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Molecular characterization of *Pseudomonas sp.* isolated from milk samples by using RAPD-PCR

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ABSTRACT

Degradation of milk and its products is one of the major breakthroughs what many dairy based industries are facing. Despite of recent advances in antibiotic therapy, the industries are still facing the issue seriously. The spoilage of the milk and reduction in the shelf life of processed milk takes place through various enzymatic activities associated with the contamination of dairy products by Pseudomonas *spp. High diversity might be one of the possible reason for a strain to adapt to the new environment, which is useful for its propagation and domestication of wild species. In this study, we attempted to determine the use of RAPD-PCR as an effective tool to detect genetic variation between P. aeuroginosa species isolated from the different milk samples. Milk samples were obtained from different areas both from the local field and dairy farms. The samples were screened for the pseudomonas and DNA was extracted. The DNA thus obtained was used for RAPD PCR using the OPZ-8 and OPD-20 primers to reveal the genetic diversity among the variants. The cluster analysis based on the RAPD analysis was done separately for both OPD-20 and OPZ-8 and in case of OPZ-8 cluster analysis, Major group A was divided into two sub groups, first subgroup containing only four accessions, namely, 13, 10, 7 & 8 and Second subgroup containing nine accessions as 6,4,514,3,9,2,11 & 12. In case of the primer OPZ-8 cluster analysis, Major group A was divided into only one sub group containing only four accessions, namely 7, 5,8 & 1. Group B containing ten accessions, namely 6, 3, 4, 14, 11, 9, 12, 2 & 10. All bands showed polymorphism and there was no monomorphic band observed common to all the samples. The number of bands and banding pattern were variable depending upon the primer and type of species tested.*

Key words: Pseudomonas, Milk samples, RAPD, Dendrogram analysis.

INTRODUCTION

Spoilage Bacteria originating in refrigerated raw milk mostly come from the family Pseudomonadaceae [1, 2]. They are called as psychrotrophs, growing well at common refrigeration temperatures (0-15^{0}C) [1]. Pseudomonades such as *Pseudomonas flourescens* are capable of producing heat stable lipases and proteases[3], which are responsible for spoilage of milk with characteristic bitterness, rancidity, fruity flavor, casein breakdown, and ropiness due to production of slime and coagulation of proteins [1,3,4].

Pseudomonas aeruginosa is a gram-negative, motile, extracellular, aerobic, and rod-shaped bacterium that is able to consume a broad range of organic compounds. It is ubiquitous in the natural environmental settings for it can be

isolated from different living organisms comprising plants, animals, and humans [5]**.** *Pseudomonas aeruginosa* is an opportunistic pathogen capable of infecting virtually all tissues. Pulmonary tract colonization with mucoid *P. aeruginosa* is a major cause of morbidity and mortality in patients with cystic fibrosis. *P. aeruginosa* infections in hospitals mainly affect the patients in intensive care units and those having catheterization, burn, and/or chronic illnesses [6].The risk of *P. aeruginosa* pneumonia is increased in immunosuppressed patients or those who require mechanical ventilation knowing that *P. aeruginosa* is the leading cause of ventilator-associated pneumonia (VAP). Furthermore, around 20% of pneumonia conditions in intensive care units (ICU) are due to *P. aeruginosa* [7, 8]

Pseudomonads, particularly *Pseudomonas aeruginosa*, have been the critical cause in majority of outbreaks of intramammary infections. Clinical mastitis is often severe with gangrene and death loss [9, 10]. Subclinical and recurrent mild clinical mastitis have also been reported [11]. Intramammary infections due to *P. aeruginosa* are generally quite refractory to treatment. Control of outbreaks of *P. aeruginosa* mastitis generally requires culling of chronically infected cows and identification and elimination of the source of the organisms.

Water and soil are also the primary sources of *Pseudomonas* sp. [11, 3, 1]. Hose nozzles and milking equipment can become colonized by Pseudomonads. Under stressful conditions, such as the presence of low levels of iodine-based disinfectants, these organisms produce a slimy glycocalyx [12]. This slime favors adherence to and colonization of pipe and hose surfaces, and offers increased resistance to surfactants, phagocytes, and certain antibiotics [11]. Polymorphism in RAPD may be due to deletion, addition or substitution of base [13]. High diversity might be one of the possible reasons for a strain to adapt to the new environment, which is useful for its propagation and domestication of wild species. Geographically, isolated individuals tend to accumulate genetic variations during the course of environmental adaptations [13].

This study is an attempt to establish the genetic diversity background. High levels of polymorphism reported in our study reveal that RAPD markers as a suitable tool for genetic diversity studies. This study will pave the way for detailed research to understand all the aspects of the divergence in the milk contaminants. In this study, we attempted to determine the use of RAPD-PCR as an effective tool to detect genetic variation between *P. aeuroginosa* species isolated from the different clinical sample. Milk samples were obtained from different areas both from the local field and dairy farms. The samples will be screened for the pseudomonas and extracted for the genomic DNA. The DNA thus obtained will be used for RAPD PCR using the OPZ-8 and OPD-20 primers to reveal the genetic diversity among the variants.

MATERIALS AND METHODS

Sample collection: Milk samples were collected from different areas in sterile containers and stored at 4 °C until further use. The milk samples were serially diluted and 10^{-3} and 10^{-4} dilutions were plated onto nutrient agar plates to obtain isolated colonies. Colonies were selected and streaked onto Cetrimide agar plates for selective isolation of *Pseudomonas.*

Cetrimide agar is a selective and differential medium for the identification of *Pseudomonas aeruginosa in* which Cetrimide acts as a detergent which inhibits most bacteria and enhances the production of two pigments pyocyanin, pyoverdine. 13 Pseudomonas isolates were selected and purified on Cetrimide Agar plates.

BIOCHEMICAL CHARACTERIZATION: The different isolates obtained were screened for grams staining. Different mediums were used for the biochemical characterization of the isolated and selected bacteria for their identification according to *Bergey's Manual of Determinative Bacteriology***.** Determination of the physiological properties of strains was performed according to the biochemical tests recommended. These tests included, among other assays, aerobic or anaerobic growth, pH range of growth, test for motility, methyl-red and Voges–Proskauer tests, Oxidase, Catalase, hydrolysis of casein, citrate utilization, nitrate reduction, Indole production, gelatin hydrolysis, and hydrolysis of polysaccharides and fermentation of various sugars.

MOLECULAR CHARACTERIZATION OF *Pseudomonas*

DNA isolation: The isolated colonies were then cultured in Luria-Bertani broth and incubated at 37°c for 48 hours. Following the incubation, 2ml bacterial culture was centrifuged at 6000 rpm for about 10 minutes. To the pellet 1ml of lysis buffer (10mM Tris HCl, pH 8; 0.5M EDTA; 0.5% SDS; 1M NaCl) was added and vortexed properly and incubated at 45 ^{0}C in boiling water bath for 10 minutes.

Following incubation, 1ml of phenol: chloroform mixture (1:1) was added to the mixture and centrifuge at 10,000 rpm for 10 minutes. The upper aqueous layer was transferred and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and then 1/10th volume of 3M sodium acetate was added. The contents are mixed properly and centrifuged at 10,000 rpm for 10 minutes. To the upper aqueous layer double the volume of chilled ethanol was added to precipitate the DNA and later centrifuged at 12,000 rpm for 10 minutes. The DNA pelleted was the stored in 20-50ml of TE buffer and stored at 4°C for further use. The extracted DNA was then quantified using the Nano drop spectrophotometer (ND-1000) to check for the purity. The pure DNA obtained thus obtained was run on 0.8% agarose gel to check for the DNA bands.

RAPD-PCR: RAPD-PCR profiles were used to differentiate strains and were generated using the primer OPZ-8 (5′-GGGTGGGTAA-3′) and OPD-20 (5′- ACCCGGTCAC-3′) [14]. The total reaction size was 25µl, which contained 2µl of template DNA and 47.5µM primers. PCR was performed using the following protocol: 94°C for 5min, followed by 30 cycles of 94°C for 45sec, 55°C for 1 min, and 72°C for 1min, followed by 72°C for 5min. PCR products were visualized on 1.5% agarose gel electrophoresis.

The molecular size (bp) of each potential band position was determined across all RAPD-PCR profiles. At each band position, two possible alleles were considered either present (a score of 1) or absent (a score of 0). Different RAPD profiles were designated by different scores and classified as different genotypes. Phylogenetic variation were determined by converting RAPD data into a frequency similarity and analyzed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a phylogenetic tree.

RESULTS

ISOLATION OF *Pseudomonas sp.:* 13 *Pseudomonas* colonies were isolated from various clinical samples. The strains were sub cultured and maintained on Cetrimide Agar plates. Pseudomonas cultures appeared fluorescent yellow or green color on the Cetrimide Agar plates (fig 1).

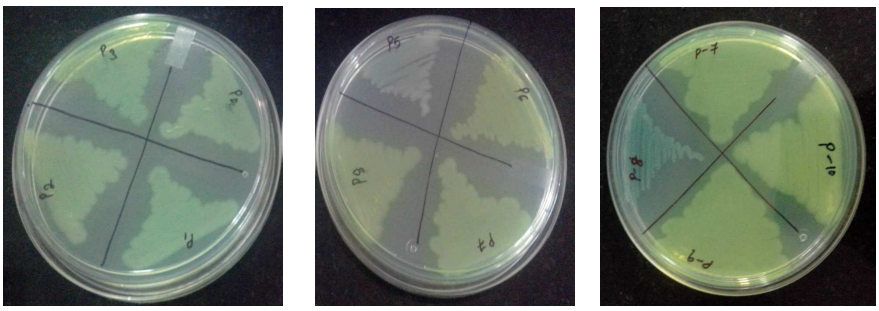

Figure 1: *Pseudomonas* isolates subcultured on Cetrimide Agar plates

BIOCHEMICAL CHARACTERIZATION: The isolated strains were morphologically characterized for further confirmation by Gram's Staining. The isolated colonies were all observed to be Negative for gram's staining and were short rods. The isolated *Pseudomonas* strains were biochemically characterized by IMViC. The test results were observed and tabulated. Of all the 13 isolates, all of them showed positive to Citrate utilization and Catalse test. All the 13 isolates were found negative to Indole test, Methyl red test and VP test.

MOLECULAR CHARACTERIZATION OF *Pseudomonas sp*:
The qualitative analysis of DNA by gel electrophoresis is shown in following pictures. The obtained DNA showed sharp single bands on 0.8% agarose gel without any degradation or RNA contamination. The following graphs show the DNA quantification by Nanodrop ND 1000. The graphs represent the DNA quantity in ng/µl. Also it shows the 260/280 and 260/230 ratios which represent the purity of DNA. All the samples (13 isolates) showed good amount and purity of DNA (fig 2).

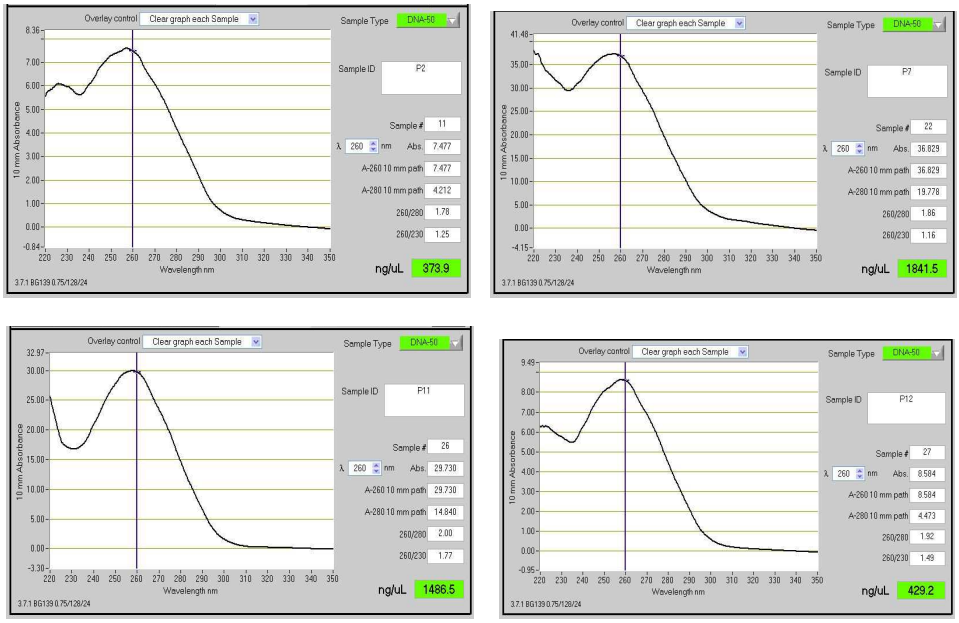

Figure 2: Quantification of the isolated DNA using NanoDrop Spectrophotometer

RAPD PCR: The samples were amplified with two arbitary primers namely OPZ- 8 and OPD- 20. The amplified product was run on a 1 % agarose gel. After which the bands were analysed and used further for dendogram analysis.

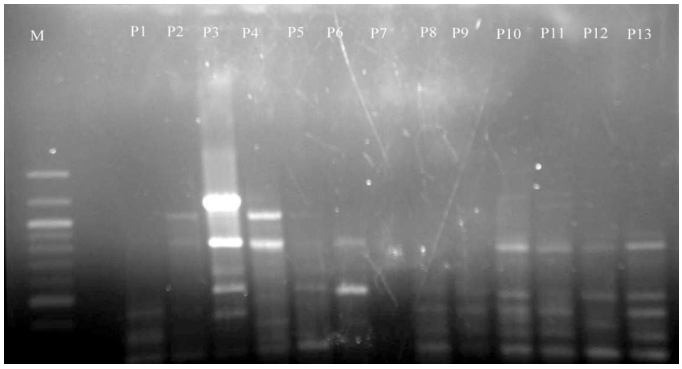

Figure 3: DNA fingerprinting result for OPZ 8. Lane M: Standard marker. P1-P13 sample isolates

Cluster analysis was performed on the basis of similarity co-efficient generated from RAPD profiles. The cluster analysis based on the RAPD analysis was done separately for both OPD-20 and OPZ-8 (Fig 4 & 7). In case of the primer OPZ-8 cluster analysis, Major group A was divided into two sub groups, first subgroup containing only four accessions, namely, 13, 10, 7 & 8. Second subgroup contains nine accessions as 6,4,514,3,9,2,11 & 12. Group B containing only one accession namely 1. These accessions are diverse from other accessions and placed at end of the cluster. But in contrary the cluster analysis of OPD-20 showed different results. In case of the primer OPZ-8 cluster analysis, Major group A was divided into only one sub group containing only four accessions, namely 7, 5,8 & 1. Group B containing ten accessions, namely 6, 3, 4, 14, 11, 9, 12, 2 & 10. These accessions are diverse from other accessions and placed at end of the cluster.

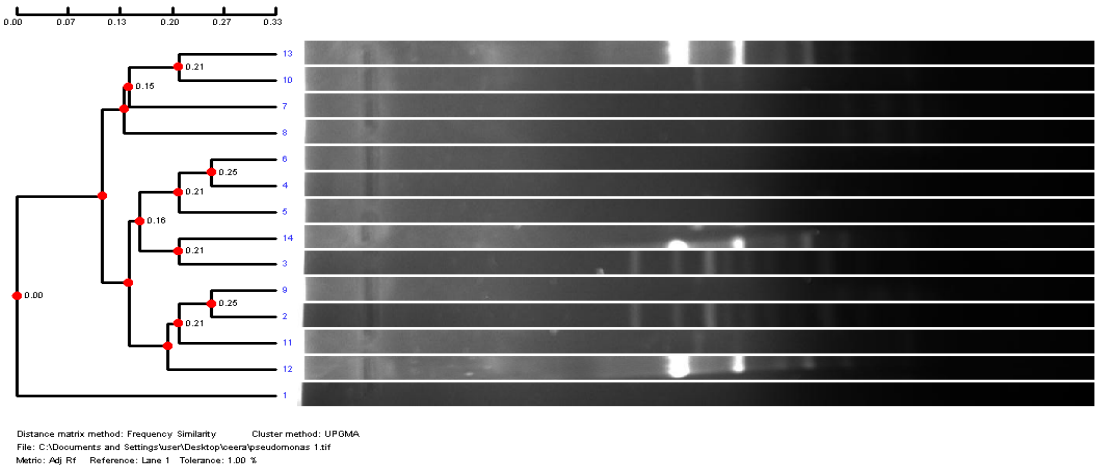

Figure 4: Dendrogram analysis for OPZ 8. Dendrogram on the basis of RAPD similarity matrix data by unweighted pair group method with average (UPGMA) cluster analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100.00	41.67	8.33	33.33	41.67	33.33	33.33	41.67	25.00	50.00	33.33	33.33	41.67	16.67
2	41.67	100.00	66.67	58.33	66.67	75.00	58.33	50.00	83.33	75.00	75.00	75.00	50.00	58.33
3	8.33	66.67	100.00	75.00	66.67	75.00	58.33	50.00	66.67	41.67	58.33	58.33	50.00	75.00
4	33.33	58.33	75.00	100.00	75.00	83.33	50.00	25.00	58.33	50.00	50.00	50.00	41.67	50.00
5	41.67	66.67	66.67	75.00	100.00	75.00	58.33	50.00	66.67	41.67	58.33	58.33	33.33	58.33
6	33.33	75.00	75.00	83.33	75.00	100.00	66.67	41.67	75.00	66.67	66.67	50.00	58.33	66.67
7	33.33	58.33	58.33	50.00	58.33	66.67	100.00	58.33	58.33	66.67	66.67	66.67	58.33	66.67
8	41.67	50.00	50.00	25.00	50.00	41.67	58.33	100.00	66.67	58.33	41.67	58.33	66.67	58.33
9	25.00	83.33	66.67	58.33	66.67	75.00	58.33	66.67	100.00	75.00	75.00	75.00	66.67	75.00
10	50.00	75.00	41.67	50.00	41.67	66.67	66.67	58.33	75.00	100.00	66.67	66.67	75.00	50.00
11	33.33	75.00	58.33	50.00	58.33	66.67	66.67	41.67	75.00	66.67	100.00	66.67	41.67	66.67
12	33.33	75.00	58.33	50.00	58.33	50.00	66.67	58.33	75.00	66.67	66.67	100.00	58.33	66.67
13	41.67	50.00	50.00	41.67	33.33	58.33	58.33	66.67	66.67	75.00	41.67	58.33	100.00	75.00
14	16.67	58.33	75.00	50.00	58.33	66.67	66.67	58.33	75.00	50.00	66.67	66.67	75.00	100.00

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100.00	45.45	45.45	45.45	63.64	54.55	63.64	72.73	45.45	27.27	36.36	27.27	27.27	36.36
2	45.45	100.00	63.64	63.64	45.45	72.73	27.27	36.36	63.64	63.64	72.73	63.64	63.64	72.73
3	45.45	63.64	100.00	81.82	63.64	90.91	63.64	18.18	81.82	63.64	90.91	81.82	81.82	90.91
4	45.45	63.64	81.82	100.00	63.64	90.91	45.45	36.36	63.64	63.64	72.73	63.64	63.64	72.73
5	63.64	45.45	63.64	63.64	100.00	54.55	63.64	54.55	63.64	45.45	54.55	45.45	45.45	54.55
6	54.55	72.73	90.91	90.91	54.55	100.00	54.55	27.27	72.73	54.55	81.82	72.73	72.73	81.82
7	63.64	27.27	63.64	45.45	63.64	54.55	100.00	36.36	63.64	27.27	54.55	63.64	63.64	54.55
8	72.73	36.36	18.18	36.36	54.55	27.27	36.36	100.00	18.18	36.36	9.09	0.00	0.00	9.09
9	45.45	63.64	81.82	63.64	63.64	72.73	63.64	18.18	100.00	63.64	90.91	81.82	81.82	90.91
10	27.27	63.64	63.64	63.64	45.45	54.55	27.27	36.36	63.64	100.00	72.73	63.64	63.64	72.73
11	36.36	72.73	90.91	72.73	54.55	81.82	54.55	9.09	90.91	72.73	100.00	90.91	90.91	100.00
12	27.27	63.64	81.82	63.64	45.45	72.73	63.64	0.00	81.82	63.64	90.91	100.00	100.00	90.91
13	27.27	63.64	81.82	63.64	45.45	72.73	63.64	0.00	81.82	63.64	90.91	100.00	100.00	90.91
14	36.36	72.73	90.91	72.73	54.55	81.82	54.55	9.09	90.91	72.73	100.00	90.91	90.91	100.00

Fig 5: Similarity Matrix Calculated by Frequency Similarity. Left: OPZ8; Right: OPD20

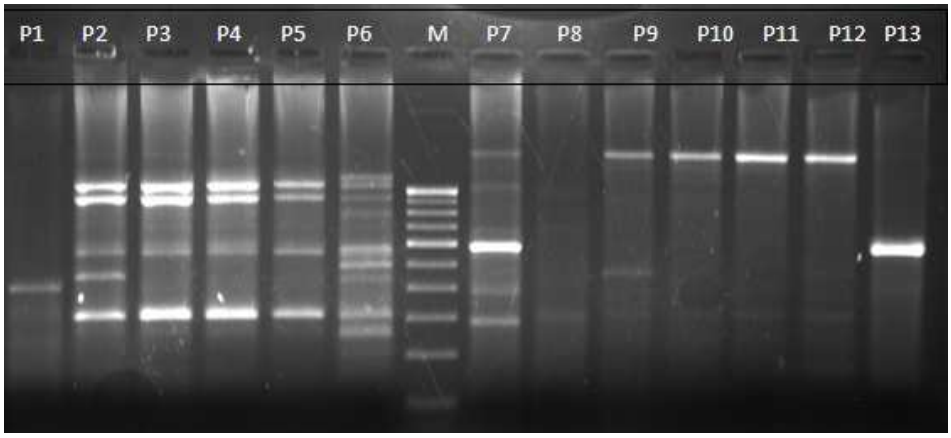

Figure 6: DNA fingerprinting result for OPD-20. Lane M: Standard marker. P1-P13 sample isolates

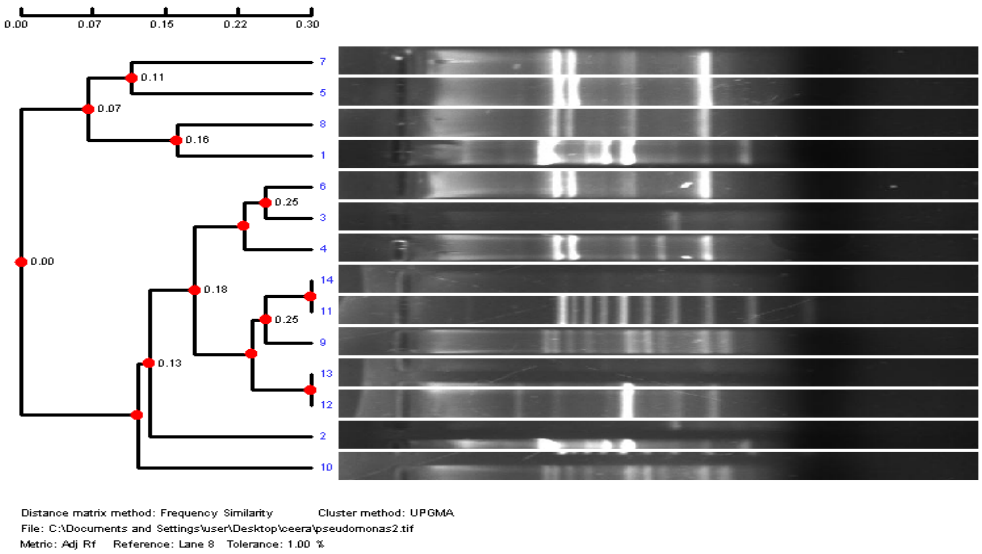

Figure 7: Dendrogram analysis for OPD20. Dendrogram on the basis of RAPD similarity matrix data by unweighted pair group method with average (UPGMA) cluster analysis

RAPD have been widely employed in the assessment of genetic relationships both within and between several plant samples [15]. RAPD and ISSR are simple, provide a quick screen for DNA polymorphism and very small amounts of DNA are required. In addition, information on template DNA sequence is not needed [16]. The genetic variation through RAPD markers has also been highlighted in a number of medicinal plants [17]. The results show that both the marker systems are efficient to distinguish 13 accessions of Pseudomonas and to reveal molecular relationship among them. The cluster analysis of 13 accessions of pseudomonas with molecular approaches revealed that accessions collected from local field samples fell into different clusters and those from the packets obtained from different brands fell in the same cluster. Occasionally, some of the accessions from dairy farms fell in the same cluster. These results imply that multiplicity of factors including the geographical locations were responsible for the selection of genotypes that might have got naturalized at the site of collection.

DISCUSSION

It can be concluded that RAPD marker might be a more useful tool for the identification of milk contaminants. The present findings can help the genetic variation analysis among different accessions of Pseudomonas. Hence, further information and studies are required to know the patterns of gene flow within and between population and its effects on its progression and demographic ways, to assess its impact on population viability [19]. The accessions showed a considerable level of genetic diversity, indicating a high genetic variability in the population.

Similarly to previous studies [19]. *Pseudomonas* spp. were found to be the most prevalent microorganisms, from those isolated from the milk plant. Species belonging to this genus, particularly *P. fluorescens*, are common inhabitants of milk plants and have also been previously found in this sort of facility after disinfection [20].

The present study deals with the determination of genetic diversity among *Pseudomonas sp.* isolated from various milk samples using RAPD markers. The selected *Pseudomonas* colonies were subcultured and purified on Cetrimide Agar plates and were further maintained on Nutrient Agar slants. For further confirmation the selected *Pseudomonas* strains were morphologically characterized by Gram's staining and were found to be gram negative, short rods. They were further biochemically characterized by tests such as Indole test, Methyl Red Test, Voges Proskauer test, Citrate test and Catalase test. All the strains were negative for Indole test, Methyl Red test and Voges Proskaeur test and were positive for Citrate Utilization test and Catalase test. Hence from these tests it was further confirmed that the isolated strains belonged to the genus *Pseudomonas*.

For molecular characterization of the *Pseudomonas* strains, genomic DNA was isolated from 13 *Pseudomonas sp*. by Phenol-chloroform extraction method. In this study DNA fingerprinting was performed using two RAPD primers (OPZ- 8 and OP-D20). OP-Z8 and OP-D20 produced clear banding patterns. All the observed bands were polymorphic and no monomorphic bands were observed. DNA fingerprinting, a tool widely used in forensic science is also useful in a variety of applications with plants. It is used to identify cultivars; to positively identify and differentiate accessions; genetic diversity within breeding populations, and species that might be difficult to characterize due to similar morphological characteristics or indistinct traits. A number of molecular tools and procedures are being employed to establish DNA fingerprinting profiles and each of these procedures has its strengths and weaknesses. The use of molecular markers to study genetic diversity will help in characterizing the *Pseudomonas sp* isolated from milk samples.

CONCLUSION

In the present study, *Pseudomonas sp*. were isolated from various milk samples. These isolates were further morphologically and biochemically characterized. The strains were randomly amplified using two arbitrary primers namely OP Z8 and OP D20. This variation in the number of bands may be due to the sequence of primer, availability of annealing sites in the genome or template quality. All bands showed polymorphism and there was no monomorphic band observed common to all the samples. The number of bands and banding pattern were variable depending upon the primer and type of species tested. The RAPD, analysis in this study has proven to be useful in characterization and differentiation of various *Pseudomonas sp.* from clinical isolates. Despite the enormous and similar discriminating potential of the two markers used they showed some differences in their discrimination capacities. Since, the banding pattern by RAPD was variable depending upon the primer. The genetic data collected during this work will guide the choice of genotypes to cross according to their lineage belonging or their level of diversity.

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