DEVELOPMENT OF DNA DIAGNOSTIC MARKERS FOR CERTAIN MICROBES USING PCR AND LAMP TECHNOLOGIES

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CERTIFICATE

This is to certify that the work entitled, "Development of DNA diagnostic markers for certain bacteria using PCR and LAMP technologies" submitted by Kiran Narta and Pratibha Panwar in partial fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Fig. 3.18 Agarose gel showing amplifications with primer set2 using their respective microbes

LIST OF ABBREVIATIONS

ΔG	Change in free energy
AA	*Acetobacter aceti*
AL	*Alcaligenes latus*
AR	*Agrobacterium rhizogenes*
bp	Base pairs
BS	*Bacillus subtillis*
CV	*Chromobacterium violaceum*
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EC	*Escherichia coli*
EDTA	Ethylenediaminetetra acetic acid
LAMP	Loop-mediated isothermal Amplification
KR	*Kocuria rhizophila*
Mb	Mega basepairs
$MgCl_2$	Magnesium Chloride
NaCl	Sodium chloride
nts	Nucleotides
PCR	Polymerase Chain Reaction
PA	*Pseudomonas aeruginosa*

PF	*Pseudomonas flourescens*
PP	*Pseudomonas putida*
SDS	Sodium dodecyl sulphate
ST	*Streptococcus thermophillus*
TAE buffer	Tris acetic acid EDTA buffer
TE buffer	Tris EDTA buffer
Tris HCl	Tris hydrogen chloride
UV	Ultra violet
WE	*Waterisia eutropa*
XCC	*Xanthomonas campestris pv. campestris*
ZM	*Zymomonas mobilis*

ABSTRACT

Most of the microbes are associated with a variety of diseases and their virulence differs. There were two objectives of this work. For the first objective, the microbes chosen were not essentially pathogenic and were chosen to demonstrate a software that can identify unique sequences in microbes so that they can be used as diagnostic markers. This verification was done using PCR technique. The second objective was to use LAMP for microbial diagnostics. The microbes chosen for the latter objective were Xanthomonas campestris pv. campestris (causes black rot of crucifers) and Magnaporthe grisea (causes rice blast). To determine the uniqueness of the sequence to be used as a marker, the specific primers developed from the sequence were used on DNA of other microbes (negative microbes for that primer set). The gel electrophoresis of the PCR product showed that there was non-specific binding of the primer and, hence, amplification. However, on comparing the amplicon sizes seen on gel run, it was deduced that most of the sequences could be used as markers as the desired amplicon size was known to us. For LAMP technique, no turbidity was seen after the amplification which suggested no amplification had occurred. This was verified by performing gel run which also showed no result.

CHAPTER ONE : INTRODUCTION

Microbes Used

Bacillus subtilis

Bacillus subtilis is a Gram-positive, rod-shaped and endospore-forming aerobic bacterium. It is found in soil and rotting plant material and is non-pathogenic. It is one of the most studied gram-positive bacteria. One feature that has attracted a lot of interest in *B. subtilis* is its ability to differentiate and form endospores.
Several strains related to *B. subtilis* are used in the commercial production of extracellular enzymes, such as B. amyloliquefaciens alpha-amylase. Other strains produce insect toxins, peptide antibiotics and antifungals, some of which have been used in agricultural crop protection.
The *B. subtilis* genome contains several genes that are predicted to code for proteins that belong to the cupin superfamily. Cupins are proteins that are related to plant seed storage proteins that fold into small beta-barrels.

Its genome of 4,214,810 bp comprises 4,100 protein-coding genes.

Bacillus subtilis is a bacterium that is used as a fungicide on flower and ornamental seeds, and on agricultural seeds.

Chromobacterium violaceum

Chromobacterium violaceum is a gram-negative, facultative anaerobic, non-sporing coccobacillus. It is part of the normal flora of water and soil of tropical and sub-tropical regions of the world.

It produces a natural antibiotic called violacein, which may be useful for the treatment of colon and other cancers. It grows readily on nutrient agar, producing distinctive smooth low convex colonies with a dark violet metallic sheen (due to violacein production). Its full genome was published in 2003.

C. violaceum rarely infects humans, but when it does it causes skin lesions, sepsis and liver abscesses that may be fatal. Currently there are no vaccines against it. Burkholderia pseudomallei is commonly misidentified as *C. violaceum* by many common identification methods.
C. violaceum has adapted to life with a scarcity of nutrients, and high levels of radiation and other toxic threats. *C. violaceum* strains are also used for the extraction of gold from soil.

Pseudomonas aeruginosa

It is a gram-negative, aerobic, rod-shaped bacterium with unipolar motility. *P. aeruginosa* is also an opportunistic pathogen of humans and plants.

It is found in soil, water, skin flora and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and has thus colonised many natural and artificial environments. It uses a wide range of organic material for food, this versatility enables the organism to infect damaged tissues or people with reduced immunity. The symptoms of such infections are generalised inflammation. If such colonizations occur in critical body organs, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipments including catheters, causing cross infections in hospitals and clinics. It is implicated in hot-tub rash.

An opportunistic, nosocomial pathogen of immunocompromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns wounds, and also causes other blood infections. *Pseudomonas aeruginosa* is a leading cause of opportunistic infection among persons with compromised immune systems.
With plants, *P. aeruginosa* induces symptoms of soft rot with *Arabidopsis thaliana* and *Lactuca sativa*. It is a powerful pathogen with *Arabidopsis* and with some animals: *C.elegans, Drosophila and Galleria mellonella.*

Pseudomonas fluorescens

Pseudomonas fluorescens is being researched as a biological control organism

It has multiple flagella. It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration.

Optimal temperatures for growth of *Pseudomonas fluorescens* are 25-30 °C. Pseudomonas fluorescens is also a nonsaccharolytic bacteria.

Heat stable lipases and proteases are produced by *Pseudomonas fluorescens*. These enzymes cause milk to spoil, by causing bitterness, casein breakdown, and ropiness due to production of slime and coagulation of proteins.

The genomes of *P. fluorescens* strains SBW25, Pf-5 and PfO-1 have been sequenced.

P. fluorescens is an unusual cause of disease in humans, and usually affects patients with compromised immune systems (e.g., patients on cancer treatment).

Pseudomonas putida

It is a gram-negative, rod-shaped saprotropic soil bacterium.

It is the first patented organism in the world. Because it is a living organism the patent was disputed and brought before the United States Supreme Court in the historic court case Diamond v. Chakrabarty which the inventor, Ananda M. Chakrabarty, won. It demonstrates a very diverse metabolism, including the ability to degrade organic solvents such as toluene. This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil. Use of *P. putida* is preferable to some other Pseudomonas species capable of such

degradation as it is a safe strain of bacteria, unlike *P.aeruginosa* for example, which is an opportunistic human pathogen.

Pseudomonas putida has the potential to help clean up organic pollutants, it is used as a soil inoculant to remedy napthalene contaminated soils.

P. putida is capable of converting styrene oil into the biodegradable plastic PHA. This may be of use in the effective recycling of polysterene foam.

P. putida has the most genes of any known species involved in breaking down aromatic hydrocarbons, like TNT. Aromatic hydrocarbons are hazardous chemicals generated by the burning of coal, gas, tobacco, meat and other organic matter.
The petroleum industry is investigating *P. putida* as a cheap means of purifying fuel, while the pathogen's resistance to antibiotics is allowing crop scientists to study its ability to protect plants from pests and help them grow.

Streptococcus thermophilus

Streptococcus thermophilus is one of the most commercially important of all lactic acid bacteria. Its a gram-positive facultative anaerobe. It is non-motile, non-spore forming bacteria. Streptococcus thermophilus is an alpha-hemolytic species of the viridans group. It is also classified as a lactic acid bacterium (LAB). *Streptococcus thermophilus* is found in fermented milk products. It is not a probiotic (it does not survive the stomach in healthy humans).

Streptococcus thermophilus is used, along with *Lactobacillus* spp., as a starter culture for the manufacture of several important fermented dairy foods, including yogurt and Mozzarella cheese.

The genome of *S. thermophilus* is 1.8 Mb, making it among the smallest genomes of all lactic acid bacteria.

Xanthomonas campestris pv.campestris

Xanthomonas campestris pv. campestris (Xcc), a gram-negative aerobic rod, is the causal agent of black rot, which affects crucifers such as Brassica and Arabidopsis. Symptoms include marginal leaf chlorosis and darkening of vascular tissue, accompanied by extensive wilting and necrosis. Full leaf yellowing, wilting, and necrosis occur as the disease advances.

The Xcc bacterium also infects weeds, including *Arabidopsis thaliana*, Researchers now easily test hypotheses about genes associated with plant infections using the publicly available genome sequences of the plant pathogen (Xcc) and one of its hosts (Arabidopsis).

Xanthomonas campestris pv. campestris is grown commercially to produce the exopolysaccharide xanthan gum, which is used as a viscosifying and stabilising agent in many industries.

Magnaporthe grisea

M.grisea also known as rice blast fungus, rice rotten neck, rice seedling blight, blast of rice, oval leaf spot of graminea, pitting disease, ryegrass blast, and Johnson spot, is a plant-pathogenic fungus that causes an important disease affecting rice.

Causes diseases called blast disease or blight disease. Rice blast causes economically significant crop losses annually. Each year it is estimated to destroy enough rice to feed more than 60 million people. The fungus is known to occur in 85 countries worldwide. Strains of the fungus can infect domesticated grasses such as barley, wheat, rye, pearl millet, and turf grasses in addition to rice. Thus, even when crops are burned to destroy fungal infection, grass weeds can act as a disease reservoir. The fungus has been able to develop resistance to both chemical treatments and genetic resistance developed by plant breeders in some types of rice. It is thought that the fungus can achieve this by genetic change through

mutation. There are also concerns that *M. grisea* may be used as a biological weapon by a terrorist organization.

Apart from these *Alcaligenes latus, Acetobacter aceti, Agrobacterium rhizogenes, Escherichia coli, Kocuria rhizophila, Watersia eutropha* and *Zymomonas mobilis,* were used as negatives for all primer sets.

Molecular techniques

As a large number of microbial genomes are being mapped and the thousands of individual genes are sequenced. With this vast knowledge, the potential commercial impact on DNA diagnostic technologies has become more apparent. This project entails diagnosis of microbes by amplifying their unique DNA sequences. Two methods are used for amplification: PCR and LAMP.

PCR

Polymerase Chain Reaction (PCR) is recognized as one of the most sensitive methods used by molecular biologists in various diagnostic approaches.

PCR is a technique, which uses a DNA polymerase enzyme to make a huge number of copies of virtually any given piece of DNA or gene. It facilitates a short stretch of DNA (usually fewer than 3000bp) to be amplified by about a million-fold. In practical terms it amplifies enough specific copies to be able to carry out any number of other molecular biology applications e.g. size determination (in bases) and its nucleotide sequence. The particular stretch of DNA to be amplified, called the target sequence, is identified by a specific pair of DNA primers, oligonucleotides usually about 20 nucleotides in length which designate the outer limits of the amplification product. The primers are designed that can be extended by DNA polymerase. The 3'ends of the hybridised probe are oriented to one another. Isolated DNA containing the segment to be amplified is

heated briefly to denature it, and then cooled in the presence of primers. The template is then replicated in the presence of the 4 dNTPs. The cycle of heating and cooling is repeated 20-30 times over a few hours in an automated process. PCR uses Taq polymerase which remains active after every heating and cooling step.

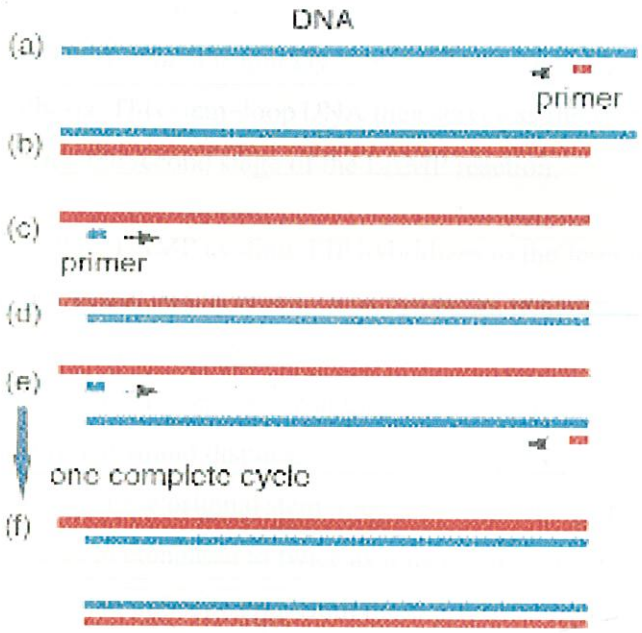


Fig. 1.1 Polymerase Chain Reaction.

LAMP

Loop mediated isothermal amplification (LAMP) is a novel DNA amplification method that allows reactions to occur under isothermal conditions. Four primers are required in the LAMP reaction. It has higher specificity then conventional PCR method. There is a large product accumulation of varying length, making the product detection easier.

The LAMP method

The mechanism and expected reaction steps of LAMP are illustrated in Figure1. Inner primer FIP hybridizes to F2c in the target DNA and initiates complementary

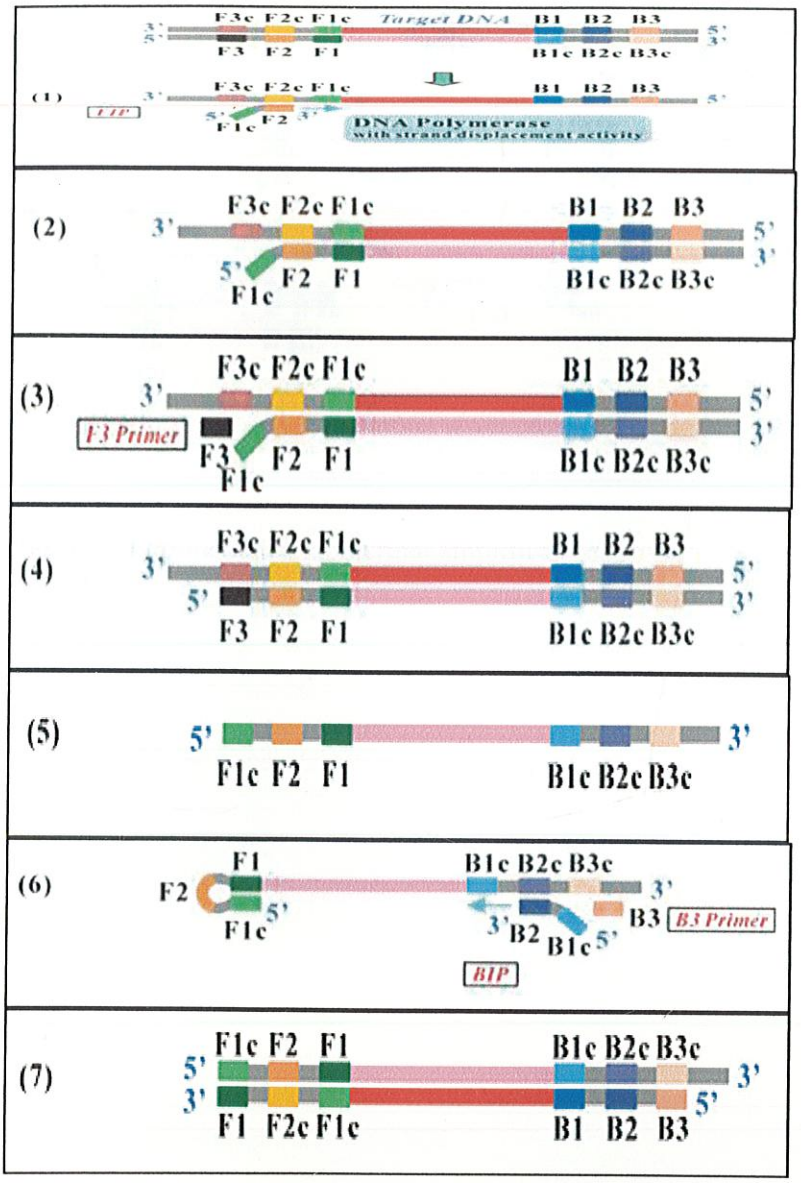
strand synthesis. Outer primer F3, which is a few bases shorter and lower in concentration than FIP, slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end. This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA, which is quickly converted to a stem–loop DNA by self-primed DNA synthesis. This stem–loop DNA then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction.

To initiate LAMP cycling, FIP hybridizes to the loop in the stem–loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem–loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem–loop DNA and one gap repaired stem–loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end. Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles, a part of which is designated the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

The final products are a mixture of stem–loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand.

The use of four primers (recognition of six distinct sequences) in the initial steps of LAMP and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for target amplification. Moreover, in LAMP four primers (six distinct recognition sequences) are simultaneously used to initiate DNA synthesis from the original unamplified DNA to generate a stem–loop DNA for subsequent LAMP cycling, during which the target is recognized

by four sequences. Therefore, target selectivity is expected to be higher than those obtained in PCR.



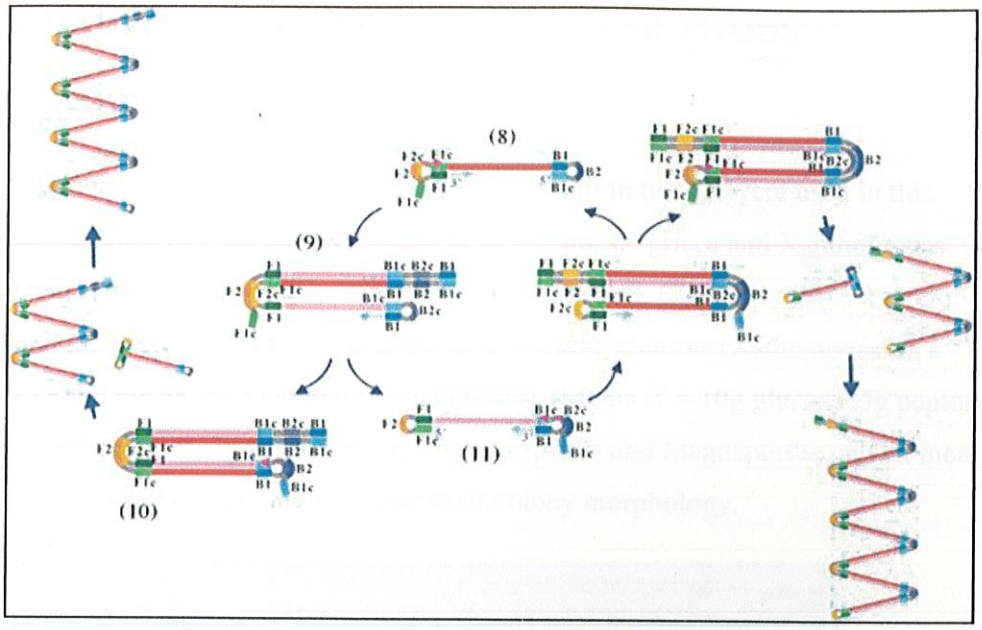


Fig. 1.2 Loop-mediated isothermal amplification.

CHAPTER TWO : MATERIALS AND METHODS

Culture collection

The permanent stock cultures of bacteria present in the lab were used in this study. Also, the sub-cultured slants of Magnaporthe grisea and Xanthomonas campestris pv. campestris were provided by Dr. R.S. Chauhan. These 2 microbial cultures were verified by preparing their working cultures (Xathomonas on a specific media for Xanthomonas campestris (Media X = 10g glucose, 5g peptone, 3g malt extract, 3g yeast extract, 15g agar, pH 7) and Magnaporthe on Oat meal agar and M9 media) and checking their colony morphology.

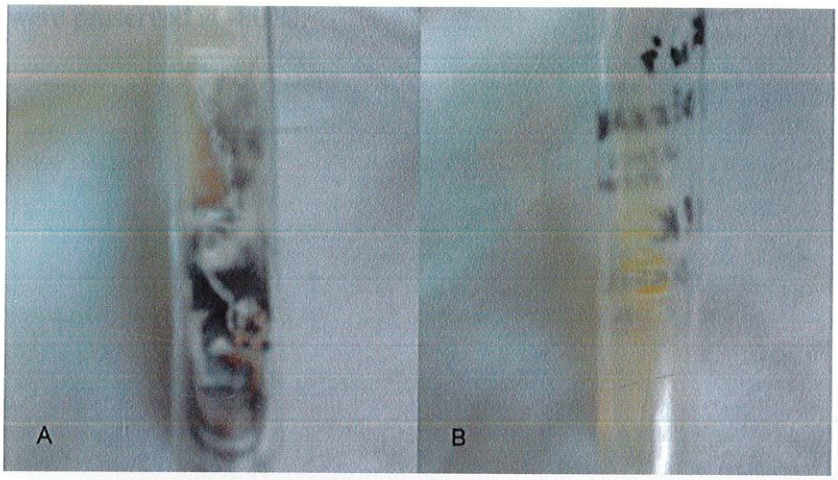


Fig. 2.1 A) Seed culture of *Magnaporthe grisea,* B) Seed culture of *Xanthomonas campestris pv campestris.*

Culture Maintenance and Storage

Working cultures were prepared in nutrient broth at 30°C. However, for Xanthomonas campestris pv. campestris Media X broth and for Magnaporthe grisea Oat meal broth was used, at the same temperature. The bacterial cultures were kept overnight and then were used in DNA isolation. Magnaporthe grisea,

being a fungus, took a week to grow and we got its conidia from which we isolated DNA.

For all bacterial microbes, except Xanthomonas, permanent glycerol cultures were already present, stored at -80°C. Xantomonas and Magnaporthe were sub-cultured again in nutrient broth and oat meal broth, respectively, at 30°C. Xanthomonas was allowed to grow overnight before preservation while Magnaporthe was grown for days. For storage 80% glycerol was used in the ratio 1:4 (glycerol:cultured broth). For Xanthomonas, two culture vials were prepared with 2.75mL storage culture media each. For Magnaporthe, three culture vials were prepared with 3mL storage culture media, each, containing conidia. All were preserved at -80°C.

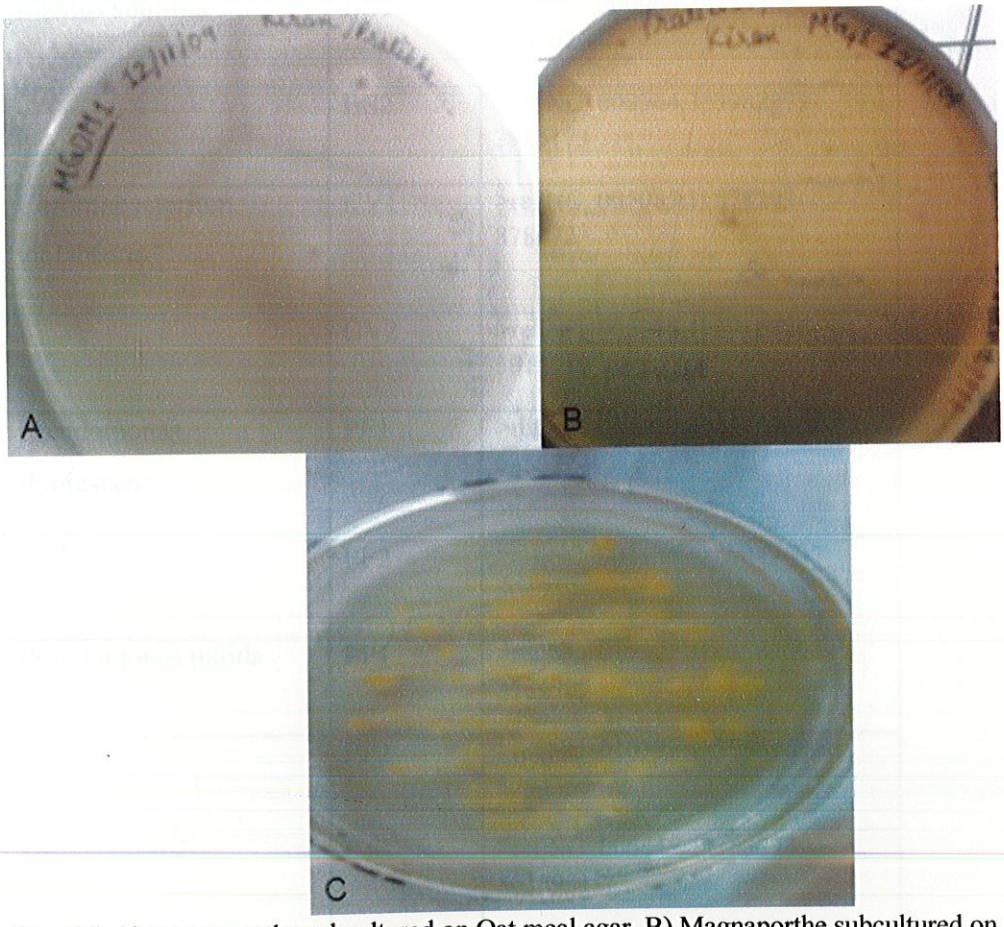


Fig. 2.2 A) Magnaporthe subcultured on Oat meal agar, B) Magnaporthe subcultured on M9 media, C) Xanthomonas campestris pv. campestris subcultured on Media X.

Unique sequence identification

PCR

For each microbe two unique sequences were identified.

Microbe name	Sequence name	Sequence reference number
Pseudomonas aeruginosa	PA1	>ref|NC_002516.2|:c622538-622023
	PA2	>ref|NC_002516.2|:3170682-3171062
Bacilus subtillis	BS1	>ref|NC_000964.3|:753265-753702
	BS2	>ref|NC_000964.3|:3982973-3983173
Chromobacterium violaceum	CV1	>ref|NC_005085.1|:878293-878472
	CV2	>ref|NC_005085.1|:3915716-3915919
Pseudomonas fluorescens	PF1	>ref|NC_004129.6|:c5255667-5255224
	PF2	>ref|NC_004129.6|:c5049685-5049494
Pseudomonas putida	PP1	>ref|NC_010322.1|:c3585094-3584909
	PP2	>ref|NC_010322.1|:3963963-3964355
Streptococcus thermophilus	ST1	>ref|NC_006448.1|:1633749-1633931

	ST2	>ref|NC_006448.1|:879567-879800
Xanthomonas campestris pv. campestris	XCC1	>ref|NC_003902.1|:3549751-3550698
	XCC2	>ref|NC_003902.1|:c2493431-2492436

Table 2.1 Microbes and their unique sequences.

LAMP

For Xanthomonas campestris pv. campestris one unique sequence was identified (>ref|NC_003902.1|:3549751-3550698). This was done using the NCBI database. Protein sequence of *Xanthomonas campestris pv. campestris* ATCC 33913 was taken from NCBI database and blasted (blastx) against protein sequence of all bacterial species. Lowest similarity protein sequence of *Xanthomonas campestris pv. campestris* ATCC 33913 was identified and the corresponding nucleotide sequence was taken as the unique sequence.

For Magnaporthe grisea mif23 gene was taken directly since PCR had already been done for it (Chanda and Gopalakrishna, 2006)

Primer designing

PCR

Primers were designed using a tool available online, namely, Primer 3. Conditions applied were T_m 60°C, primer length 20 nts and amplicon size 200-300 bp. A forward and a reverse primer was obtained for each sequence.

Sequence name	**Primer name**	**5`-3` Primer sequence**

PA1	PA1f	TCGACCTGGTCTACCTCAGC
	PA1r	GGGTGTACTGGGCGAAACT
PA2	PA2f	CAGCCTCGTTTCTCCTCCT
	PA2r	TCAGGTTGTCCTGCATCTGT
BS1	BS1f	AACTGGAAGCTATCCGAGCA
	BS1r	TAACCGTCTTTTCCCCAGTG
BS2	BS2f	CAGCCCTCGAAAAACAAAAG
	BS2r	CTGAAACCTCCGTCACTGCT
CV1	CV1f	AGCCCCTGTCTTCCTTTTTC
	CV1r	AAAAACCCATCCACGGTTTC
CV2	CV2f	AAACTGCTGACCGCTCTGAT
	CV2r	GCTTTCTTGTGCTTTTCATGC
PF1	PF1f	TGGTCAGCATCACTTCCATC
	PF1r	TGCAATTGCTTTTGCAGTTC
PF2	PF2f	ATGGGTTCCACCTTCAATGG
	PF2r	GATGATCAGCCCCAGCAC
PP1	PP1f	TTACAGCCCTGGTCACATTG
	PP1r	TAGTCATTCTGGGGGTCCTG
PP2	PP2f	CGTTGTTGGTCAACAGCTTG
	PP2r	CTACGGTCGAACCGGTCTT

ST1	ST1f	CTTGCCGGCGTTTATCTTAC
	ST1r	TGGCTAACTCTCCGTTGAAAA
ST2	ST2f	CAACGACCCTATCGACCCTA
	ST2r	GGTGTTGCAGACTCGTTTGA
XCC1	XCC1f	CAGAGTTGAGCGGCATAACA
	XCC1r	TCCACCATTCACGAAACAAA
XCC2	XCC2f	GCTGGTGGCTCAATAGCTTC
	XCC2r	GTTTCCGCATCAGGATGATT

Table 2.2 Unique sequences and their primers.

LAMP

Primers were designed using a tool available online, namely, PrimerExplorer V4. A set of four primers i.e. two outer primers (forward and reverse) and two inner primers (forward and reverse) were obtained for each microbial sequence.

Name of microbe	**Primer name**	**5`-3` Primer sequence**
Xanthomonas campestris pv. campestris	XCCf3	AAAAGCTCCCAAGCTTCCA
	XCCb3	CAAATGTGCTTCAGCTGGAC
	XCCfip	GCATGTGAGTAGGTCGTCAGCTTTTTCCTACGACT CGCGGACTT
	XCCbip	TTCACCTCAAGATAAGCGCCGTTTTTGCGTGCTGA

		AAGACATTGTC
Magnaporthe grisea	MPf3	GCTGGTACTCGAGCAACAG
	MPb3	GGTTGCTAGAGCTGTCGC
	MPfip	ATCTTTGCCAGTGCAGACGGGTTTTCGGTGCTGTC GCAATGTC
	MPbip	CACTGCCACCAAGGGCTCTTTTTTGCTGTGGCACT ACCTGATC

Table 2.3 Unique sequence and the set of four LAMP primers for each.

Phenol equilibration

Before use phenol must be equilibrated to pH > 7.8 because the DNA partitions into the organic phase at acid pH. Phenol was melted and equal volume of 1M tris HCl (pH 8) was added to it. The mixture was stirred on a magnetic stirrer for 15 minutes, after which it was kept at 4°C overnight. Next day, the upper aqueous layer was aspirated out and equal volume of 0.1M tris HCl (pH 8) was added to the phenol. The mixture was again stirred on magnetic stirrer for 15 minutes, after which it was kept at 4°C overnight. Next day, the upper aqueous layer was aspirated out and the pH of phenol was checked with a pH paper. The pH appeared > 7.8. To this phenolic phase, 0.1 volume of 0.1M tris HCl (pH 8) containing 0.2% β-mercaptoethanol was added. This mixture was transferred to a dark reagent bottle and stored at 4°C.

DNA extraction

Two different methods were used for isolation of bacterial and fungal DNA.

Bacterial DNA Isolation

Working culture (broth) was transferred to 2mL eppendorf tubes in laminar air flow and centrifuged at 6000 rpm for 10 minutes. Supernatant was carefully removed and the pellet was resuspended in 1mL lysis buffer (10mM tris HCl (pH 8), 1mM EDTA, 0.5% SDS, 1M NaCl). Tubes were incubated at 45°C for 10 minutes after which equal volume of phenol : chloroform (1:1) was added to it and centrifuged at 10000 rpm for 10 minutes. The upper aqueous layer was taken and equal volume of chloroform : isoamyl alcohol (24:1) and one-tenth volume of 3M sodium acetate (pH 5.2) was added to it. After centrifugation at 10000 rpm for 10 minutes, the upper aqueous layer was taken and double the volume of chilled ethanol was added to it. Tubes were incubated at -20°C for 20 minutes and then centrifuged at 12000 rpm for 10 minutes. Supernatant was discarded and pellet was air dried. For storage, 50 µL TE buffer (10mM tris HCl (pH 8), 1mM EDTA) was added and eppendorf tubes were stored at -20°C.

Fungal DNA Isolation

Working culture (broth) containing conidia was filtered using Whatman's filter paper. The conidia obtained was treated with liquid N_2 and ground to powder in pestle and mortar kept at -80°C overnight. 200 mg of the powdered conidia was added to 2mL extraction buffer (100mM tris HCl (pH 8), 10mM EDTA, 2% SDS, 100µg/mL Proteinase K, 1% β-mercaptoethanol) in a test tube and sonicated for 90 seconds at 150 Hz on ice. After sonification, equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added and the mixture was mixed at room temperature for 10 min. After centrifugation at 10000g for 10 minutes at 4°C, upper aqueous layer was aspirated and equal volume of isoproponol was added along with one-tenth volume of 3M sodium acetate (pH 4.8). The tubes were then allowed to stand at room temperature for 5 minutes after which they were centrifuged at 10000g for 10 minutes at room temperature. Recovered pellet

was washed with 0.5mL 70% ethanol. After removing all traces of ethanol, pellet was resuspended overnight at 4°C in 100µL TE buffer (10mM tris HCl (pH 8), 1mM EDTA) containing 50µg/mL DNase free RNase A.

DNA Amplification

Polymerase Chain Reaction

PCR was performed on the positive and negative sets of microbes. 3µL 10xPCR buffer, 4.9µL autoclaved water, 2µL magnesium chloride, 1µLdNTPs, 1µL primers (forward and reverse each), 2µL template DNA and 0.1µL Taq polymerase were added to PCR tubes and PCR was performed under conditions : 95°C for 5 minutes followed by 30 cycles of 94°C for 5 minutes, annealing temperature (depending on primer) for 1 minute, 72°C for 5 minutes; followed by 72°C for 10 minutes.

Loop-mediated isothermal amplification

LAMP was performed on Xanthomonas and Magnaporthe after their DNA isolations. 4µL 2xbuffer, 2.5µL autoclaved water, 4µLbetaine, 1µL 0.2M $MgSO_4$, 3.5µL dNTPs, 3.11µL inner primers, 0.39µL outer primers and 2µL template DNA were added to PCR tubes and placed in thermocycler at 95°C for 5 minutes. After this the mixture was chilled on ice to lower the temperature. 1µL Bst polymerase (large fragment) was added and the PCR tubes placed in thermocycler at 65°C for 90 minutes followed by 80°C for 2 minutes.

Agarose gel electrophoresis

The amplification products were run on 1.5% agarose gel. 50xTAE buffer (242g/L tris base, 57.1mL/L glacial acetic acid, 100mL/L 0.5M EDTA, pH 8-8.2) stock was prepared. Agarose was weighed and mixed in 1xTAE buffer and the mixture was heat dissolved in microwave. On cooling a bit, ethidium bromide was added and mixed. The agarose gel was poured into the caster with combs in position and allowed to solidify. The solidified gel was placed in electrophoretic unit and completely submerged in 1xTAE buffer. Wells were loaded with 4µL

loading dye and 6μL amplification product and run with 4μL DNA ladder at 100V. After gel run, the gel was viewed in Gel Doc under trans UV rays.

CHAPTER THREE : RESULTS

A molecular diagnostic using PCR technique for *Bacillus subtilis, Pseudomonas putida, Streptococcus thermophillus, Pseudomonas flourescens, Pseudomonas aeurigunosa, Chromobacterium violaceum, Xanthomonas campestris pv. Campestris* has been developed by this study.

Design of primers

After the specific DNA sequences for the microbes were submitted for analysis using online program 'PrimerExplorer V4' several sets of LAMP primer sequences were presented. Primer sets were selected according to suitable parameters. The primer sequences are given in the Table 2.2 along with their location in the genome.

The primers for PCR were designed for specific sequences using Primer3. These primers are given in Table 2.3.

Gel-Electrophoresis analysis for PCR product

Below are the PCR products on the agarose gel. First lane represents the microbial template for which the respective primers were designed. Other lanes represent the negatives for that primer.

Bacillus subtilis

BS1

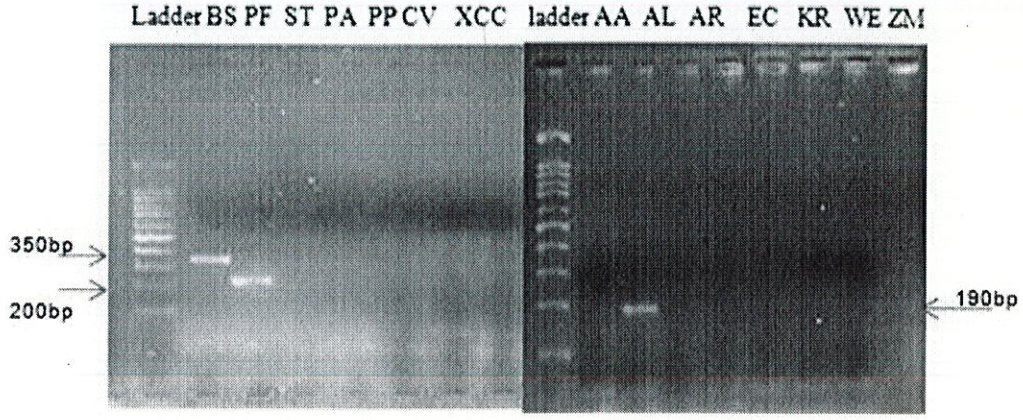


Fig. 3.1 Agarose gel showing amplification for *B. subtilis* primer set1.

Amplification of 350bp observed in *B. subtilis*, 200bp *P. fluorescens* and also 190bp *Alcaligenes latus*.

BS2

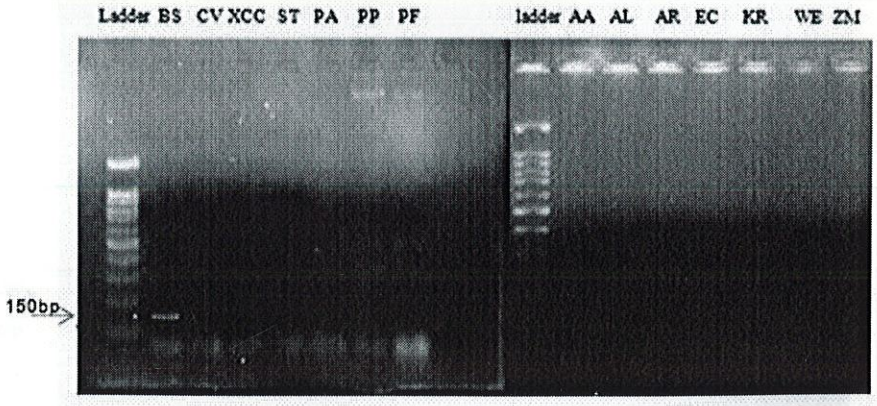


Fig. 3.2 Agarose gel showing amplification for *B. subtilis* primer set2.

Amplification of 150bp observed in *B. subtilis.*

Chromobacterium violaceum

CV1

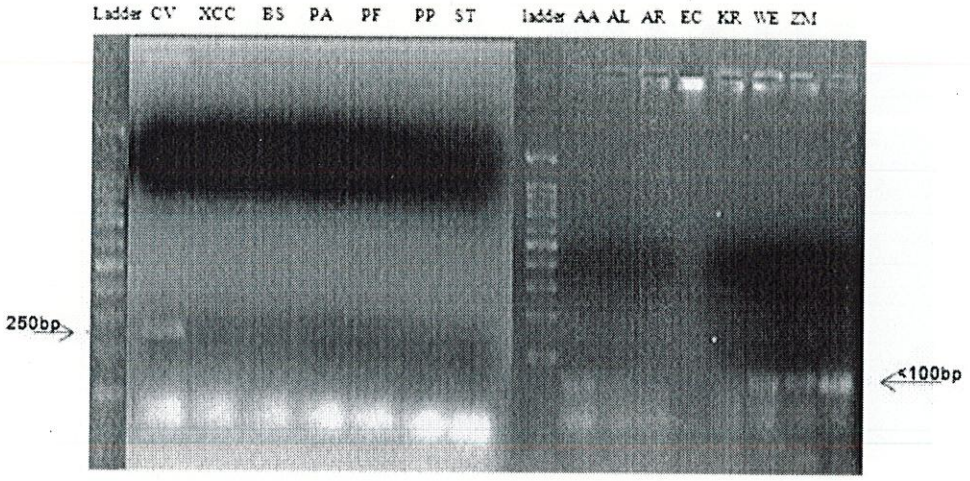


Fig. 3.3 Agarose gel showing amplification for *C. violaceum* primer set1.

Amplification of 250bp observed in *C. violaceum.*

CV2

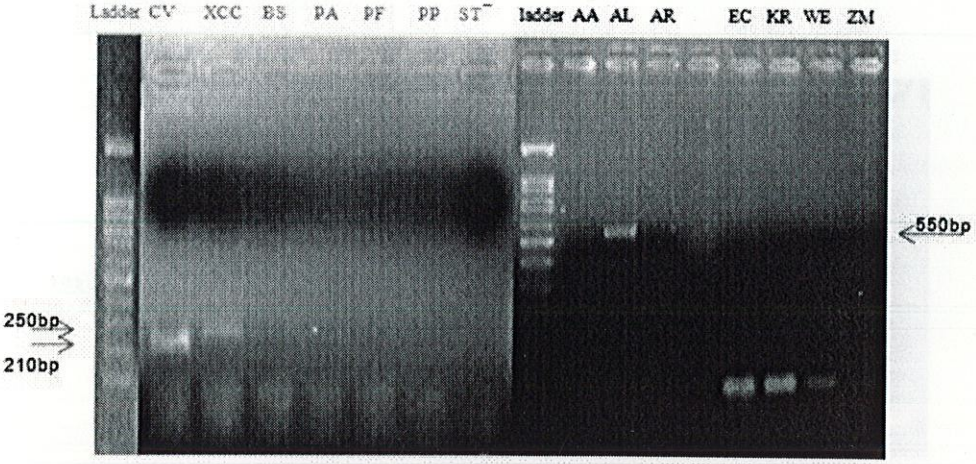


Fig. 3.4 Agarose gel showing amplification for *C. violaceum* primer set2.

Amplification of 200bp observed in *C. violaceum*, 250bp in *X. campestris* and 550bp *A. latus*

Pseudomonas aeruginosa

PA1

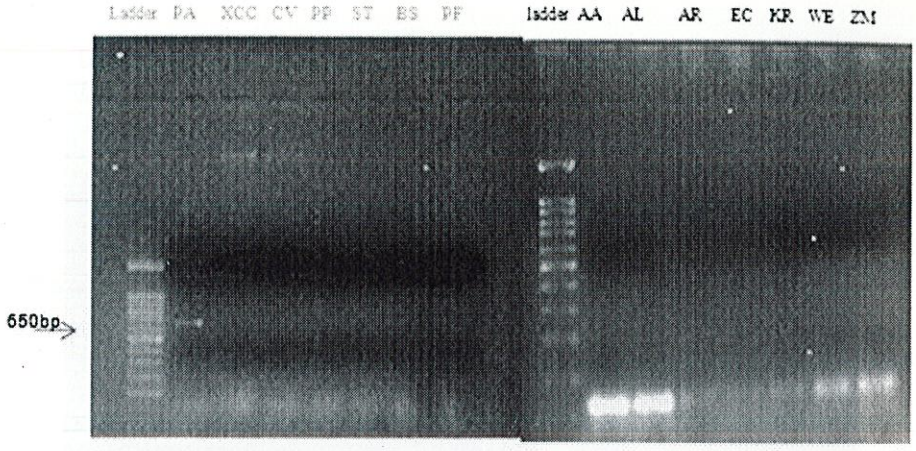


Fig. 3.5 Agarose gel showing amplification for *P.aeruginosa* primer set1.

Amplification of 650bp observed in *P. aeruginosa*

PA2

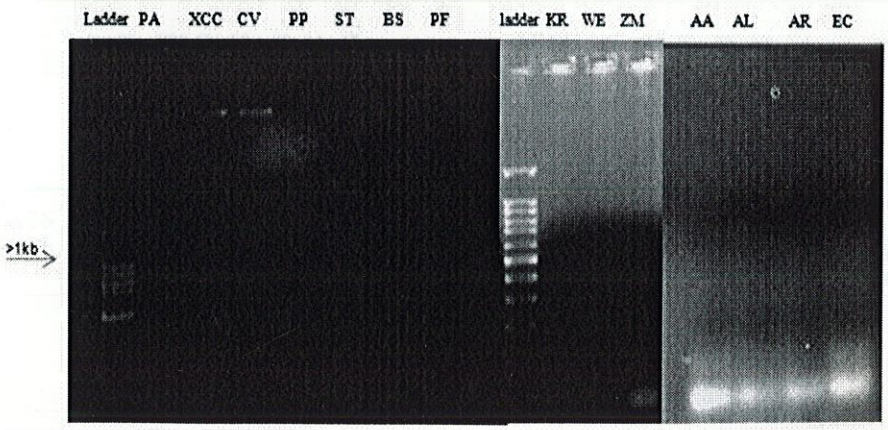


Fig. 3.6 Agarose gel showing amplification for *P. aeruginosa* primer set2.

Amplification of more than1kb observed in *P. aeruginosa, X. campestris pv. Campestris* and *C. violaceum.*

Pseudomonas fluorescens

PF1

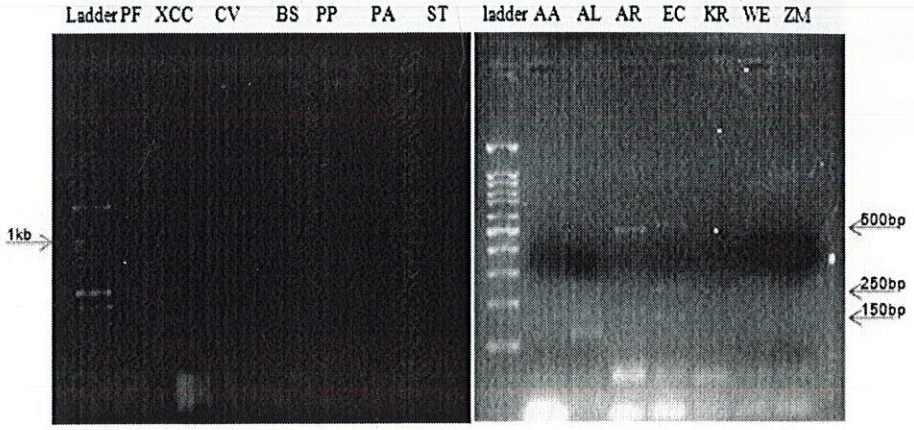


Fig. 3.7 Agarose gel showing amplification for *P.fluorescens* primer set1.

Amplification of 1kb observed in *P. fluorescens,* 120bp in *A. latus*, 500bp in *A.rhizogenes*, 500bp, 250bp and 150bp in *E. coli*

PF2

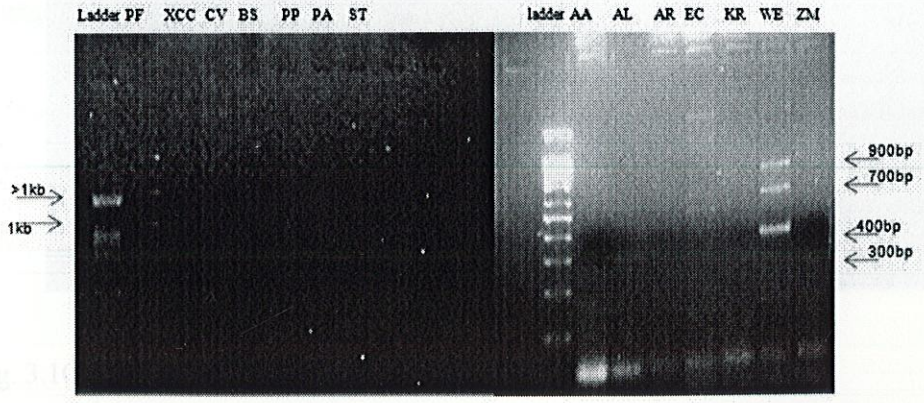


Fig. 3.8 Agarose gel showing amplification for *P. fluorescens* primer set2.

2 Amplifications of more than 1kb observed in *P. fluorescens,* amplification of 300bp in *A. latus*, multiple amplifications in *E. coli* and *W. eutropa.*

Pseudomonas putida

PP1

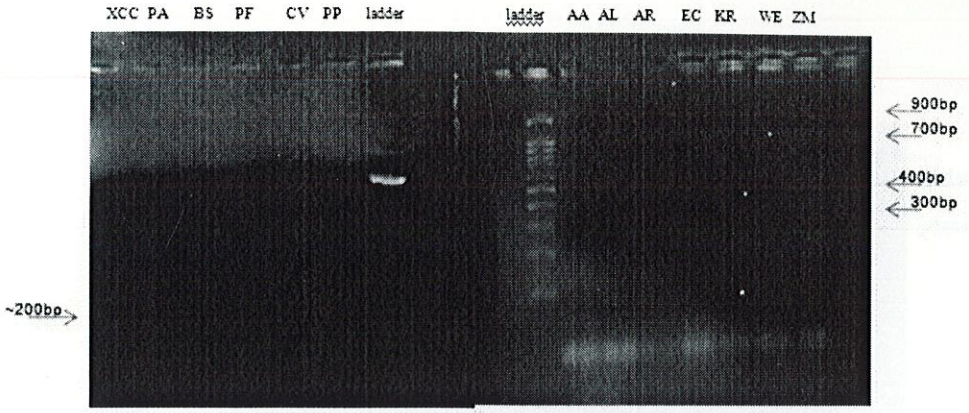


Fig. 3.9 Agarose gel showing amplification for *P.putida* primer set1.

Amplification in the form of smear of around 200bp in *P.putida.*

PP2

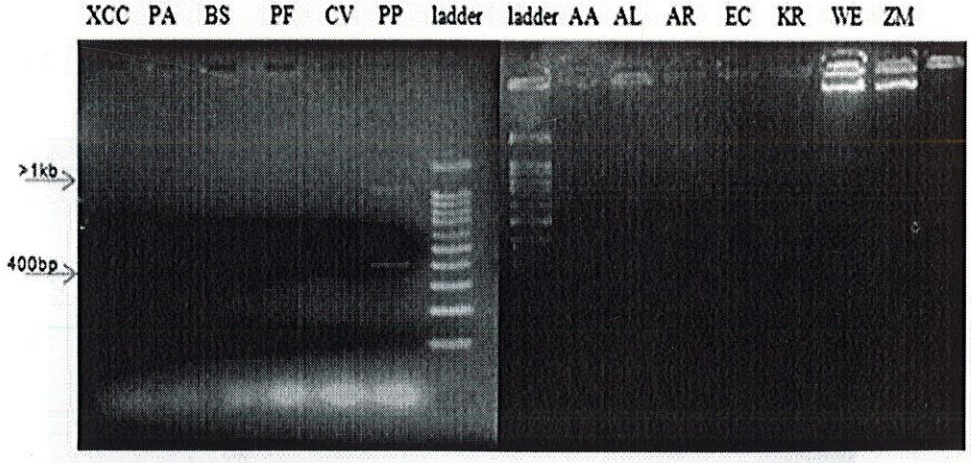


Fig. 3.10 Agarose gel showing amplification for *P. putida* primer set2.

Amplification of 400bp and 1kb in *P. putida.*

Streptococcus thermophilus

ST1

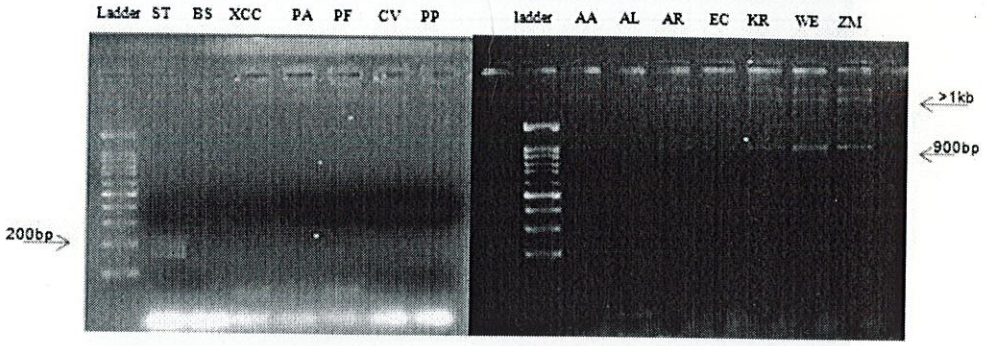


Fig. 3.11 Agarose gel showing amplification for *S. thermophilus* primer set1.

Amplification of 200bp in *S. thermophilus,* and amplifications of more than 1kb in *A. aceti, A. latus, A. rhizophila, E. coli, K. rhizophila, W. eutropa, Z. mobilis.*

ST2

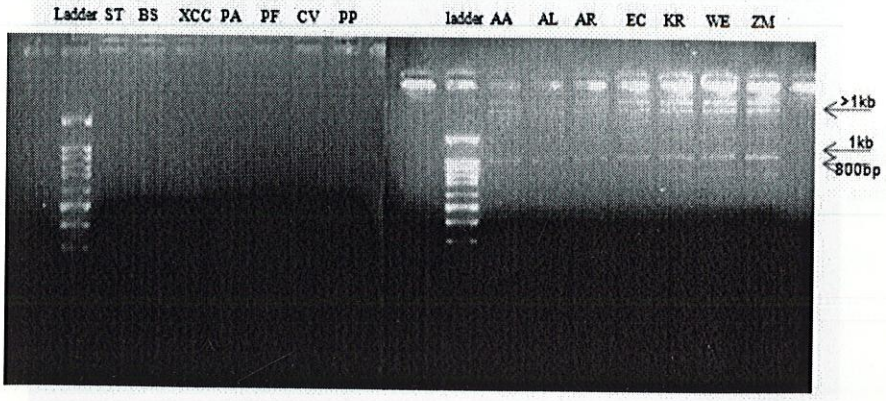


Fig. 3.12 Agarose gel showing amplification for *S. thermophilus* primer set2.

No amplification in *S. thermophilus,* and ladder like structure from 500bp upto wells in *A. aceti, A. latus, A. rhizophila, E. coli, K. rhizophila, W. eutropa, Z. mobilis.*

Xanthomonas campestris pv. campestris

XCC1

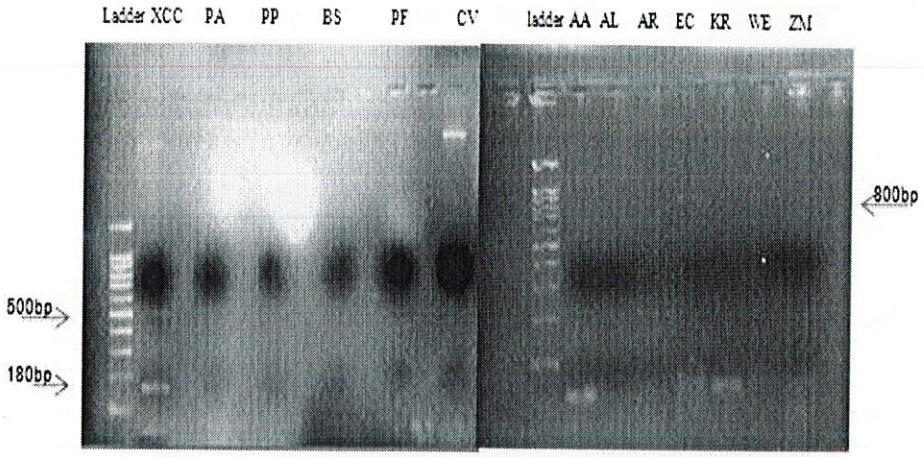


Fig. 3.13 Agarose gel showing amplification for *X. campestris* primer set1.

Amplification of 180bp and 500bp in *X. campestris,* and 800bp in *E. coli*

XCC 2

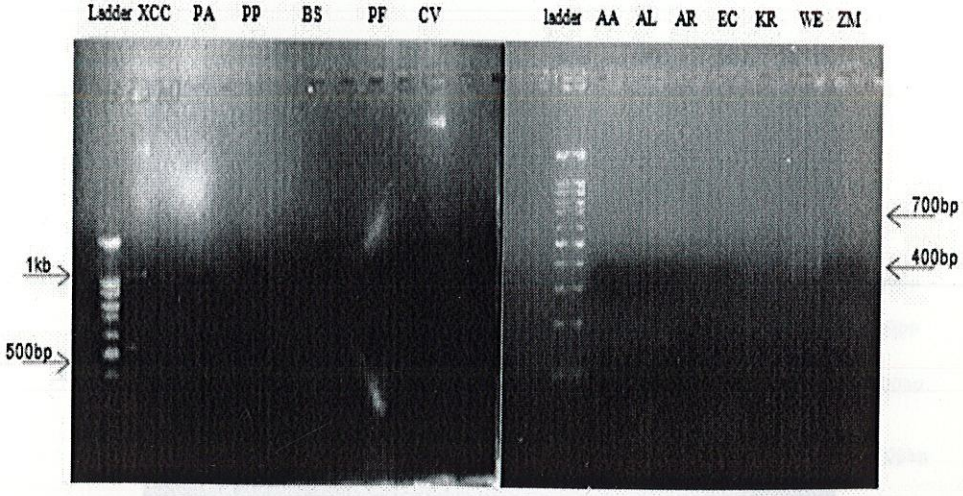


Fig. 3.14 Agarose gel showing amplification for *X. campestris* primer set2.

Amplification of 1kb and 500bp in *X. campestris, and* a smear from 400bp to 700bp in *W. eutropa*

All positives

Primer set 1

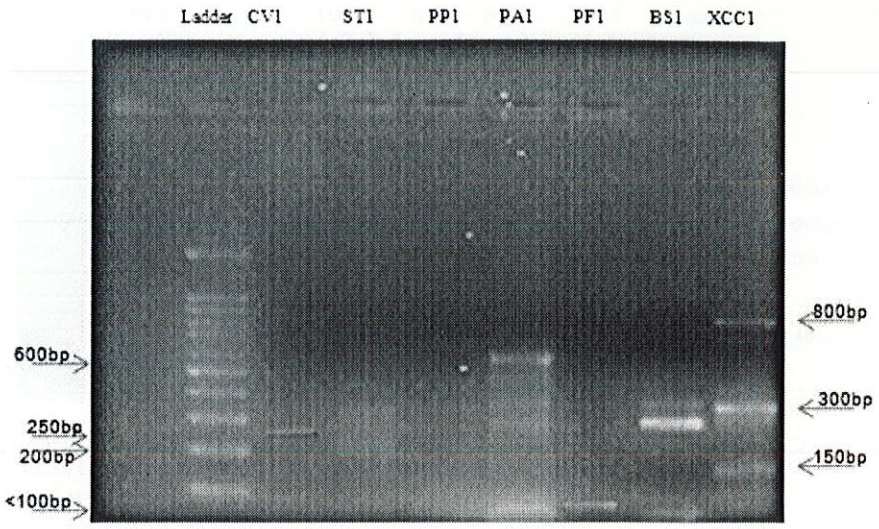


Fig. 3.15 Agarose gel showing amplifications with primer set1 using their respective microbes

Primer set 2

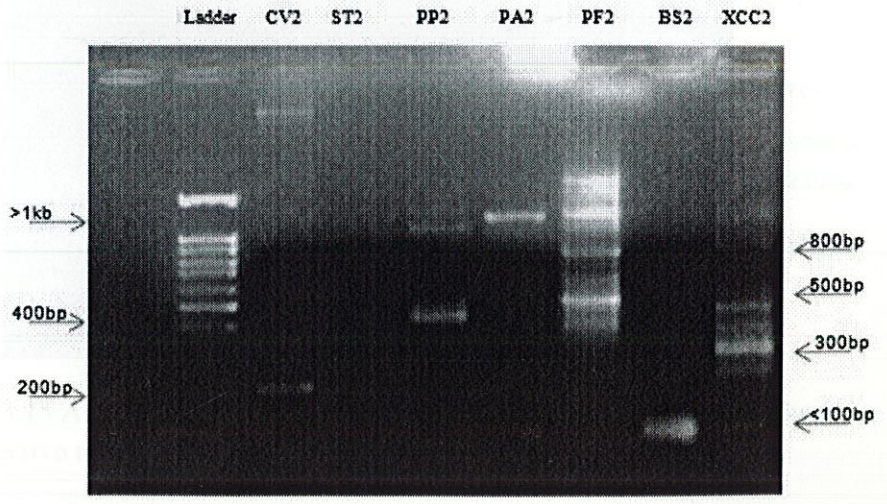


Fig. 16 Agarose gel showing amplifications with primer set2 using their respective microbes

All positives

Primer set1

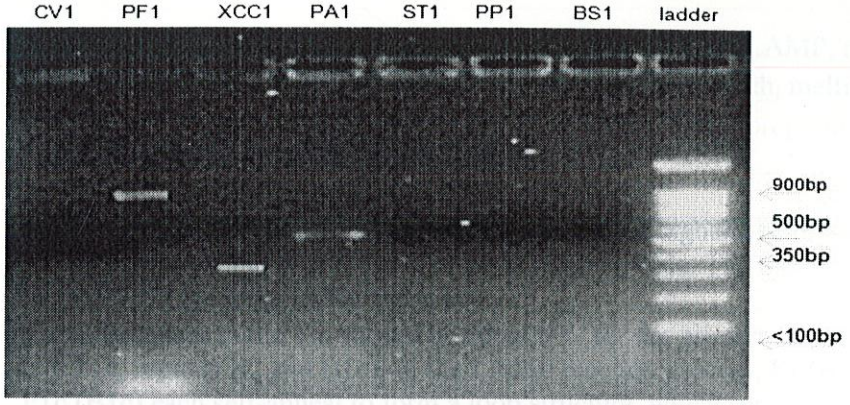


Fig. 3.17 Agarose gel showing amplifications with primer set1using their respective microbes.

Primer set 2

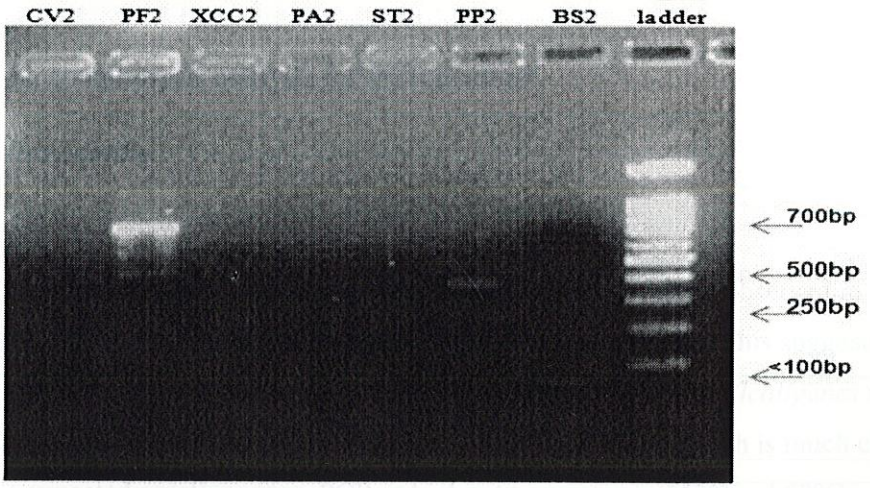


Fig. 3.18 Agarose gel showing amplifications with primer set2 using their respective microbes.

LAMP

No amplification product could be obtained for LAMP reactions.

No turbidity was observed in the tubes, and no ladder like pattern was observed in the agarose gel.

CHAPTER FOUR : DISCUSSION

Design of Primers

The different sets of primers obtained from PrimerExplorer V4, for LAMP, are given alongwith parameters like 5` position, 3` position, primer length, melting temperature, 5` and 3` ΔG which suggests stability, GC rate. The appropriate set was selected on the basis of following parameters:

- A low value of the change in free energy results in a higher likelihood of dimer formation and thus the primer set is unacceptable. The more negative ΔG the more stable end it is.
- The differences in the Tm should be about 5°C for the primers (regions F2 and F1c, regions B2 and B1c). In the LAMP reaction process, F1 (B1) and F1c (B1c) each self-anneal to form a loop structure, which serves as the starting structure for amplification. To facilitate forming this loop, F1c (B1c) was set at a Tm value around 5°C higher than those of the other primers. Also, best results will be obtained if the Tm's match between regions F2 and B2, regions F1c and B1c, and regions F3 and B3.

Gel-Electrophoresis analysis for PCR product

Bacillus subtilis

BS1

The expected amplicon size was 189bp. Amplification of 350bp observed in *B. subtilis*, 200bp *P. fluorescens* and also 190bp *Alcaligenes latus*. This suggests that the primer set showed non-specific binding in *Bacillus*. Also, in *Alcaligenes* and *P. fluorescens* showed amplicons of sizes 190bp and 200bp, which is much closer to the expected amplicon size. PCR at higher temperatures, 55°C and 58°C, showed no amplification in *Bacillus*. Hence, the sequence cannot be used as a diagnostic marker.

BS2

The expected amplicon size was 104bp. Amplification of 150bp was observed in *B. subtilis.* Hence, the sequence can be used as a diagnostic marker.

Chromobacterium violaceum

CV1

The expected amplicon size was 154bp. Amplification of 250bp was observed in *C.violaceum.* PCR at higher temperatures, 55°C and 58°C, showed no amplification in *Chromobacterium*. This sequence can be used as a marker as it shows amplification only in the desired microbe.

CV2

The expected amplicon size was 179bp. Amplification of 200bp observed in C.violaceum, 250bp in X. campestris and 550bp A.latus. PCR at higher temperatures, 55°C and 58°C, showed no amplification in *Chromobacterium*. Hence, this sequence can be used as a diagnostic marker for detection of *C. violaceum.*

Pseudomonas aeruginosa

PA1

The expected amplicon size was 213bp. Amplification of 650 bp observed in *P. aeruginosa.* Hence, the sequence can be used as a diagnostic marker as there is no amplification seen in other microbes.

PA2

The expected amplicon size was 231bp. Amplification of more than1kb observed in *P. aeruginosa, X. campestris pv. campestris* and *C. violaceum.* PCR at higher temperatures, 55°C and 58°C, showed no amplification in *P. aeruginosa.*

Pseudomonas fluorescens

PF1

The expected amplicon size was 247bp. Amplification of 1kb observed in *P. fluorescens,* 120bp in *A. latus*, 500bp in *A. rhizogenes*, 500bp, 250bp and 150bp in *E. coli*. Hence, the sequence cannot be used as a diagnostic marker.

PF2

The expected amplicon size was 153bp. Two amplifications of more than 1kb observed in *P. fluorescens,* amplification of 300bp in *A. latus*, multiple

amplifications in *E. coli* and *W. eutropa.* Hence, the sequence cannot be used as a diagnostic marker as there are no desire amplifications.

Pseudomonas putida

PP1

The expected amplicon size was 153bp. Amplification in the form of smear of around 200bp in *P. putida.* At higher temperature, 55°C, an amplicon of 950bp was observed. But at 58°C, no amplification was seen. The sequence can be used as a diagnostic marker because it shows the desired amplification in the desired microbial DNA.

PP2

The expected amplicon size was 153bp. Amplification of 400bp and 1kb observed in *P. putida.* Sequence cannot be used as a marker because the desired amplification was not seen.

Streptococcus thermophilus

ST1

The expected amplicon size was 155bp. Amplification of 200bp in *S. thermophilus,* and amplifications of more than 1kb in *A. aceti, A. latus, A. rhizophila, E. coli, K. rhizophila, W. eutropa, Z. mobilis.*

ST2

The expected amplicon size was 178bp. No amplification in *S. thermophilus,* and ladder like structure from 500bp up to wells in *A. aceti, A. latus, A. rhizophila, E. coli, K. rhizophila, W. eutropa, Z. mobilis.* Hence, sequence cannot be used as marker.

Xanthomonas campestris pv. campestris

XCC1

The expected amplicon size was 203bp. Amplification of 180bp and 500bp in *X. campestris,* and 800bp in *E. coli.* PCR at higher temperatures, 55°C and 58°C,

showed no amplification in any microbe. Hence, this sequence can be used as a marker.

XCC2

The expected amplicon size was 212bp. Amplification of 1kb and 500bp in *X. campestris,* and a smear from 400bp to 700bp in *W. eutropa.* Hence, sequence cannot be used as marker.

All positives

PCR was performed for all positive microbes with their respective primers, thrice. This was done to confirm that the primers are working on their respective microbial DNA. Though, each time the result was variable, but amplifications were seen.

LAMP

No result was obtained possibly because the strain used to design the primers was different from the strain used in lab for amplification.

CHAPTER FIVE : CONCLUSION

The development of reliable DNA diagnostic markers requires us to have unique sequences that will only be amplified in the microbe of our choice. The length of the amplicon is very important in this study since spurious amplification can be seen. On the basis of the length of the amplicon we can decide whether those spurious amplifications are of any significance. Therefore, on the basis of results it can be said that most of the markers were designed successfully.

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