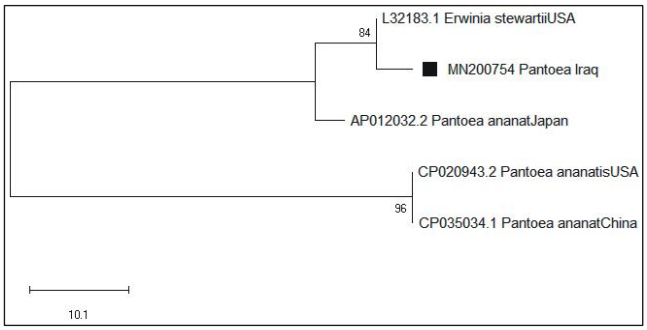


Fig. 2: Agarose gel electrophoresis image of PCR product analysis for *esaI* gene in pantoea sp. isolates. (Marker ladder 2000-100bp). Lane (1-3) positive *esaI* gene, PCR product size was (127bp) product size.



Phylogenetic analysis of *esaI* gene for *pantoea* sp. isolates. Nucleotide sequence of *pantoea* sp. from 1isolates and 4 unpublished pantoea strains were used in this analysis, the tree constructed using MegaX version 10 by the neighbor-joining method with 1000 bootstrap replicates the one isolate strains marked with the black square

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