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MOLECULAR CHARACTERISATION OF PSEUDOMONAS ISOLATES FROM FOREGUT OF CAMEL

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ABSTRACT

The C1 compartment of the camels is the most investigated part of the GIT being rich in microbial suspension. The C1 compartment fluid of single humped camel (*Camelus dromedarius*) was cultured and three pure cultures showing similar colony morphology were isolated and subjected to biochemical and molecular characterisation. Based on the nucleotide homology and phylogenetic analysis, the cultures showed similarity for *Pseudomonas aeruginosa* strain *WJQ No. 1, Pseudomonas nitroreducens* strain *HP2* and *Pseudomonas aeruginosa* strain *DKH-3* on BLAST which were submitted to the NCBI gene portal having accession number: KJ789927, KJ789925, KJ789926, respectively. The study suggests that *Pseudomonas* do exists in the foregut of camel as seen in other ruminants.

Key words: C1 compartment, camel, *Pseudomonas*

The rumen is a complex ecosystem comprised of bacteria, archaea, protozoa, and fungi that are specifically adapted to allow the breakdown of starch and fiber constituents through anaerobic fermentation resulting in the production of Volatile Fatty Acids (VFA) that are, in turn, used by the ruminant as an energy source (Von Soest, 1994). The digesta, suspended in the bulk anaerobic phase of the gut contents, is coated with a biofilm of strict anaerobes; in contrast the biofilm attached to the gut wall provides ideal conditions for facultative bacteria which metabolise oxygen perfusing from the bloodstream (Cheng *et al,* 1979). Obligate anaerobes are bacteria that cannot survive in the presence of a high oxidation-reduction potential. Most of the studies in the area of gut microbiome are limited to the common herbivores like cattle, sheep and goat. The gut community structure of other herbivore like camels need more exploration as the studies in this regard are limited only to the enzymatic assays (Mohamed *et al,* 2000a, b and 2002) and isolation of common bacterial species like *Streptococcus bovis* (Ghali *et al,* 2004). The present paper reports the isolation and molecular characterisation of *Pseudomonas* isolates from C1 compartment fluid of camel.

Materials and Methods

Sample collection: C1 compartment fluid sample from a clinically healthy animal fed on guar phalgati (*Cyamopsis tetragonoloba*) was collected in a sterile pre gassed CO_2 jar using foregut fluid extraction unit designed for camels as per the ethical approval at NRC on Camel, Bikaner.

Culturing: The C1 compartment fluid was processed for the culturing as described by Bryant and Burkey (1953). Briefly, the samples were cultured on to the medium for microcrystalline degrading bacteria and incubated anaerobically at 37°C for 24 hours. Repeated sub-culturing was done till pure cultures were obtained. Three cultures showing the similar colony morphology were further characterised for molecular identification. These three pure cultures were also used to study the morphological and certain biochemical characteristics.

DNA Isolation: Bacterial DNA from pure cultures was isolated using bacterial DNA isolation Kit (Xcelgen). Quality and purity of DNA were checked by agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer (Sambrook and Russel, 2001) was used for submarine gel electrophoresis. Ethidium bromide (1%) was added @ 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at room temperature. DNA was visualised under UV using UV transilluminator. The DNA was used further for PCR.

Polymerase Chain Reaction (PCR): For Bacteria 16S RNA gene fragment was amplified by PCR from genomic DNA using 16S gene universal primers: 8F (AGAGTTTGATCCTGGCTCAG) and 1492R

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(ACGGCTACCTTGTTACGACTT). Composition of reaction mixture for PCR was as follows:

Components	Quantity	Final concentration
DNase-RNase free water	7.50 µl	–
2X PCR master mix (MBI Fermentas)	12.50 µl	
Forward Primer	1.00 µl	10 pmole
Reverse Primer	1.00 µl	10 pmole
Diluted DNA	3.0 µl	30ng/ µl
Grand Total	25.00	

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. PCR tubes containing the mixture were tapped gently and spinned briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given below:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Final Denaturation	94°C	30 Sec	30
Annealing	52°C	30 Sec	
Extention	72°C	90 Sec	
Final Extention	72°C	10 min	1

Visualisation of PCR Product

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2% agarose gel containing ethidium bromide (1 per cent solution @10 µl/100 ml) at constant 5V/ cm for 30 min in 0.5 X TAE buffer. The amplified product was visualised as a single compact band of expected size under UV light and documented by gel documentation system (Biorad). Purification of PCR product using Xcelgen Gel extraction kit according to the manufactures protocol was done.

Sequencing of Purified 16S rDNA Gene Segment:

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyser (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturers instructions.

Cycle Sequencing

Cycle sequencing was performed following the instructions supplied along with BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction was carried out in a final reaction volume of 20µl using 200µl capacity thin wall PCR tube. The cycling protocol was designed for 25 cycles as below with the thermal ramp rate of 1°C per second.

Step	Temperature	Time
1. Denaturation	96°C	10 sec
2. Annealing	52°C	5 sec
3. Extension	60°C	4 min

* Repeat step 1 to 3 for 25 cycles

After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on shaker for 30 minutes at 300xg. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5 min, snap chilled and loaded into autosampler of the instrument.

Electrophoresis and Data Analysis

Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matri Purification.

Results

The three pure cultures from C1 compartment fluid having identical colony morphology of pale, glistening, opaque, eye shaped colonies with irregular edges were selected.

The cultures showed non-lactose fermentation on McConkey agar. The biochemical characteristics of these three pure cultures revealed gram negative rods. The gelatin liquefaction, oxidase and catalase tests were positive with no gas production and H_2S production for these three isolates. The results of sugar utilisation tests were shown in Table 1. All the three isolates showed utilisation for the sugars of mannose, D-mannitol, raffinose, trehalose, sucrose and galactose.

The quality of isolated bacterial DNA was evaluated on 0.8% agarose gel showed a single band of high molecular weight genomic DNA (Fig 1) and after amplification using universal bacterial primers a single discrete band of 1500 bp was observed on 1.2% agarose gel for all the three isolates (Fig 2).

A consensus sequence of 1346 bp , 1252 bp and 1290 bp 16S rDNA gene was generated from forward and reverse sequence data, respectively using aligner software for the three isolates. The 16 S rDNA gene sequence was used to carry out BLAST with the nrddatabase of NCBI genbank database. After sequencing, based on nucleotide homology and phylogenetic analysis the three isolates were

Table 1. Sugar utilisation tests and microbial identification of cultures from C1 compartment fluid of camels.

FEATURES		Isolate 1	Isolate 2	Isolate 3
A	Sugar utilisation tests			
1	Cellobiose	-	+	-
2	Arabinose	+	+	-
3	Glucose	-	+	-
4	Mannose	+	+	+
5	Lactose	-	-	-
6	Maltose	+	-	-
7	D Mannitol	+	+	+
8	Raffinose	+	+	+
9	Ribose	+	-	+
10	Salicin	+	+	-
11	Trehalose	+	+	+
12	Xylan	+	-	+
13	Xylose	-	+	-
14	Sucrose	+	+	+
15	Melezitose	-	-	+
16	Glycerol	-	-	+
17	Galactose	+	+	+
B	Microbe identification (16srDNA) with accession no.	*Pseudomonas aeruginosa strain WJQ No. 1* (KJ789927)	*Pseudomonas nitroreducens strain HP2* (KJ789925)	*Pseudomonas aeruginosa strain DKH-3* (KJ789926)

found similar to the *Pseudomonas aeruginosa strain WJQ* No.1 (Genbank Accession Number: HM142820.1), *Pseudomonas nitroreducens strain HP2* (Genbank Accession Number: KC961632.1), *Pseudomonas aeruginosa* strain DKH-3 (Genbank Accession Number: JQ773477.1), respectively. Information about other close homologs for the microbes was shown in the alignment view tables 2-4. The evolutionary history was inferred using the Neighbor- Joining method as described by Saitou and Nei, 1987. The bootstrap consensus tree inferred from 500 replicates was used to represent the evolutionary history of the taxa (Felsenstein, 1985). The evolutionary distances were computed using Kimura 2 - parameter method (Kimura, 1980). The analysis involved 11 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 (Tamura *et al,* 2007). The three *Pseudomonas* isolates were submitted to the NCBI gene portal having accession number: KJ789927, KJ789925 and KJ789926 respectively. The evolutionary analysis generated using MEGA 5 software were shown in Figs 3, 4 and 5, respectively.

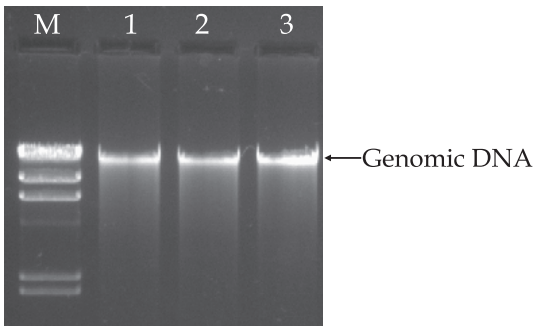

Fig 1. Genomic DNA isolation from 3 isolates on 0.8% agarose gel. M: Marker; 1, 2, 3 are Isolate1, Isolate2, Isolate 3.

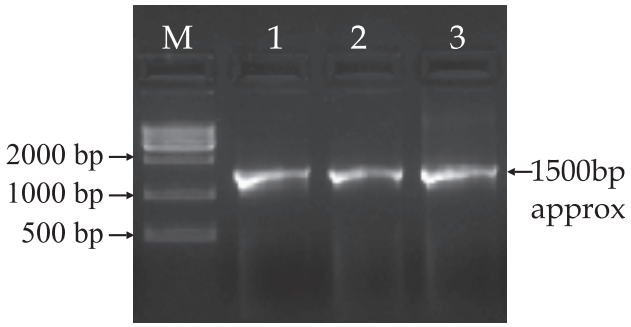

Fig 2. Amplified PCR product of 3 isolates on 1.2% agarose gel. M: Marker; 1, 2, 3 are Isolate1, Isolate2, Isolate 3.

Discussion

Camels were sought for their value as a source of draft, milk, meat, hide and sports racing in various parts of the world. In Indian agriculture, the rearing of camels was mostly confined to north western arid parts. An extensive understanding of the microbial ecology of the camel gastrointestinal tract will contribute to the sustainable farming of

camels well into the future. Preliminary study on the numbers of cellulolytic bacteria and fermentation products from camels has been reported (Hungate *et al,* 1959). This paper reports the cultural isolation and molecular characterisation of *Pseudomonas* isolates from the C1 compartment of dromedary camel. *Pseudomonas aeruginosa* is environmentally versatile microorganism and has an ability to adapt and thrive in a wide variety of ecological niches i.e., terrestrial, aquatic, animal, human and plant host associated environments. This bacterium has diversity of metabolic pathways and physiological responses, allowing it to acclimatise to diverse environments (Rahme *et al,* 2000 and Kimata *et al,* 2004). *Pseudomonas aeruginosa* is gram-negative aerobic organism, and are opportunistic pathogens capable of infecting both humans and animals (Khan and Cerniglia, 1994). *P. aeruginosa* was also identified to be epidemiologically important human pathogen responsible for pneumonias, urinary tract infections (UTIs), blood stream infections, and surface skin infections (Driscoll *et al,* 2007). Since the organism exists in a wide variety of ecological niches, it can cause infections in various hosts, is resistant to many antibiotics, can transmit antibiotic resistance; it is proposed to study the genotype and phenotype of *Pseudomonas aeruginosa* organism in various environmental settings. In addition the clinical and non-clinical *P. aeruginosa* strains might be functionally equivalent in several traits relevant for their virulence or environmental properties. In the year 2008, Oyeleke and Okusanmi also reported the isolation of *Pseudomonas aeruginosa* from cow, sheep and goat which were able to hydrolyse cellulose. Lynd *et al,* 2002 also isolated *Pseudomonas* along with other organisms from the rumen which were implicated in the hydrolysis of cellulose. The rumen represents a dynamic habitat, where the hydrolysis of cellulose takes place. *Pseudomonas* although detected in milk-fed calves (Jayne, 1979), it has not previously been reported as a component of the rumen flora of healthy adult ruminants. Its isolation from sheep fed different diets and at different locations suggests that it may be more widespread in ruminants than previously thought. The difference in specificity of the *P. aeruginosa* bacteriocins from different animals suggests a potential role in the gut ecology of this species, though the in vivo role of pyocins in unclear (Govan, 1986). The carriage of *P. aeruginosa* by ruminants, if widespread, must give some cause for concern, as this species can cause infections in the community and hospitals and multi-drug-resistant

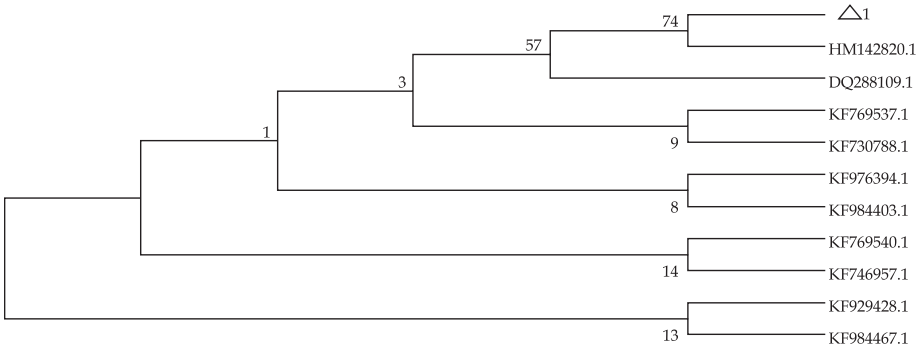

Fig 3. Phylogenetic tree of Isolate 1 is similar to *Pseudomonas aeruginosa* strain WJQ No.1 (HM142820.1).

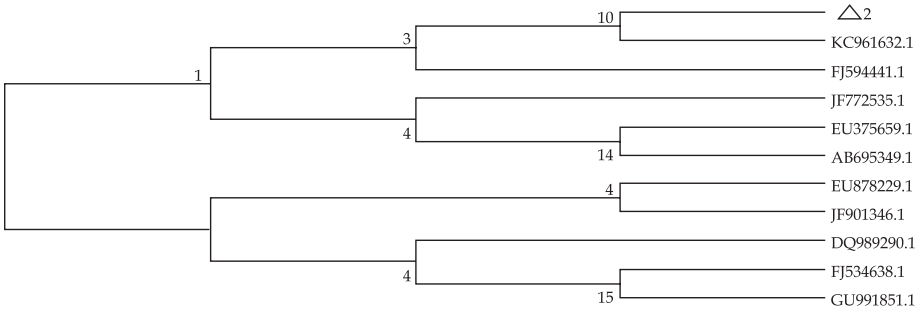

Fig 4. Phylogenetic tree of Isolate 2 is similar to *Pseudomonas nitroreducens* strain HP2 (KC961632.1).

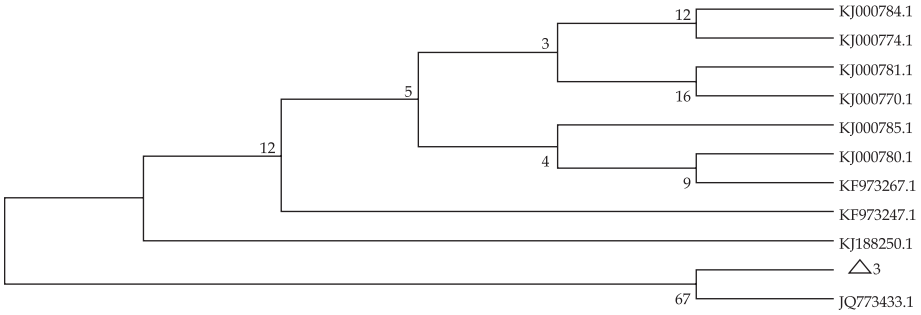

Fig 5. Phylogenetic tree of Isolate 3 is similar to *Pseudomonas aeruginosa* strain DKH-3 (JQ773433.1).

Table 2. Sequence producing significant alignments for isolate 1.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HM142820.1	*Pseudomonas aeruginosa* strain WJQ No.1	2481	2481	100%	0.0	99%
DQ288109.1	*Pseudomonas* sp. HF3-5	2475	2475	100%	0.0	99%
KF976394.1	*Pseudomonas aeruginosa* strain C1501 16S	2470	2470	100%	0.0	99%
KF929428.1	*Pseudomonas aeruginosa* strain AR01	2470	2470	100%	0.0	99%
KF769540.1	*Pseudomonas aeruginosa* strain PPS02	2470	2470	100%	0.0	99%
KF769537.1	*Pseudomonas aeruginosa* strain FA02	2470	2470	100%	0.0	99%
KF746957.1	*Pseudomonas aeruginosa* strain ET6	2470	2470	100%	0.0	99%
KF984467.1	*Pseudomonas* sp. BAB-3358	2470	2470	100%	0.0	99%
KF984403.1	*Pseudomonas* sp. BAB-3048	2470	2470	100%	0.0	99%
KF730788.1	*Pseudomonas aeruginosa* strain HF5	2470	2470	100%	0.0	99%

Table 3. Sequence producing significant alignments for isolate 2.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KC961632.1	*Pseudomonas nitroreducens* strain HP2	2307	2307	100%	0.0	99%
AB695349.1	*Pseudomonas nitroreducens* gene,strain: 4APA	2307	2307	100%	0.0	99%
JF901346.1	Endophytic bacterium 90P-1	2307	2307	100%	0.0	99%
JF772535.1	*Pseudomonas* sp. bD39(2011)	2307	2307	100%	0.0	99%
FJ594441.1	*Pseudomonas* sp. CAT1-8	2307	2307	100%	0.0	99%
FJ534638.1	*Pseudomonas* sp. PGB2	2307	2307	100%	0.0	99%
EU878229.1	*Chryseobacterium* sp. PNP8	2307	2307	100%	0.0	99%
DQ989290.1	*Pseudomonas* sp. Q3	2307	2307	100%	0.0	99%
GU991851.1	*Pseudomonas* sp. 1GW5	2302	2302	100%	0.0	99%
EU375659.1	*Pseudomonas* sp. m41	2302	2302	100%	0.0	99%

Table 4. Sequence producing significant alignments for isolate 3.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ773433.1	*Pseudomonas aeruginosa* strain DKH-3	2377	2377	100%	0.0	99%
KJ188250.1	*Pseudomonas aeruginosa* strain N17-1	2372	2372	100%	0.0	99%
KJ000785.1	*Pseudomonas* sp. SCU-B99	2372	2372	100%	0.0	99%
KJ000784.1	*Pseudomonas* sp. SCU-B97	2372	2372	100%	0.0	99%
KJ000781.1	*Pseudomonas* sp. SCU-B90	2372	2372	100%	0.0	99%
KJ000780.1	*Pseudomonas* sp. SCU-B88	2372	2372	100%	0.0	99%
KJ000774.1	*Pseudomonas* sp. SCU-B80	2372	2372	100%	0.0	99%
KJ000770.1	*Pseudomonas* sp. SCU-B73	2372	2372	100%	0.0	99%
KF973267.1	*Pseudomonas aeruginosa* strain JQZSG-4	2372	2372	100%	0.0	99%
KF973247.1	*Pseudomonas aeruginosa* strain XLSG-4	2372	2372	100%	0.0	99%

strains may be untreatable (Quinn, 1998). Duncan *et al,* 1997 while screening facultative sheep-rumen bacteria which inhibit growth of *Escherichia coli* produced 11 strains of *Pseudomonas aeruginosa* localising in the biofilm associated with the ovine rumen wall. Bouraoui *et al* (2011) observed 65% clones belonging to *Pseudomonas* genus with *Pseudomonas lutea* appeared the most frequent homology hit in a BLAST GenBank on taxonomic analysis of suspended bacterial fraction of dromedary rumen fluid. In the present study two isolates of *Pseudomonas aeruginosa* and one isolate of *Pseudomonas nitroreducens* were characterised from C1 compartment of dromedary camel as also seen in sheep and other ruminants.

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