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Molecular Identification of *Pseudomonas aeruginosa* from food borne isolates

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A B S T R A C T

A research was done for the isolation and identification of undesirable microorganisms in food products using molecular approach the result revealed the presence of *Pseudomonas aeruginosa* in various food products by means of miss handling during harvesting and storage. Many of these isolates were resistant or reduced susceptibilities to multiple antimicrobial agents.The resistant bacteria isolates were selected for 16S rRNA identification of *Pseudomonas aeruginosa.* 16S rRNA is a significant target to the molecular level identification of *Pseudomonas aeruginosa.* The results obtained were found to be a novel food borne pathogens, which were further named *Pseudomonas aeruginosa* strain HV17 and *Pseudomonas aeruginosa* strain HV77, after characterization the sequence of isolate was deposited in GenBank with accession numbers 'KU982961' and 'KU982962' respectively. 16S rRNA is the fundamental molecular method to identify organism *Pseudomonas aeruginosa* at strain level. Identified strain HV17 and HV77 and sequences will help for further research in the field of food borne pathogens.

Introduction

Food borne diseases are globally important, as they result in considerable morbidity, mortality, and economic costs. Many different diseases, including those due to bacteria, viruses, parasites, chemicals, and prions, may be transmitted to humans by contaminated food (Kirk *et al.,* 2015) (Saranraj *et al.,* 2012). According to Centers for Disease Control and Prevention, food borne illness is known to be a ubiquitous, costly, yet preventable public health concern. However, the statistical data of food borne illness on a global scale is fragmented due to the unrecognized or unreported outbreaks particularly in the developing countries(Endersen *et al.,* 2014) (Han *et al.,* 2014) (Carvalho *et al.,* 2012)

World Health Organization stated that food safety remains a continuous challenge to everyone especially in the management of both infectious and non-infectious food borne hazards (Chibeu, 2013**)(**Sillankorva *et al* 2012). Current effective technologies and the good manufacturing practices, the food safety is constantly threatened by the factors related to changes in lifestyle, consumer eating habits, food and agriculture manufacturing processes and also the increased international trade.

A high diversity of saprotrophic and pathogenic microorganisms is recorded in food raw materials and in processed food products. Microorganisms get on vegetables, fruit and other food samples like milk and its product, poultry products from air, soil, packaging materials, people's hands, during harvesting and storage (Rūta Tekorienė, 2008) (Kakarla *et al.,* 2015) (Mohamed *et al.,* 2012).

Control of microbial spoilage of agro-products is crucial for the quality and safety of foods which requires an understanding of a number of factors including the knowledge of possible hazards, their likely occurrence in different products, their physiological properties and the availability and effectiveness of different preventative measures (Bhattarai *et al.,* 2015). Milk is a significant food of human nutrition owing to its high nutritional value. It is naturally a good medium for growth of microorganisms (Rangel *et al.,* 2013). The predominant microbiological populations in ready-to-eat salads are psychrotrophs including *Pseudomonas* spp. Pectolytic strains of *Pseudomonas* have been reported to cause fruits and vegetables deterioration during storage (Bali *et al.,* 2013) (Bhattarai *et al.,* 2015).

In the present study, we examined the prevalence and antibiotic-resistance profiles of the organism and the presence of various virulence factors in *P. aeruginosa* isolated from food samples obtained from local market in and around Bagalkot district, Karnataka, India.

Materials and Methods

Isolation of *P. aeruginosa* from different food sources

Samples are collected from local market in and around Bagalkot district, Karanataka, India, in sterile plastic bags and microbial analysis are done for those samples by using specific media Cetrimide agar was used for the identification of food borne *Pseudomonas* which is selective media for the bacterium.

Morphological characterization

Gram's reaction- Gram's staining, Motility determination- Hanging drop method and Soft agar stabbing (Tube Method) Endospore staining test, Motility test were carried out for the morphology of cell.

Biochemical characterization

Catalase, Oxidase, Nitrate Reduction, IMVIC test, Carbohydrate Utilization, Urease production, Gelatin Hydrolysis, Coagulase Test and DNase Test were performed for the confirmation of the Bacterial isolates according to the bergey's manual.

Identification of *Pseudomonas aeruginosa* by 16S rRNA

Isolation of genomic DNA

Among of 53 *Pseudomonas aeruginosa* isolates 20 resistant bacterial isolates were selected for 16S rRNA identification; among 20 resistant bacterial isolates only two bacterial isolates were selected for 16S rRNA sequence analysis based on purity of the DNA as well as purity of PCR amplified product. DNA were isolated from bacterial culture in 1.5ml tube centrifuge 2min at 14,000rpm at RT and discard supernatant and wash pellet three times with sterial water, add 550µl of TE buffer + lysozyme +2ml of RNase 20mg/ml incubate suspension for 30min at 37°c. Add 76µl of 10% SDS + proteinase k incubate for 15min at 65°c. Add chloroform/ isoamylalcohol

mix the content for 15 sec and centeifuge 5min at 14,000rpm at RT The supernatant was discarded. And 300µl of 70% ethanol was added to it for washing. The pellet was centrifuged at 10,000rpm for2mins and the supernatant was discarded. The pellet was allowed to air dry. The pellet was dissolved in 30µl of T.E buffer and the pellet was stored at -20^0 c for further use.

DNA Quantification

The DNA concentration will be determined by measuring the absorbance at 260nm using Nano Drop$^{TM.}$ Pure Link Genomic elution buffer was used as blank. Purity of the sazmple will be also checked by measuring the 260/280 ratio.

DNA was quantified based on bands obtained in Agarose gel electrophoresis. DNA samples were amplified and checked on 1%Agarose gel electrophoresis and bands were observed under gel Documention.

PCR amplification

PCR reaction was performed in a Thermal cycler (MJ Research PTC200). The reaction mixture 50µl consisted of 20ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5µl of 10 X Taq buffer (100 mM Tris-HCl, , 500 mM KCl pH-8.3) , 200µM dNTP, 10 pmoles each universal primers (forward primer PS16SF1: 5'CGTAACTGGTCTGAGAGG AT3', PS16SF2:5'GTCACACTGGAA CTGAGACA3'and reverse primer PS16SREV3: 5'ACCGTATGCGCTTCTT CATTGACC3') and 2.0 mM MgCl2 was used. Amplification includes initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation 94°C for 30 seconds, annealing temperature of primers at 50°C for 30 seconds and extension at 72°C for 1minute. A final extension at 72°C for 15 minutes was used. 5µl of the amplified product was then analyzed by submarine agarose gel electrophoresis in 1.2 % agarose gel with ethidium bromide at final concentration of 0.5µg/ml for 15min. visualize the gel under Gel doc/UV transilluminator. Amplified PCR product were commercial sequenced at Chromous Biotech Pvt.Ltd

Results and Discussion

Isolation of *P. aeruginosa* from different food sources

Cetrimide agar was used for the isolation and identification of food borne *Pseudomonas* which is selective media for the bacterium. Organisms were identified based on color change in media and colony morphology and microscopic characterization.

Morphological and Biochemical characterization

Pseudomonas aeruginosa, a member of the Gamma Proteobacteria class of bacteria, is a motile, Gram negative, facultative, rod-shaped bacterium measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm in size. Its optimum temperature for growth is 37°C, but retains a growth potential at temperatures as high as 42°C. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. *P. aeruginosa* strains produce two types of soluble pigments, a fluorescent pigment pyoverdin, and a blue pigment, pyocyanin.

Identification of *Pseudomonas aeruginosa* by 16S rRNA

Isolation and Quantification of genomic DNA

Among of 53 *Pseudomonas aeruginosa* isolates 20 resistant bacterial isolates were selected for 16S rRNA identification; among 20 resistant bacteria isolates only two

bacterial isolates were selected for 16S rRNA sequence analysis based on purity of the DNA :-Quantification of DNA by Nano Drop ™ as well as purity of the DNA was checked by 1% Agarose Gel Electrophoresis and bands were observed under gel Documentation.

PCR amplification

Purified DNA product was used for amplification by using specific primers and PCR product was loaded on 1% Agarose Gel Electrophoresis and bands were observed under gel documentation.

Amplified PCR product was sent for sequence analysis at Chromous Biotech Pvt.Ltd

Two sequences obtained were compared against the sequences available in the NCBI, database using the BLAST. The results obtained were found to be a novel food borne pathogens, which were further named *Pseudomonas aeruginosa* strain HV17 16S ribosomal RNA gene, partial sequence with accession numbers 'KU982961' and *Pseudomonas aeruginosa* strain HV7716S ribosomal RNA gene, partial sequence with accession numbers 'KU982962', after characterization the sequence of isolate was deposited in GenBank with accession numbers 'KU982961' and 'KU982962' respectively.

Current study was aimed to identify novel strains like *Pseudomonas aeruginosa*. It is a multi drug resistant isolate from food samples and resistant bacterial isolates were selected for Molecular Characterization i.e 16S rDNA sequence analysis was carried out, In order to identify the strain, extraction and amplification of genomic DNA, 16S rRNA sequence analysis was carried out. Both the sequences obtained were compared against the sequences available in the NCBI, nr database using the BLASTn. The results obtained were found to be a novel foodborne pathogens, which were further named *Pseudomonas aeruginosa* strain HV17 and *Pseudomonas aeruginosa* strain HV77, after characterization the sequence of isolate was deposited in GenBank with accession numbers 'KU982961' and 'KU982962' respectively. *L. monocytogenes* from retail chicken, beef meat and seafood samples was carried out. Phylogenetic trees were constructed using dnapars and dnaml available in Phylip. The secondary structures of 16S rRNA gene sequence were predicted using UNAFOLD, a Linux based software (Pyde *et al.*, 2013). *Enterobacteriaceae* are recognised as some of the most important food borne pathogens worldwide new strains of E*nterobacteriaceae* based on PCR method for *Enterobacteriaceae* in egg cuisines. 16sRNA sequencing was applied to the isolated DNA and the new sequence was submitted to Genbank. Out of 35 samples 2 strains found to be novel.

The results obtained from blast were found to be a novel food borne pathogens, which were further named *Enterobacteriaceae* bacterium Pyde1 and *Enterobacteriaceae* bacterium Pyde2 (Nagarjun *et al.*, 2015). Present study was done to find rapid and accurate identification of bacteria by PCR amplification with 16S rRNA gene has been evaluated for *Aeromonas sobria*, *A. schubertii* and *A. jandaei* and for *A. hydrophila* and *A. veronii*. *A. veronii* has been isolated from the septic arthritis patient and identified by 16S rRNA PCR.

Fig.1 Growth of bacterial colonies on specific media

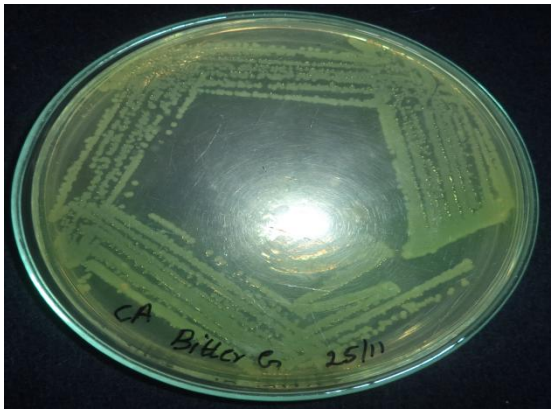


Fig.2 Genomic DNA Loaded on 1% Agarose Gel

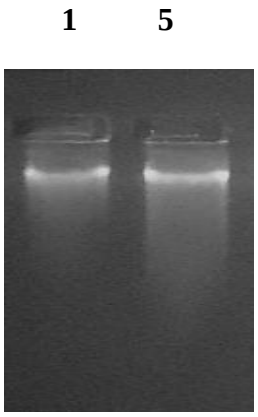
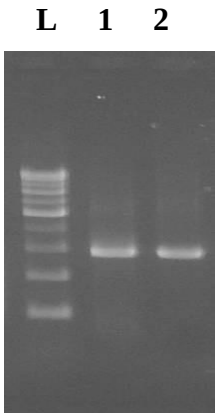


Fig.3 PCR Product loaded on 1% Agarose Gel



Therefore, 16S rRNA is a significant target to the molecular level identification of *Aeromonas veronii* which will help in prevention and cure of diseases in various aquatic animals (Vijai Singh *et al.*, 2012). Bacterial foodborne pathogens, such as

Bacillus spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., and *Vibrio* spp. were detected by a 16S rRNA based oligonucleotide array (Böhme *et al.*, 2014).

In conclusion, among the 53 *Pseudomonas* isolates 20 different resistant bacteria have shown resistance to different antibiotics, among 20 resistance bacteria two were highly resistance strains namely HV17 and HV77, these 2 are chosen for 16sR DNA sequencing for identification of the *Pseudomonas aeuroginosa* in strain level. After amplification with specific primers the PCR amplicon was sent for sequencing to the sequencer and sequenced products are deposited in the Genbank with the gen bank accession number 'KU982961' and 'KU982962' for HV17& and HV77 strains respectively. The 16s rRNA is a significant target to the molecular level identification of *Pseudomonas aeruginosa.* The effort made through this studies will help to identify organisms in strain level. These techniques will be useful for species identification of bacteria and should be applicable in the studies of epidemiology, diagnosis, virulence and molecular taxonomy.

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