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Isolation and identification of pathogenic microbes from tomato puree and their delineation of distinctness by molecular techniques

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ABSTRACT

The present investigation is on isolation and identification of pathogenic microbes of tomato puree. On the basis of results of the gram's staining, growth on selective and differential media and biochemical analysis of sample S-1 and S-2 of tomato puree, total 12 pathogenic species i.e., *Proteus mirabilis, Aeromonas hydrophila, Klebsiella sp., Vibrio furnissii, Vibrio sp., Erwinia herbicola, Vibrio furnissii, Serratia marcescens, Vibrio furnissii, Proteus sp., Pseudomonas sp.* and *Klebsiella sp.* were recorded. Pathogenic *Vibrio sp., Klebsiella sp., Serratia sp.*, and *Pseudomonas sp.* were seen in unsterilized sample S-2 and negligible in sterilized sample S-1. The amplification of genomic DNA from the seven bacterial species with the four primers showed the species have different RAPD profiles. The bands obtained ranged in size from 100-3000bp. DNA fingerprints were obtained using 04 random primers and gave 151 bands of which 71 are polymorphic, 64 monomorphic and 16 are unique bands representing 47.01%, 42.38% and 10.59% respectively. RAPD analysis successfully identified and 04 RAPD-PCR markers accurately distinguish between these seven species of bacteria. The technique is reliable, reproducible and relatively easy to use with these species. The result reported in this work is the first step in the genetic characterization of the one bacterial species, further step will include the use of RAPD in studies on population genetics.

1. INTRODUCTION

The globalization of the food trade offers many benefits to consumers, as it results in a wider variety of high quality food that are accessible, affordable and safe, meeting consumer's demands. However, factors such as trading features, poor local infrastructure, characteristics of the products sold in the lack of sanitary surveillance increase concerns about the potential for food poisoning due to microbiological contamination [1]. Food science is the study of physical and chemical characteristics of food, whereas food technology includes the total sequence of operations from the selection of raw material through processing, preservation and distribution. Therefore, the food scientist must understand both the processing and storage of foods as well as know about agricultural production and consumer desire. Since, it is essential to feed the world and the only way this can be done as through the preservation of food. On the other hand during processing and packaging of some foods, its micro-flora is reduced by process of sterilization. This is done to increase the self-life of the food and also to prevent food borne diseases [2]. Food processing is usually designed as such to decrease or eliminate microbial population in the food. Many methods have been adopted like pasteurization; chemical preservatives etc. to increase the shelf life of the perishable food items. Still, there are chances of introduction of microbes causing spoilage of foods during post-processing handling of the food. So, different processed food before coming to the consumer level should be microbiologically analyzed for any food borne pathogen in it. These analysis, therefore are called detection method. Detection of pathogens in food requires the execution of multi step microbiological analytical methods or protocol for detection of pathogens in food includes cultural, biochemical or genetic technique or combination of all these techniques. The study is highlighted the need for improved strategies for food safety, in particular appropriate hygienic precautions to avoid contamination of tomato puree during the manufacturing process and appropriate preservation technique, during storage and transportation to prevent transmission of pathogenic microbes to consumers.

2. MATERIALS AND METHODS

Two samples of tomato puree were collected from Council's Laboratory, MPCST, Bhopal, out of which one sample was sterilized while another was unsterilized. Sample S-1 in brown in colour, while sample S-2 was red in colour. Both samples were in semisolid in nature.

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2.1 Isolation and enumeration of microorganisms

The serial dilution-agar plating method or viable count method is one of the commonly used procedures for the isolation and enumeration of microorganisms. This method is based upon the principle that, when material containing microorganisms are cultured each viable microorganism will develop into a colony. Hence, this method was adopted which appeared colonies on the plates represents the number of living organisms present in the samples. The number of colonies appearing on dilution plates were counted, averaged and multiplied by the dilution factor to find the number of cells/spores per gram (or milliliter) of the sample. Entire procedures for Isolation and enumeration of microorganisms were adopted as methods given by [3].

2.2 Identification of microbes by biochemical tests and PIB-Win (2007)

After isolating the pure cultures, the microorganisms were identified using various methods provided by [4] using selective and differential media. The Probabilistic identification of bacteria window (PIB Win, 2007) is a windows version of a DOS program PIB (also called Bacterial Identifier). The program has three major functions, (i) the identification of an unknown isolate, (ii) the selection of additional tests to distinguish between possible strains if identification is not achieved, and (iii) storage and retrieval of results. It also has some utility functions for assessing the usefulness of identification matrices and for converting matrices into different formats. The program makes use of Excel files to store identification matrices and archives results for this, although other file formats are supported to allow backwards compatibility with the DOS version of the program. Up to date information on the program can be found on the PIBWin web site www.som.soton.ac.uk/staff/tnb/pib.htm, which can also be accessed from the Help menu.

2.3 Genetic variability among and between isolated pathogenic bacteria

A single cell colony of bacterial species was grown overnight on Nutrient Agar Medium (NAM) at 37^0C for extraction of genomic DNA. The extraction of genomic DNA was done by protocol described by [5]; [6] with slight modifications. 1.5µl of this solution was put in the vial of Nano-Drop UV-Spectrophotometer (ND-1000) and the absorbance recorded. The absorbance ratio 260/280 should be 1.8 for pure DNA and 2.0 for pure RNA [5]. Deviations from these ratios indicate contamination of protein, phenol and RNA. If the absorbance ratio of DNA template is more than 2.0 then, RNase extraction is necessary.

The concentration of DNA required in the mixture for the RAPD-PCR process to obtain better amplification lies between 50-100ŋg/µl but the amount of DNA obtained per µl is not uniform hence it is diluted to obtain uniform concentration according to the quantity of the DNA isolated. Prepared the PCR mix was prepared as per the protocol of [6]Janerthanan and Vincent (2007) for PCR amplification. The PCR reactions were performed in 25 µl was composed of 10X Taq buffer-A (2.50 µl), 10 mM dNTP (1.00 µl), RAPD Primer (1.00 µl), Taq DNA Polymerase 3U/µl (0.50 µl) Sterile Water (19.00 µl) Template DNA (0.50 µl). The PCR amplification was performed in a Thermal Cycler make Eppendorf (Germany) with setting of the following cycles consisted as mentioned above.

94^0C	94^0C	35^0C	72^0C	94^0C	38^0C	72^0C	72^0C
5.0 min.	45 sec.	1.0 min.	1.5 min.	45 min.	1.0 min.	1.0 min.	10.0 min.
Denaturation	X8 Cycles			X35 Cycles			Final extension

The amplified DNA fragments were separated on 1.2% agarose gel and stained with ethidium bromide. A low range DNA marker was run with each gel (100, 200, 300, 600, 1000, 1500, 2000, 2500 and 3000 bp, make Bangalore Genei, India). The amplified pattern was visualized on a UV transilluminator and photographed by gel documentation system (Alpha-Innotech, USA) and after that scoring of the fingerprints and molecular weight was also performed by gel documentation system.

The DNA fingerprints were scored as presence (1) or absence (0) of fragments on the gel photographs and RAPD fragments. The Similarity Indices were calculated with each primer and a matrix was developed. Finally, all matrices were analyzed as an average. The graphic phenogram of the genetic relatedness among the twenty accessions was produced by means of UPGMA (Un-weighed pair group method with arithmetic average) cluster analysis of averaged similarity index. RAPD banding patterns were recorded on spreadsheets, which were used to determine gene diversity, gene flow, number of polymorphic loci and genetic distance through a construct by an un-weighted pair group method of arithmetic mean (UPGMA) as per [7] using Past 1.91 ver software Popgene 32 ver software.

3. RESULTS AND DISCUSSION

In the present investigation, using the serial dilutions, 10-1 of the samples S1, the total 04 colonies were recorded and CFU was 40 cells ml-1, whereas, in dilution 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, the total colonies were 02 in each and CFU was 200, 2000, 2×10^4, 2×10^5, 2×10^6 and 2×10^7 respectively (Table 1). In the dilution 10-8, 10-9 there was no colonies and CFU were observed (Table 2). Similarly, in sample S2 dilutions 10^{-1} and 10^{-2} smear was observed, therefore, no CFU counts was recorded, whereas, in dilution 10^{-3}, 10^{-4}, 10^{-4}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}, the total colonies and CFU 65 (65×10^3), 53 (53×10^4), 21 (21×10^5), 16 (16×10^6), 09 (9×10^7), 07 (7×10^8) and 03 (3×10^9) respectively (Table 3). Microbial counts get reduced to 10^3 on pasteurization of the natural sour soup puree [8] . After analyzing and comparing with above statement, the microbial count of the samples S2 and S1, it found reduced from 10^5 to 10^3. Sample S1 also qualifies the both BIS (IS: 3882) and FPO (1995) standard of microbial count which is not more than 10^4. Some selective and differential media were used to identify some particular group of microorganisms. They were Mannitol agar, MacConkey and Eosine methylene blue.

Table. 1: Morphology and CFU counts of colonies of sample S1.

Dilution	Colony morphology				Bacterial ID	Total No. of colonies	CFU (No. of cells/ml)
	Color	Margin	Form	Elevation			
10^{-1}	Yellow	Entire	Circular	Convex	Bac 018	4	40
	White	Lobate	Irregular	Raised			
	White	Entire	Circular	Flat	Bac 019		
	White	Undulate	Circular	Raised	Bac 020		
10^{-2}	Yellow	Entire	Circular	Convex		2	200
10^{-3}	Yellow	Entire	Circular	Convex		2	2000
10^{-4}	White	Entire	Circular	Flat	Bac 021	2	2×10^{4}
10^{-5}	White	Undulate	Circular	Flat	Bac 022	2	2×10^{5}
	Yellow	Entire	Circular	Convex			
10^{-6}	Yellow	Entire	Circular	Convex		2	2×10^{6}
10^{-7}	Powdery Yellow	Entire	Circular	Raised	Bac 023	2	2×10^{7}
10^{-8}	-	-	-	-	-	No colony	-
10^{-9}	-	-	-	-	-	No colony	-

Table. 2: Morphology and CFU counts of colonies of sample S2.

Dilution	Colour	Colony morphology			Bacterial ID	Total No. of colonies	CFU (No. of cells/ml)
		Margin	Form	Elevation			
10^{-1}	-	-	-	-	-	Smear	-
10^{-2}	-	-	-	-	-	Smear	-
10^{-3}	White	Filamentous	Rhizoid	Flat	Bac 024	65	65×10^{3}
	White	Lobate	Irregular	Raised	Bac 025		
	White	Entire	Circular	Raised			
10^{-4}	White	Lobate	Irregular	Raised	Bac 026	53	53×10^{4}
	White	Entire	Circular	Raised	Bac 027		
10^{-5}	White	Lobate	Irregular	Raised	-	21	21×10^{5}
	White	Entire	Circular	Raised	-		
10^{-6}	White	Undulate	Circular	Flat	-	16	16×10^{6}
	Yellow	Entire	Circular	Convex	-		
10^{-7}	White	Entire	Circular	Flat	-	9	9×10^{7}
	Yellow	Entire	Circular	Raised	-	7	7×10^{8}
10^{-8}	Yellow	Undulate	Irregular	Flat	Bac 028		
10^{-9}	Orange	Entire	Circular	Raised	Bac 029	3	3×10^{9}

Table. 3: Growth of different bacteria isolated from samples S1 and S2.

S. No.	Bac-ID	Mannitol agar	Mac conkey	EMB
1.	Bac 018	-	-	-
2.	Bac 019	-	-	-
3.	Bac 020	-	-	-
4.	Bac 021	-	-	-
5.	Bac 022	-	-	-
6.	Bac 023	-	-	-
7.	Bac 024	-	-	-
8.	Bac 025	-	-	-
9.	Bac 026	-	-	-
10.	Bac 027	-	-	-
11.	Bac 028	+	+	+
12.	Bac 029	-	-	-

Note: + = Growth was found, - = Growth was not found

Table. 4: Gram's staining of bacterial culture isolated from samples S1 and S2.

S. No.	Bacterial ID	Status
1.	Bac 018	G (-), *Staphylococcus*
2.	Bac 019	G (-), *Diplococcus*
3.	Bac 020	G (-), *Streptococcus*
4.	Bac 021	G (-), *Streptococcus*
5.	Bac 022	G (-), *Streptococcus*
6.	Bac 023	G (-), *Staphylococcus*
7.	Bac 024	G (-), *Diplococcus*
8.	Bac 025	G (-), *Streptococcus*
9.	Bac 026	G (-), *Staphylococcus*
10.	Bac 027	G (-), *Streptococcus*
11.	Bac 028	G (-), *Staphylococcus*
12.	Bac 029	G (-), *Staphylococcus*

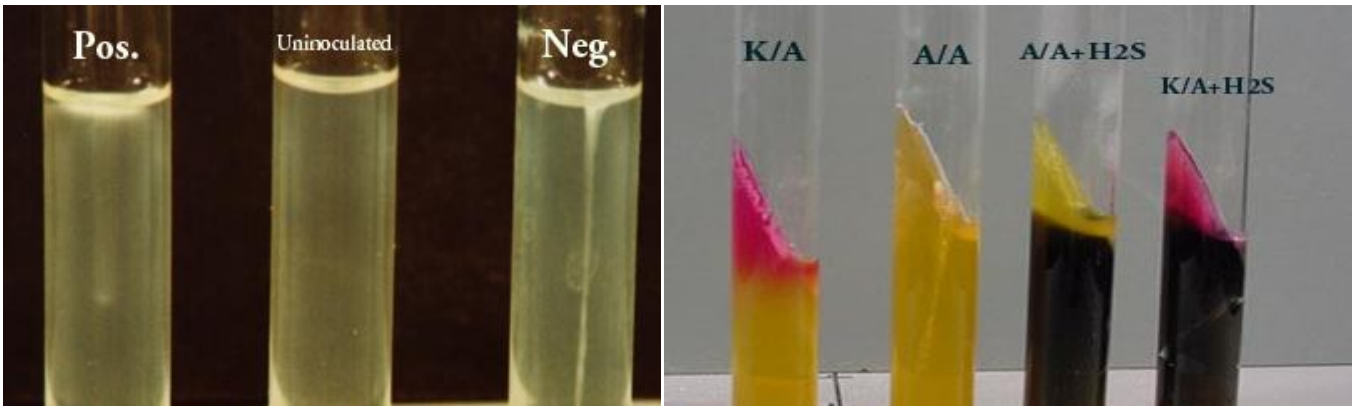

Fig. 1: Photographic representation of different biochemical test.

Table. 5: Biochemical results of the bacteria isolated from samples S1 and S2

BAC ID	Fermentation			IMVic				Motility	Urease	Starch	H2S Prod.	Casein	Catalase	TSIA			
	Glu	Suc	Lac	Indole	MR	VP	Citrate							Acid	Gas	Pb	H2S
Bac 018	+	+	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-
Bac 019	+	+	+	-	-	+	-	-	-	+	+	+	-	-	+	-	+
Bac 020	+	+	+	-	-	+	+	-	+	+	-	+	-	+	+	-	-
Bac 021	+	+	-	-	-	+	+	-	+	-	-	-	-	+	-	-	-
Bac 022	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-
Bac 023	+	+	+	-	-	+	-	+	+	-	-	+	+	+	-	-	-
Bac 024	+	+	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-
Bac 025	+	+	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-
Bac 026	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Bac 027	+	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
Bac 028	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Bac 029	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-

Note: + = Result Positive, - = Result Negative.

All 12 isolated cultures were poured on the above mentioned media of which only one culture i.e., Bac 028 was showed their growth on all three media (Table 3, Fig. 1). The results of gram staining of isolated bacterial culture of S1 and S2 samples of tomato puree were recorded showed all bacterial species were found gram negative. Bac 018, Bac 023, Bac 026, Bac 028 and 029 were Statphylococcus and Bac 020, Bac 021, Bac 022, Bac 025 and Bac 027 were Streptococcus whereas Bac 019 and Bac 024 were Diplococcus (Table 4). The biochemical studies on the bacteria isolated from the sample S1 and S2 of the tomato puree were done with several biochemical tests (Table 5 and Fig. 1).

Some bacteria oxidize tryptophan by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. In the present study, all the 12 cultures showed negative results with indole production (Table 5). Bac 025 culture showed positively with Methyl-Red test and rest of the culture showed negatively. While Bac 025 culture showed negatively with Voges-Proskauer test and rest of the culture showed positively (Table 5). Citrate test is used to differentiate enteric bacteria on the basis of their ability to utilize the citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. Bac 019, Bac 023, Bac 027 and Bac 029 showed negative and Bac 018, Bac 020, Bac 021, Bac 022, Bac 024 Bac 025, Bac 026 and Bac 028 showed positively with citrate test (Table 5).

Table. 6: Bacterial species identified by PIBWin software from sample S1 & S2.

S. No.	Bac ID	Species
1.	Bac 018	*Proteus mirabilis*
2.	Bac 019	*Aeromonas hydrophila*
3.	Bac 020	*Klebsiella sp.*
4.	Bac 021	*Vibrio furnissii*
5.	Bac 022	*Vibrio sp.*
6.	Bac 023	*Erwinia herbicola*
7.	Bac 024	*Vibrio furnissii*
8.	Bac 025	*Serratia marcescens*
9.	Bac 026	*Vibrio furnissii*
10.	Bac 027	*Proteus sp.*
11.	Bac 028	*Pseudomonas sp.*
12.	Bac 029	*Klebsiella sp.*

Fermentation degradation of various carbohydrates such as glucose (a monosaccharide), sucrose (disaccharide) by microbes, under anaerobic condition is carried out in a fermentation tube. The glucose and sucrose showed positively with all except Bac 028. Bac 018, Bac 021, Bac 022, Bac 024, Bac 025, Bac 026 and Bac 028 negatively showed with lactose (Table 5). Bac 019, Bac 020, Bac 023, Bac 027 and Bac 029 showed positively with lactose fermentation.

Hydrogen sulfide (H2S) production can be detected by incorporating a heavy metal salt containing cysteine and sodium thiosulfate as the sulfur substrates. Hydrogen sulfide, a colourless gas, when produced reacts with the metal salt (ferrous sulfate) forming visible insoluble black ferrous sulfide precipitates. In the present study, Bac 019 culture showed positive result with H2S production, while other cultures gave negative result (Table 6).

The urease is a hydrolytic enzyme, which attacks the carbon and nitrogen bond amide compounds (e.g. urea) with the liberation of ammonia. It is a useful diagnostic test for identifying bacteria, especially to distinguish members of the genus Proteus from the gram-negative pathogens. In the test Bac 019, Bac 029, showed positive colour and Bac 018, Bac 020, Bac 021, Bac 022, Bac 023 Bac 024, Bac 025, Bac 026, Bac 027 and Bac 028 showed negatively with urease test (Table 5).

Casein is the major protein found in milk. Some microorganisms have the ability to degrade the protein casein by producing proteolytic exoenzyme, called proteinase (caseinase) which breaks the peptide bond CO-NH by introducing water into the molecule, liberating smaller chains of amino acids called peptides. Formation of a clear zone adjacent to the bacterial growth, after inoculation and incubation of agar plate cultures, is an evidence of casein hydrolysis. Pathogenic bacteria (e.g. species of Staphylococcus and Streptococcus), aquatic bacteria (e.g. Peudomonas and some Proteus species), and soil bacteria (e.g. speices of Bacillus and Clostridium) are known to produce extra cellular proteinases. In the test Bac 018, Bac 019, Bac 020and Bac 023, showed positive and Bac 021, Bac 022, Bac 024, Bac 025, Bac 026 Bac 027, Bac 028 and Bac 029 showed negatively with Casein hydrolysis test (Table 6).

Amylase is an exoenzyme that hydrolyses (cleaves) starch, a polysaccharide (a molecule which consists of eight or more monosaccharide molecules) into maltose a disaccharide (double sugars, i.e. composed of two monosaccharide molecules) and some monosaccharide such as glucose. In the test Bac 018, Bac 019, Bac 020, Bac 022, Bac 025 and Bac 029 showed positive and Bac 021, Bac 023, Bac 024, Bac 026, Bac 027 and Bac 028 showed negatively with amylase production test (Table 5).

Motility test is used to differentiate between motile and non-motile bacteria. Flagella and cilia are the locomotary organ in the bacteria. In this test motile bacteria diffused from the stab line whereas non-motile bacterial remains confined to its stab line. In the present study Bac 019, Bac 020 and Bac 021 culture showed negative results with Motility test, while other culture showed positive result (Table 5).

Triple sugar iron agar (TSIA) medium is composed of three sugars: lactose, sucrose and very small amount of (1%) glucose, iron (ferrous sulphate) and phenol red as indicator. The indicator is employed for the detection of fermentation of sugars indicated by the change in colour of the medium due to the production of organic acid and hydrogen sulfide (H2S). If an organism ferments any of the three sugars or any combination of them, the medium will become yellow due to the production of acid as end product of fermentation. Production of gas from the fermentation of a sugar by an organism is indicated by the appearance of bubbles or splitting in the butt or pushing up of the entire slant from the bottom of the tube. Hydrogen sulfide (H2S) production by an organism is indicated by the reduction of ferrous sulfate of the medium to ferric sulfide, which is manifested as a black precipitate. Bac 019, Bac 022, Bac 026, Bac 027, Bac 028 and Bac 029 showed negatively with acid production test, while Bac 018, Bac 020, Bac 021, Bac 023, Bac 024 and Bac 025 showed positive result. Bac 018, Bac 019 and Bac 020 culture showed positive and rest of the cultures showed negative results with gas production test (Table 5). On the basis of results of the gram's staining, growth on selective and differential media and biochemical analysis the isolated bacterial from sample S1 and S2 of Tomato puree are identified using the software PIBWin-2007. The results of analysis are referring in table no. [6]. According to [9], the microflora founded in sterilized tomato puree is B. stearothermophilus, which is not founded in both the sample S1 and S2. The pathogenic microbes detected in tomato puree by [10] using PCR technique are Salmonella and E. coli. Both the microbes are found to be absent in given sample of tomato puree. [11] founded some aerobes, Staphylococcus spp., pyschoytropes and members of Enterobacteriaceae in tomato puree. Though most of these microbes are not founded in the sample S1 and S2, but some different species of microbes has been founded. Few are pathogenic like Vibrio spp, Klebsiella sp, Serratia sp, and Pseudomonas sp. But these are mainly present in unsterilized sample S2 and negligible in sterilized sample S 1.

Table. 7: Quantification of isolated genomic DNA from 07 species of bacterial culture.

Sample ID	Quality			Quantity ng/µl
	A-260	A-280	260/280	
Bac-019	7.922	4.025	1.97	396.08
Bac-020	4.457	2.403	1.85	222.86
Bac-022	3.967	2.091	1.90	198.36
Bac-023	1.472	0.809	1.82	18.42
Bac-025	0.672	0.373	1.80	33.61
Bac-027	18.215	9.307	1.96	910.76
Bac-028	1.472	0.809	1.82	73.61

A single cell colony of bacterial species was grown overnight on Nutrient Agar Medium (NAM) at 370C for the isolation of total genomic DNA. The growth culture of 15 ml was then centrifuged at 5500 rpm for 10 minutes and supernatant was discarded and repeated the process once more. Resuspended the pellet with 1 ml TE buffer and added 90 µl 10% SDS, then 700µl phenol: chloroform was added, mixed gently and centrifuged it at 10000 rpm for 10 minutes. The supernatant was then transferred to a fresh eppendorf tube. Next 500 µl phenol: chloroform was added, mixed and centrifuged at 10000 rpm for 10 minutes. The DNA was precipitated with 50µl sodium acetate and 400 µl of chilled isopropanol. DNA pellet washed with 70% ethanol, dried, dissolved in 50µl deionised water and stored at -20 0C. Genetic diversity of the selected pathogenic microbes, the genomic DNA was isolated of which results are shown in table 7. The quantity of DNA isolated from the bacterial culture encoded Bac-019 was 396.08ng/µl. The ratio of absorbance by wavelength 260/280 was 1.97, which shows little contamination of RNA in the isolated DNA because the standard absorbance of the pure DNA should be 1.80 [5]. The quantity of DNA isolated from the bacterial culture encoded Bac-020 was 222.86ng/µl. The ratio of absorbance by

wavelength 260/280 was 1.85 and quantity of DNA isolated from the bacterial culture encoded Bac-022 was 198.36ng/µl. The ratio of absorbance by wavelength 260/280 was 1.90. The quantity of DNA isolated from the bacterial culture encoded Bac-023 was 18.42ng/µl. The ratio of absorbance by wavelength 260/280 was 1.82. The quantity of DNA isolated from the bacterial culture encoded Bac-025 was 33.61ng /µl. The ratio of absorbance by wavelength 260/280 was 1.80. The quantity of DNA isolated from the bacterial culture encoded Bac-027 was 910.76ng /µl. The ratio of absorbance by wavelength 260/280 was 1.96. The quantity of DNA isolated from the bacterial culture encoded Bac-028 was 73.61 ng /µl. The ratio of absorbance by wavelength 260/280 was 1.82. The detailed banding patters has shown in figure 2 as DNA fragments obtained by different primers. Primer BP-1 (AM 911690) gave total 15 bands with their sizes ranging from 306 to 2136 bp, 13 bands with their size from 406 to 2136 bp, 14 bands from 114 to 1590 bp, from 6 bands from 483 to 1485 bp, 12 bands from 326 to 2656, 16 bands from 283 to 2136 bp, 15 bands from 283 to 2034 bp in Bac-019, Bac-022, Bac-022, Bac-025, Bac-027, Bac-028 respectively. BP-3 (AM 773772) gave total 02 bands with their sizes 491, 956, 09 bands with their size from 308 to 1403 bp, 11 bands from 198 to 1507 bp, from 9 bands from 508 to 1143 bp, 11 bands from 352 to 1810, 17 bands from 262 to 3020 bp, 11 bands from 327 to 1341bp in Bac-019, Bac-022, Bac-022, Bac-025, Bac-027, Bac-028 respectively. BP-5 (AM 911680) gave total 6 bands with their sizes ranging from 462 to 1401 bp, 8 bands with their size from 830 to 2166 bp, 8 bands from 288 to 1681 bp, 7 bands from 830 to 2166 bp, 6 bands from 684 to 1681, 8 bands from 510 to 2166 bp, 8 bands from 599 to 2444 bp in Bac-019, Bac-022, Bac-022, Bac-025, Bac-027, Bac-028 respectively. Similarly BP-6 (AM773778) gave 4 bands with their sizes were 397, 535, 648, and 1392 bp in Bac-018. Bac-020 gave total 8 bands and their sizes were 475, 636, 838, 1500, 1727, 2222, 2388 and 3010 bp. Bac-022 gave total 6 bands and their sizes were 252, 288, 475, 594, 878 and 1500 bp. Bac-023 gave total 9 bands and their sizes were 475, 594, 739, 838, 942, 1750, 2277, 2444 and 3010 bp. Bac-025 gave total 6 bands and their sizes were 211, 660, 838, 942, 1178 and 2277 bp. Bac-027 gave total 8 bands and their sizes were 445, 594, 739, 942, 1178, 1795, 2277, 2472 and 3010 bp. Bac-028 gave total 4 bands and their sizes were 356, 942, 1178, 1392 and 1818 bp. The Jaccards genetic distance was obtained represented in the table 09. Total 151 DNA fingerprints were obtained using 06 random primers and gave 151 bands of which 71 are polymorphic, 64 monomorphic and 16 are unique bands representing 47.01%, 42.38% and 10.59% respectively (Table 10). The amplification of genomic DNA from the seven bacterial species with the four primers showed the species have different RAPD profiles. The bands obtained ranged in size from 100-3000bp. A total of nine DNA diagnostic markers were found. Despite the small number of samples analyzed, as the number of specific marker increases, the capacity to identify the different species also increases [12]. Therefore, this RAPD analysis successfully identified. Nine DNA markers, that accurately distinguishes between these seven species of bacteria as shown in figure 3 as phylogenetic relationship. The technique is reliable, reproducible and relatively easy to use with these species.

Table. 8: Dilution of isolated DNA for RAPD analysis.

Sample ID	Template Conc. ŋg/µl	Dilution		Final Conc. ŋg/µl
		Water (µl)	DNA (µl)	
Bac-019	396.08	25	5	3.16
Bac-020	222.86	22	8	1.261
Bac-022	198.36	21	9	1.04
Bac-023	18.42	-	-	18.42
Bac-025	33.61	-	-	33.61
Bac-027	910.76	29	1	31.40
Bac-028	73.61	-	-	73.61

Table. 9: Distance matrix (Jaccard's and UPGMA).

Bacterial Species	*Aeromonas hydrophila*	*Klebsiella sp.*	*Vibrio furnissii*	*Erwinia herbicola*	*Serratia marcescens*	*Proteus mirabilis*	*Pseudomonas sp.*
Aeromonas hydrophila	0.00000	0.73077	0.91803	0.90566	0.89286	0.84848	0.91667
Klebsiella sp.	0.73077	0.00000	0.83582	0.81356	0.87879	0.81081	0.95946
Vibrio furnissii	0.9180	0.83582	0.00000	0.87097	0.92754	0.90000	0.97333
Erwinia herbicola	0.90566	0.81356	0.87097	0.00000	0.86207	0.78788	0.93846
Serratia marcescens	0.89286	0.87879	0.92754	0.86207	0.00000	0.88000	0.85938
Proteus mirabilis	0.84848	0.81081	0.90000	0.78788	0.88000	0.00000	0.85526
Pseudomonas sp.	0.91667	0.95946	0.97333	0.93846	0.85938	0.85526	0.00000

Table. 10: Pattern of polymorphism, monomorphism and uniqueness primer wise between 07 species of bacteria.

S. No.	Primer	Total No. of bands	Total No. of polymorphic bands	Total No. of monomorphic bands	Total No. of unique bands	Polymorphism, %	Monomorphism, %	Uniqueness, %
1.	BP1	47	18	25	4	38.56	53.19	8.51
2.	BP3	41	15	21	5	36.58	51.21	12.19
3.	BP5	35	23	9	3	65.71	25.71	8.57
4.	BP6	28	15	9	4	53.57	32.14	14.28
Overall		151	71	64	16	47.01	42.38	10.59

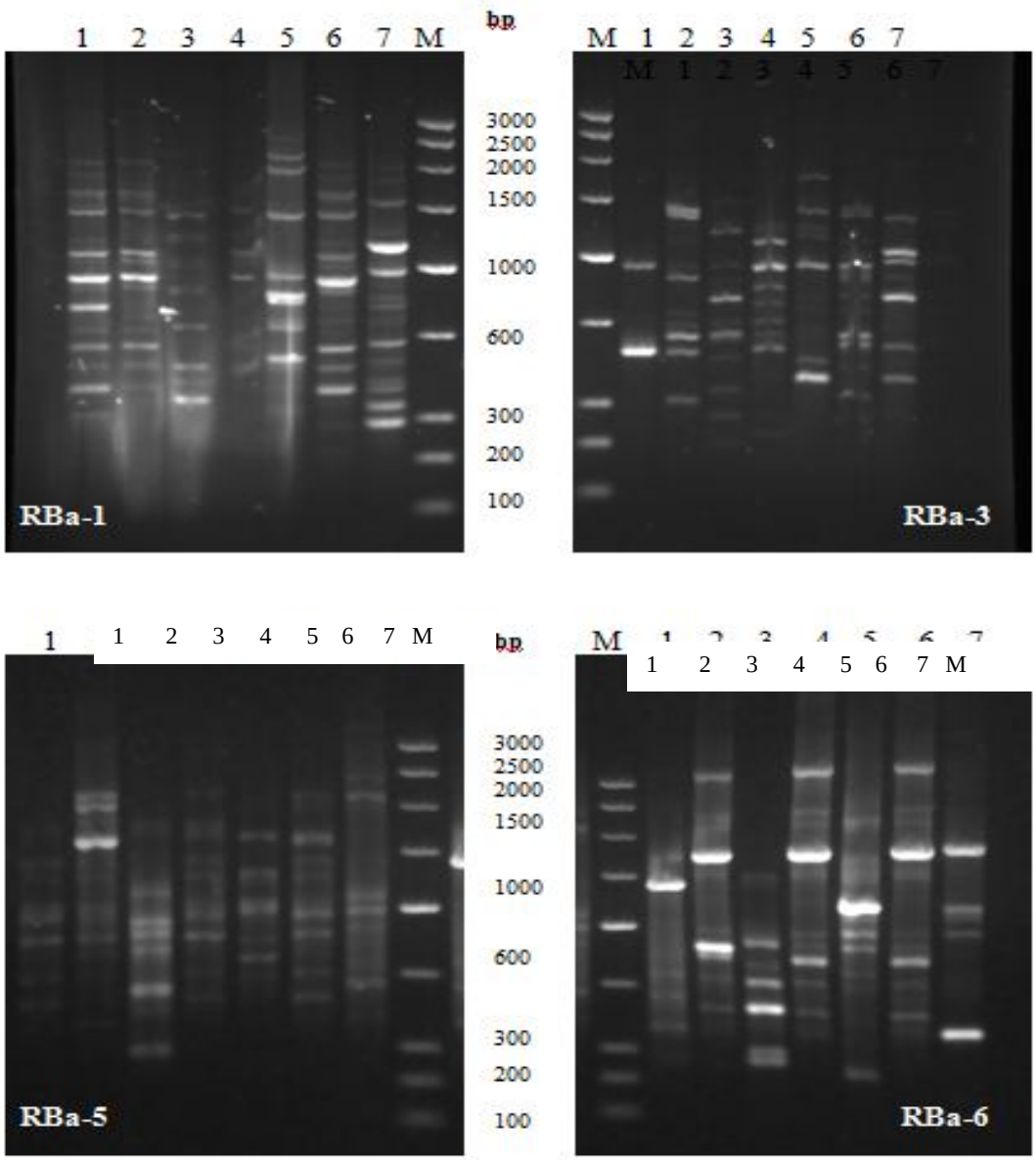

Fig 2. Random amplified polymorphic-DNA fragment patterns generated using primers RBa-1, RBa-2, RBa-5 and RBa-6. 1. *Aeromonas hydrophilla*, 2. *Klebsellia sp*., 3. *Vibrio furnisii*, 4. *Erwinia hericola*, 5. *Serratia marcescens*, 6. *Proteus mirabitis*, 7. *Pseudomonas sp*. M is molecular marker of low range DNA ladder.

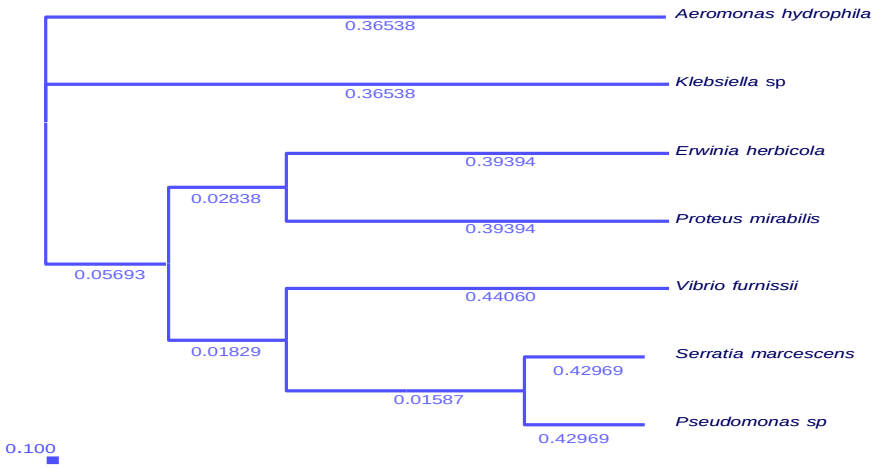

Fig. 3: Un-weighted pair group method with averages (UPGMA) phylogenetic relationship based on Nei's (1972) genetic distance (*D*) among seven species of bacteria constructed by Mega Software (version 5.0)

The result reported in this work are a first step in the genetic characterization of the one bacterial species, further step will include the use of RAPD in studies on population genetic. RAPD marker, which mainly reveals changes in non-coding DNA region [13], therefore, the resolution between species and even population may be better because of the greater polymorphism that can be detected. Knowledge of genetic structure and of the relationship among population is essential for understanding life history pattern and hence essential in the development of appropriate and efficient conservation strategies.

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