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Isolation and Identification of Cellulytic Ruminant *Pseudomonas Aeruginosa* by Classical and Molecular Methods in Basrah Province

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ABSTRACT

Cellulose is the most abundant sugar on the surface of the earth and is the main building material in plants that is the primary link in what is known as the food chain. Cellulose is also found in a variety of other organisms, such as algae, fungi, and bacteria. Cellulose is a major component of the ruminant diets and can be broken down into metabolites that can be absorbed by the host for use as an energy source. This study was conducted to isolate and diagnose some cellulose-analyzed bacterial species present inside the ruminant rumen, where the number of samples was 30 samples distributed between (3) samples of camels (10) samples from sheep (4) from buffaloes (13) samples from cows, which were collected from the Basra and Najaf slaughterhouses, The isolates were diagnosed by biochemical and phenotypic tests, and then their diagnosis was confirmed by molecular methods by means of the 16SrRNA diagnostic gene by polymerase chain reaction (PCR). The molecular and phenotypic results showed that the number of isolates was 12 isolates distributed among the bacterial species where the number of *Pseudomonas aeruginosa* was three isolates by 25%.

KEYWORDS

Cellulytic Ruminant, *Pseudomonas Aeruginosa*, Molecular Methods.

Introduction

Cellulose is a major component of plant cell walls and is the most abundant renewable energy source (Whitaker, 1990). Cellulose is one of the most abundant organic compounds present on the surface of the globe, and it is a polymer of a large number of glucose units bound together by-1,4 glycosidic bonds. Each cellulose molecule contains up to about 10,000 units of glucose. The degree of polymerization varies according to the source of cellulose and the main source of cellulose is plant tissue (Egwuatu et al., 2018; Hussain et al., 2017). Cellulose constitutes about 50% of the dry weight of plant biomass and about 50% of the dry weight of biomass for secondary sources such as agricultural waste (Haruta et al., 2003).It also constitutes a large group of carbon sources for microorganisms that are responsible for decomposition of soil organic matter (Shanker et al., 2011).The decomposition of cellulose and its subsequent use is on the one hand important as a source of global carbon and the value of cellulose made it a source of renewable energy. Also, the abundance of cellulose makes it an attractive raw material for the production of many industrially important products (Bhat et al., 2000). At present, vegetable cellulose is mainly used for fuel, animal feed, Natural fertilizers, and in the paper industry (Hendriks and Zeeman, 2009; Agbor et al., 2011). and in many industries, including the manufacture of paper, clothing, cosmetics and food, especially diet foods (Cheng et al. 2009, Joog et al., 2003; Delmer and Amor 1995). With the help of the cellulosic system, it became possible to convert cellulose into glucose which is a more affordable, versatile and biologically appropriate product (Woodward Shewale et al. 1983, Shewale 1982). Bacteria are among the microorganisms that break down cellulose and found in the intestine of herbivorous and other organisms of plant nutrition, including insects. Cellulose-degrading bacteria can be found in different areas such as the digestive tract of ants, the rumen of ruminants, the large intestine of horses, in industrial waste, in the soil, and in all four parts of the ruminant intestine (rumen, reticulum, omasum, and real abomasum) (Irfan et al., 2012), From a microbiological point of view, the rumen is a complex ecosystem containing a wide range of microorganisms that can break down feed particles (Huws et al., 2016).Not all types of cellulose degenerating bacteria have been identified yet, however, molecular analyzes help to reveal groups that contribute to the degradation of cellulose and fibers (Flint et al., 2008).Some types of bacteria secrete cellulase and celluobiase enzymes, where the first enzyme breaks down the cellulose product, while the second enzyme breaks down cellulose into glucose, which is used by microorganisms as a source of energy. Therefore, researchers were interested in many bacteria that produce cellulase due to their high growth average and their resistance to the harsh environment when compared to fungi and have good potential in producing the cellulase enzyme such as *P. aeruginosa* is a Gram-negative aerobic bacillus of the Pseudomonadaceae family of the Gammaproteobacteria variety of the protobacteria and contains 12 individuals. Another in its family, P. aeruginosa is found most commonly in soil and water, as well as in plants and humans (Pang, 2019; Govan and Deretic, 1996; Smith and Iglewski, 2003) Pseudomonas aeruginosa has an extraordinary ability to colonize a large variety of

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habitats, especially in humid and watery environments, (Bergonier et al., 2014) Magill et al., 2003) and they are in the form of motile bacilli and are found either in the form of single cells or in pairs or chains. After 24 hours of growth, colonies on the feeding medium of the clays are large, rough and slightly convex, and the colony has a distinct odor (Brooks et al., 2007). Its length ranges between 1.5-3 µm and its width between 0.5 - 0.8 µm. It is mobile in liquid media and on solid surfaces and its movement depends on a unipolar flagellum (Robertson et al., 1989).

The study aimed to isolate ruminant bacteria, measure its ability to degrade cellulose, diagnose that bacteria by conventional and molecular methods, then determine the DNA sequencing and compare it with the NCBI Blast for the purpose of diagnosing samples and drawing the phylogenetic tree of the bacteria under study using the MEGA-X program.

Materials and Methods

(30) samples were collected from the slaughterhouses in Basra and Najaf and included the study samples (sheep, cows, buffaloes and camels). Samples were taken from the rumen fluid by three samples from the front, middle and back by a sterile 5 ml syringe, then samples were culture on the solid medium Bushnell Haas medium (BHM)), The dishes were incubated in the incubator at a temperature of 37 ° C for a period of 72-24 hours (Sari et at., 2017), after which the ability of bacteria to degrade cellulose was measured, where the bacterial colonies that had grown on BHM medium were exposed to an iodine solution for 20 minutes. Then it was washed with distilled water, and the presence of cellulase secreting bacteria was found by creating a transparent halo surrounding the growing bacterial colony. After that, the cellulose-dissolving colony was isolated in pure methods, and the bacteria most likely to degrade cellulose were selected for subsequent studies.

Identification of Bacteria

The bacterial isolates were diagnosed based on their agricultural and microbiological traits, biochemical tests, and molecular characteristics as follows:

A- Morphology

The bacterial isolates growing on BHM medium were initially identified depending on their phenotypic characteristics in terms of colony size, color and shape, as well as their ability to degrade cellulose.

B- Microscopical Examination

Depending on the method. (Soni,2013)

C- Biochemical Tests

Include (the oxidase test and the Catalase test according to the method of (Hemraj et al; 2013).

D-Molecular Study

The DNA genome was extracted from the bacterial isolates according to the method of Kato et al., 1998, then the 16S rRNA gene was amplified by PCR technology according to the initiator shown in the following table.

Table 1. Primers used to Amplification the 16S rRNA gene

Reference	Gene size (bp)	Tm(^{0}C)	Primer sequence 5'-3'	Primer name
(Lane, 1991)	1500	54	GGT TAC CTT GTT ACG ACT T	F1492
	1500	60	AGA GTT TGA TCC TGG CTC AG	R 27

PCR was performed with a volume of 50 l (mastermix25ixl (Bioneer), Premier Forward 5µl, Premier Reverse 5µl, Template DNA 5µl, Distal water 10µl).

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After the additions were completed inside the PCR tube, they were mixed by a shaking device to mix the components inside the tube. Then the tubes were transferred to the Thermo cycler device and placed inside and set the device work program as in the following table, where the PCR polymerization reaction was performed to amplify the 16SrRNA gene, according to (Raji et al., 2008) As in the table below.

Table 2. The program used to Amplification the 16S rRNA gene

Stage	Steps	temperature	Time (min)	The number of cycles
First	Initial denaturation	95 C	5 min	1
	Denaturation	95 C	30 sec	
second	Annealing	55 C	30 sec	
	Extension	72 C	30 sec	35
third	Final Extension	72 C	5 min	1

Then the samples were removed on a 2% acarose gel, and the gel was tested with a UV machine, then the results were recorded and photographed directly by the camera. After that, sequencing of the PCR products was done by Macrogen Corporation in South Korea, and after obtaining the DNA sequencing results, the results were compared with the gene bank, where the studied species were diagnosed based on these results, and the results of DNA sequencing were compared using NCBI http: // www. ncbi.nlm.nih.gov For alignment of samples, an evolutionary tree was created using MEGA 5 program.

Results and discussion

The number of samples reached (30) samples, which were collected from the slaughterhouses in Basra and Najaf. They were distributed between (3) samples of camels (10) samples of sheep (4) of buffaloes and (13) samples of cows. All of these samples were grown on Haas medium Bushnell. The primary culture was conducted on this medium for the purpose of isolating and purifying the study samples. The number of *P. aeruginosa* isolates was three, which is an approach to a study by (Hossain et al; 2013) in Bangladesh where it was isolated two isolates of *P. aeruginosa* from cattle using nutrient agar and Macconkey agar. Approach to a study by (Oyeleke and Okusanmi; 2008), where *P. aeruginosa* was isolated from the rumen of three types of ruminants (cows, sheep and goats) and their average was 9%. As for Duncan et al. (1999) was able to isolate *P. aeruginosa* from sheep's rumen. Their study produced 11 strains of *P. aeruginosa* and then performed phenotypic examination and biochemical and molecular tests. The studied bacteria were identified by their phenotypic properties by growing on Bushnell Haas medium, Nutrient agar and MacConkey agar. The results showed that *Pseudomonas aeruginosa* are negative isolates of the Cram stain, and this result agrees with (Cappuccino and Welsh; (2018). Where it appeared after testing it with a light microscope under the force of magnification 100x in the form of short rods, devoid of the capsule and containing flagellates used for movement. As for the production of pigment, they were distinguished by a greenish or greenish-yellow color for colonies growing on the medium of Nutrient agar, and this indicates their production of pyocene and bioferidine pigment (fluorescent green - yellow Figure (1A). As for its growth on the medium of MacConkey agar, it was yellow-coloured, and this indicates its inability to ferment lactose (Figure 2) and this is agree with Brown et al; (2004) and Forbes et al (2007) and also agrees with the study of Taima (2019). *P. aeruginosa* grows well at 37 ° C but can survive temperatures between 4 and 42 ° C (Stover et al., 2000). Its growth at a temperature of 42 ° C is an important diagnostic feature of *Pseudomonas aeruginosa* compared to the rest of the Pseudomonas species. This agrees with Procop et al (2017).

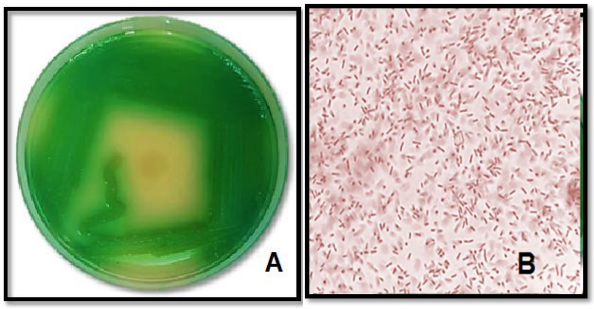

Figure 1. A: *Pseudomonas aeruginosa* growing on Nutrient agar and B image under a microscope, magnified a thousand times

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Figure 2. *Pseudomonas aeruginosa* on MacConkey medium

Table 3. Represents traits and pigments

P. aeruginosa	Character and pigments
-	Gram Staining
-	Capsule
+	Flagella
+	Pigment
-	Spore

+: positive; -: negative

Biochemical Tests

1 - The Catalase Test

This test was performed on *P. aeruginosa* isolates, and the result was positive for all isolates by the emergence of gas bubbles, thus they are able to produce the enzyme catalase and convert H_2O_2 into oxygen and water. Figure (4) Explain the test.

2 - The Oxidase Test

The results of this test showed that *P. aeruginosa* isolates are positive, where a bluish-purple color was observed within a few seconds of adding the reagent, and this indicates its ability to produce the enzyme oxidase. Figures (3,4) and table (4) Explain that. *P. aeruginosa* showed a positive result for both oxidase and catalase test, and this is consistent with Taima (2019).

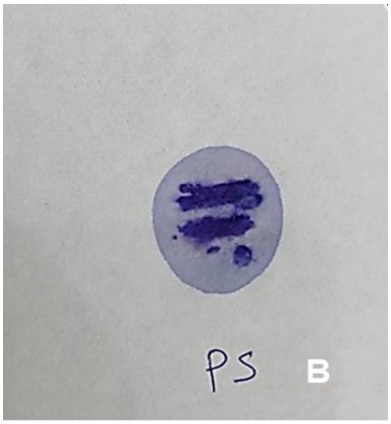

Figure 3. *P. aeruginosa*

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Table 4. Biochemical tests for *P. aeruginosa*

Biochemical tests	*P. aeruginosa*
Catalase Test	+
Oxidase Test	+
Motility test	+
Growth at 42 ^{0}C	+

+: positive; -: negative

Figure 4. Biochemical tests (Catalase Test) A; (Oxidase Test) B

Its Ability to Degrade Cellulose

P. aeruginosa isolates were grown on BHM medium, and all isolates were grown on the medium at a temperature of 37 C°. for a period ranging from 48 to 72 hours. All isolates showed their ability to degrade cellulose and were detected with iodine. Figure (5) shows. The results of the current study showed the ability of *P. aeruginosa* to degrade cellulose, showing its ability to degrade cellulose when grown on BHM medium, which is close to a study conducted by Agarwal et al; 2014) demonstrated the ability of *P. aeruginosa* to degrade cellulose due to its ability to produce the cellulase enzyme. This is identical to the study of Oyeleke and Okusanmi; (2008) who isolated some bacteria from the rumen of three different types of ruminants (cows, sheep and goats) where the percentage of *Pseudomonas aeruginosa* was 9%, Bacillus 37.8%, Micrococcus 8.1%, and Streptococcus 44.3%. These organisms were tested at a later time for their ability to degrade cellulose and they showed that the bacterial species *P. aeruginosa*, Streptococcus and Bacillus were able to decompose cellulose, and the study indicates that the ruminant rumen harbors many microorganisms that are active in breaking down cellulose and asymptotic to study Goel et al. (2019), their study was conducted to purify and characterize the cellulase enzyme from cellulosic microorganisms isolated from the landfill and from among 28 microbial isolates produced five isolates of Pseudomonas sp. Cellulase enzyme on BHM dishes containing 1% CMC.

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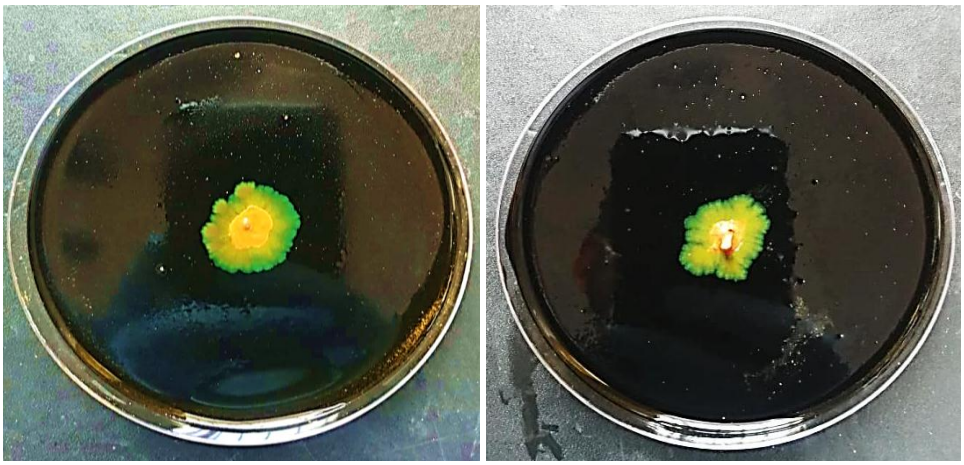

Figure 5. P. aeruginosa analyzer for cellulose on BHM medium

Molecular Study
DNA Detection

The DNA extracted from different bacterial isolates was detected by electrophoresis on a 1% Acarose gel containing ethidium pigment with a voltage difference of 80 volts (Figure 6).

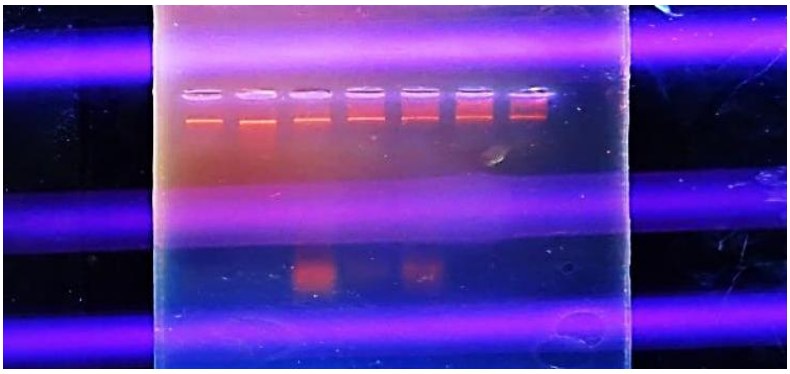

Figure 6. Electrophoresis of genomic DNA on 1% Aerosol gel

Gene Detection

The results of the amplification of the 16S rRNA gene for the isolated samples showed a positive result, and the gene size was 1600 bp. Figure (7) shows the migration for an hour at an amount of 65 V.

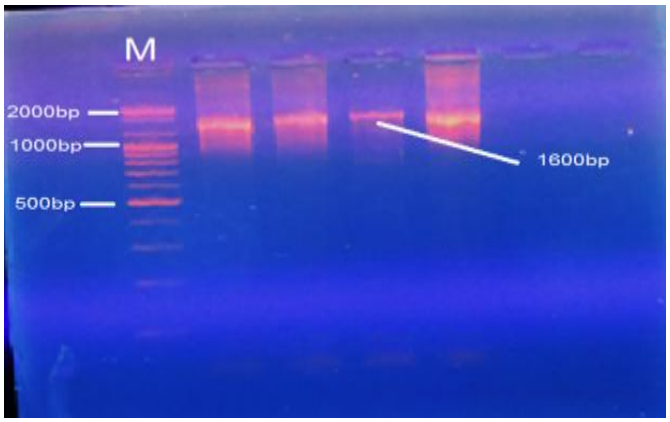

Figure 7. Electrophoresis of the PCR products of the 16SrRNA gene on an Acarose gel with a concentration of 1%

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M = Ladder

The results of the current study resulted in confirming the diagnosis of some species by matching them genetically with the reference isolates in the gene bank. The isolate *P. aeruginosa* was molecularly diagnosed and the R 45 and F 19 isolates showed a match with the reference isolates from (97.37 - 98.52), respectively. An approximation to the study Amoon et al; (2018), which indicated characterization of *P. aeruginosa* isolated from clinical samples by sequencing of the 16SrRNA gene. Sequencing analysis by BLAST program showed significant similarity and conformity with *P. aeruginosa* from China KX461910 and Australia JN609194 and with isolates of *P. aeruginosa*. Other than the gene bank database.

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