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RAPID IDENTIFICATION OF HUMAN PATHOGENIC BACTERIA (*PSEUDOMONAS AERUGINOSA, VIBRIO SPS.*) AND FUNGUS (*ASPERGILLUS NIGER*) BY USING MULTIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT

In this study, we focused on the isolation of highly pathogenic bacteria and fungus in freshwater bodies collected from Lucknow region. We have targeted mainly five *Vibrio species* such as *Vibrio cholera, Vibrio alginolyticus, Vibrio mimicus, Vibrio parahaemolyticus, Vibrio vulnificus, Pseudomonas aeruginosa* and *Aspergillus niger* that are the common pathogenic., *Aspergillus niger* caused lung infection such as cystic fibrosis patients, asthma and aspergilla are the culprits behind allergic bronchopulmonary. In our work we selected five antibiotic such as Oxytetracycline, Rifampicin, Cefotaxime, Tetracycline and Flucanozole used in which Cefotaxime showed highest activity against *Vibrio sps* and *Pseudomonas aeruginosa* with a zone of inhibition of 29 mm and 28 mm at 0.2 mg/ml concentration and the Oxyteracycline shows the lowest activity against *Pseudomonas aeruginosa* with a zone of 19 mm at 0.2 mg/ml concentration. In the fungus sps., Fluconazole showed the highest activity against *Aspergillus sps.* with a zone of inhibition of 29 mm at 0.2 mg/ml. After genomic DNA isolation, Multiplex PCR technique also provides the highest supplement to the traditional methods for more accurate monitoring of human pathogenic bacteria and fungus in freshwater and soil.

KEYWORDS: *Multiplex PCR, P.aeruginosa, amplification,antibiotic susceptibility, vibrio spp., ZOI.*

1. INTRODUCTION

Vibrio species mainly present in the aquatic environment includes fresh, coastal and marine habitats. These species are highly pathogenic in the surface of the digestive tract in fish and zooplankton **(Drake *et al.*, 2007).**

A multiplex PCR method especially accomplished for application in diagnostic laboratories, developed for diagnosing 5 human pathogen *Vibrio* species such as *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio mimicus*, and *Vibrio alginolyticus* **(Singh *et al.*, 2013)**.

Pseudomonas is a gram-negative bacterium which causes infections that are resistant to nearly all types of antibiotics. In these species, *Pseudomonas aeruginosa* are mainly human pathogenic include bloodstream infections, pneumonia (lung infection), urinary tract infection, and surgical wound infections **(Stead, 1992).**

Aspergillus niger is a human pathogenic fungal species which is ubiquitous in soil **(Denning, 1998; Morgan *et al.*, 2005).** *Aspergillus niger* is mainly food spoilage causing fungal species. These species also have the large range of plant biomass degrading enzymes used as the interesting compound for biotechnological production **(Dubey *et al.*, 2017).** Aspergilli causes lung disease such as cystic fibrosis and asthma, aspergilla which also cause aspergillosis **(Kliasova *et al.*, 2005)**.

The multiplex polymerase chain reaction method is the popular and effective method for detection of pathogenic bacterial and fungal species. In this method, specific primers are designed to detect various strains of pathogenic bacterial and fungal sps **(Singh, 2015).**

2. MATERIAL AND METHOD

2.1 Isolation of Fungus and bacteria

The *Aspergillus niger* used in this study was obtained on Sabouraud's Dextrose Agar from the soil sample. Samples were collected from Lucknow region for isolation of *Vibrio sps*. and *Pseudomonas aeruginosa*. In our work, TCBS media and Cetrimide Agar media was selected for the growth of colonies having *Vibrio sps* and *Pseudomonas aeuroginosa.* 200 µl of fresh water sample was spread on their selective media and left for 24 to 48 hours at 37^0 C. The broth was prepared for obtaining a pure culture of the colony which used in genomic DNA isolation **(Massey & charan, 2016; Kumar *et al.*, 2015**).

2.2 Fungus DNA isolation

For fungal DNA isolation, 2 ml of the fungal biomass was placed in a ceramic plate and crushed with lysis buffer (3 % SDS, 50 mM EDTA, Tris HCl). This fungal biomass homogenates in a centrifuge (REMI RM-12C) at 13,000 rpm for 10 min. RNsse A (10mg/ml) was added in the fresh centrifuge tube and then incubated at 37ºC for 15 min then Phenol: Chloroform: Isoamyl was added. After this step, the upper layer was taken in a centrifuge tube and then an equivalent volume of 100% of ethanol was add and precipitated at – 20 ^{0} C for 30 min. After centrifuge, the DNA pellet washed with 70 % of ethanol and then dissolved in 1 X TE buffer for Qualitative analysis by Agarose gel electrophoresis **(Aamir *et al.*, 2015)**.

2.3 For Bacterial DNA isolation

The DNA isolation of bacteria was accomplished by using the phenol-chloroform method **(Prapaiwong *et al.*, 2009).** An overnight culture grown in tryptone- soy broth (TSB) were centrifuge (REMI RM-12C) at 8,000 rpm for 5 min. Add 900µl 1x TE buffer, 100 µl 10 % SDS in the pellet. After this step these microcentrifuge tubes were incubated for 2 hours at 56-60ºC in water bath. After incubation adds 500µl P:C:I reagent then centrifuge at 10,000 rpm for 5 min. Add chilled ethanol to the tube and incubate it for overnight at 4ºC in the refrigerator (Celfrost). After incubation add 100 µl 1xTE buffers and store it for agarose gel electrophoresis (GeNei^{TM}).

2.4 Quantification of DNA by UV-visible Double beam spectrophotometer

The quantity of the genomic DNA was determined by comparing the measurement at 260-280 nm (the wavelength for which DNA and protein absorb) using UV visible spectrophotometer (Systronics) **(Aamir *et al.*, 2015)**.

2.5 Primer Designing

In the multiplex PCR method, specific primers are used to their corresponding gene and size of expected amplification products were ***shown in table 1*** **(Shi, 2012; Sugita, 2004; Yadav, 2014).**

Table 1: Primers for PCR amplification.

Vibrio sps.	**VM-F**	**CAGGTTTGYTGCACGGCGAAGA**
5 Reverse primer :		
V.cholera	VC- Rmm	AGCAGCTTATGACCAATACGCC
V. parehaemolyticus	VC- Rm	TGCGAAGAAAGGCTCATCAGAG
V. Vunificus	VV- Rm	GTACGAAATTCTGACCGATCAA
V. mimicus	VM- Rm	YCTTGAAGAAGCGGTTCGTGCA
Vibrio alginolyticus	V.al2- MmR	GATCGAAGTRCCRACACTMGGA
Pseudomonas aeruginosa	aroE-F	TGGGGCTATGACTGGAAACC
	aroE-R	TAACCCGGTTTTGTGATTCCTACA
Aspergillus niger	aflR-F	AACCGCATCCACAATCTCAT
	aflR-R	AGTGCAGTTCGCTCAGAACA

2.6 Multiplex Polymerase Chain Reaction Amplification

For amplification of DNA 20 µl PCR reaction mixture which contained 2.5 µl 10X PCR buffer, 2 µl dNTP mixture, 1 µl reverse and forward primer, 1 µl of Taq DNA polymerase and 5 µl of DNA templates. The amplification was performed in an Eppendrof PCR system preincubation step at 94^{o}C for 3 min; 25 cycles of denaturation at 94 ^{o}C, annealing at 60 ^{o}C and extension step at 72 ^{o}C for 60 second and final extension at the same temperature for 7 min **(*Shown in table 2*) (Shi *et al.*, 2012).**

Table 2: PCR condition.

Initial denaturation	**94 degree C for 3 min**
Denaturation	94 degree for 30 sec
Annealing	60 degree for 30 sec
Extension	72 degree for 60 sec
Final extension	72 degree for 7 min
Number of cycles	25

2.7 Antibiotic Susceptibility Test

Isolates, *Pseudomonas aeruginosa*, *Vibrio sps*, *& Aspergillus niger* were screened for their sensitivity to antibiotics because the frequency of occurrence of these pathogens was very high. Multidrug's resistant strains of these pathogens are emerging worldwide. This test was performed by well diffusion technique to determine the sensitivity of antibiotic against pathogenic microbes. Overnight grown culture of respective bacterial sps. were spread on agar media and then incubated at 37 ˚C for 24 h. Inhibition zone diameters were compared with the standard inhibition zone for resistance, intermediate and susceptible character **(Wose, 2002)**.

3. RESULT AND DISCUSSION

3.1 Isolation of Pathogenic bacteria and fungus

Isolation of *Vibrio sps Pseudomonas aeuroginosa* and *Aspergillus niger* was done by spreading the water and soil sample on their selective media for obtaining discrete colonies *(as **view in fig 1)***.

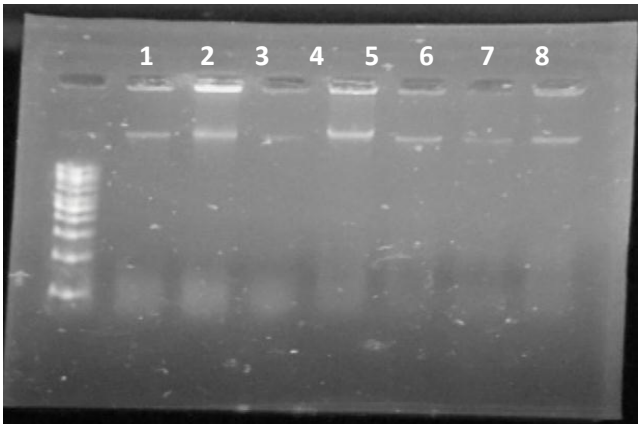
(a) ***(b)*** ***(c)***

Figure 1: ***Isolation of bacteria and fungus; (a) Vibrio sps. on TCBS Media; (b) Pseudomonas aeruginosa on Cetrimide Agar Media; (C) Aspergillus niger on Sabouraud's Dextrose Agar,***

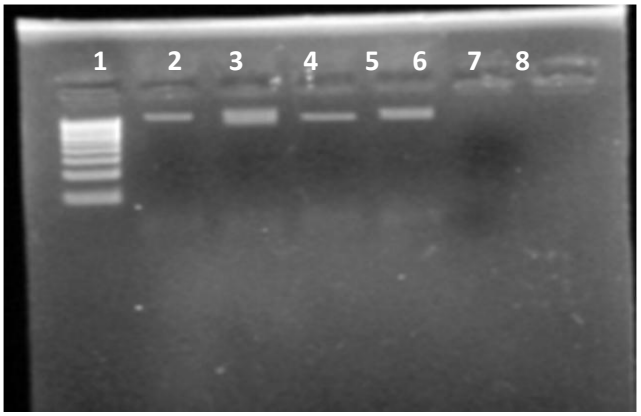
In many developed countries with inadequate sanitation and fecal contamination of environment water by pathogens are very common and river water is the major source of pathogens **(Massey, 2016).** According to **wose *et al.* (2002)** rivers of north Indian region shows the highest contamination with the microbial pathogen and *Vibrio cholera* was most abundant among all contribute. In this work 25 water samples collected from river and ponds and specific primers are designed which targeted the gene such as toxigenic of the *Vibrio* sps. The rapid detection of the selected *Vibrio sps* including *V. cholera, V. mimicus, V. alginolyticus, V. parahaemolyticus and V. vulnificus* was done by using the multiplex PCR method. After detection with multiplex PCR, they observed that three species were present i.e. *V. cholera, V. alginolyticus, V. parahaemolyticus*.

3.2 Qualitative analysis of Genomic DNA

The quantitative analysis of DNA extracts was done on 0.8 % Agarose gel stained with dye i.e. ethidium bromide as ***shown in figure 2.*** The stored genomic DNA was later used in multiplex PCR.



(a)



(b)

Fig. 2: Qualitative analysis of genomic (a)Pseudomonas aeruginosa genomic DNA; DNA; Vibrio sps. Genomic DNA; (b)Aspergillus niger genomic DNA.

Isolation of genomic DNA mainly based on the detection and quantification of specific segments of the pathogen's genome (DNA or RNA). To achieve this, the specific segments are subjected to in vitro amplification. PCR is a molecular tool that allows for the amplification of target DNA fragments using oligonucleotide primers in a chain of replication cycles catalyzed by DNA polymerase (Taq polymerase). The Molecular method used for microbial identification and surveillance with high sensitivity and specificity. Nowadays, this method also used in the detection of pathogenic bacteria in environmental and clinical samples **(Yadav *et al.*, 2014)**.

3.3 Quantitative analysis of Genomic DNA

This method is done for quantification of DNA. Through this method concentration of DNA and protein in the sample is identified by the help of spectrophotometer ***(as shown in table 5, 6, and 7)***.

Table 5: Concentration of DNA in water sample (for Vibrio species).

DNA Sample	Absorbance	DNA Concentration (ng/µl)	Protein Concentration (ng/µl)	Ratio ($O.D_{260}$ / $O.D._{280}$)
Sample 1	0.047	2.35	0.06	1.28
Sample 2	0.001	0.05	0.02	0.96
Sample 3	0.004	0.2	0.06	1.23
Sample 4	0.078	3.9	0.07	1.24
Sample 5	0.041	2.05	0.06	1.21
Sample 6	0.080	4.0	0.06	1.23

Table 6: Concentration of DNA in water sample (for Pseudomonas aeruginosa).

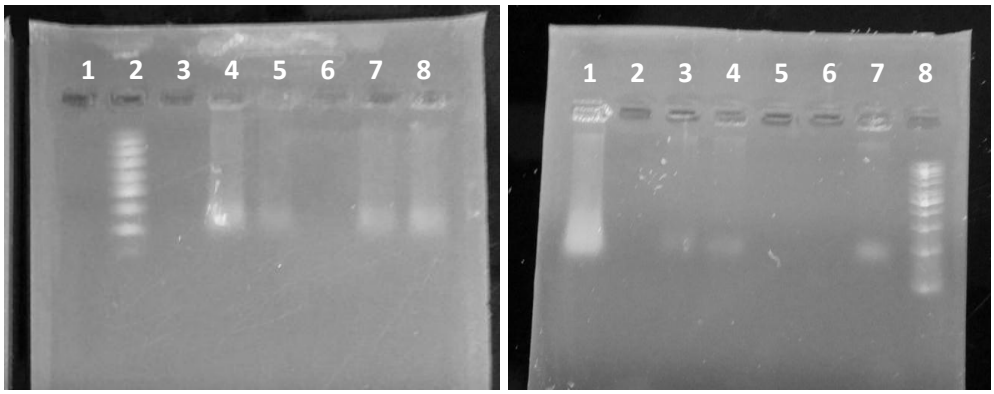
DNA Sample	Absorbance	DNA Concentration (ng/µl)	Protein Concentration (ng/µl)	Ratio ($O.D_{260}$ / $O.D._{280}$)
Sample 1	0.001	0.05	0.02	0.96

Table 7 concentration of DNA in soil sample (for Aspergillus niger)

DNA Sample	Absorbance	DNA Concentration (ng/µl)	Protein Concentration (ng/µl)	Ratio ($O.D_{260}$ / $O.D._{280}$)
Sample 1	1.00	0.14	0.14	1.28
Sample 2	1.00	0.13	0.13	1.26

3.4 Multiplex PCR optimization and amplification of DNA

By the use of a various reverse primer for *Vibrio* and single reverse primer for each *Pseudomonas aeruginosa* and *Aspergillus niger* fragments of DNA were separated in the form of bands ***(as shown in figure 3 and 4)***. This shows the pathogenic DNA fragment on the sample collected because primers used in the PCR amplification were pathogenic reverse primer ***shown in table 8 and 9***.



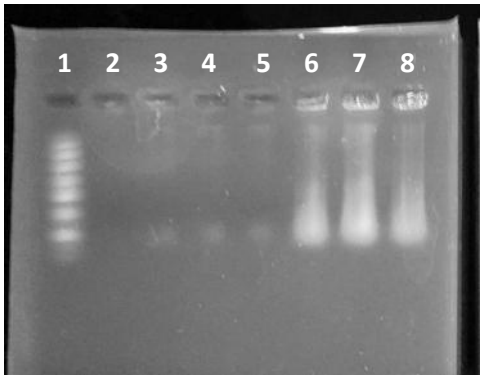


Fig. 3: Multiplex PCR amplification of bacterial DNA sample (Vibrio sps.).

Table 8: Results of PCR amplification of Vibrio sps.

Lane no.	Species	Regions
Lane 3	*V. alginolyticus*	-
Lane 4 & 5	*V. cholera*	Chart corner (Lucknow)
Lane 6	*V. fluvallis*	-
Lane 7	*V. mimicus*	Store water (Lucknow)
Lane 8	*V. parahaemolyticus*	-

Fig 4: PCR amplification of Pseudomonas aeruoginosa DNA and Aspergillus niger DNA.

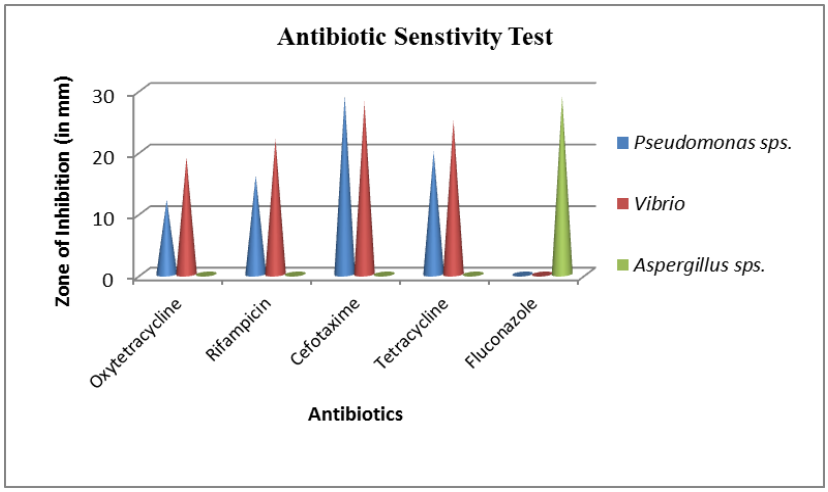
Table 9: Lane shows species of fungus and bacteria.

Lane no.	Species	Regions
Lane 2	*Aspergillus niger*	Soil (Lucknow)
Lane 3 & 4	*Aspergillus niger*	Soil (Lucknow)
Lane 5	*Aspergillus niger*	Soil (Lucknow)
Lane 6 & 7	*Pseudomonas aeruginosa*	Water (Lucknow)
Lane 8	*Pseudomonas aeruginosa*	Water (Lucknow)

This study was based on 30 freshwater bodies collected from rivers, lakes, and ponds of Indian region. In this work, they used the multiplex PCR technique for detection of *Vibrio species* including *Vibrio mimicus, Vibrio parahaemolyticus, Vibrio vulnificus* & *Vibrio alginolyticus.* This method mainly designed the specific primer used for targeting the tox gene of *Vibrio.* **(Ergin & Mutlu, 1999)**.

3.5 Antibiotic Sensitivity Test

Antibiotic sensitivity of all the isolates was analyzed by Agar well diffusion method. The antibiotics included Ox tetracycline, Rifampicin, Cefotaxime, Tetracycline, and Fluconazole. Their sensitivity to different antibiotics is represented in ***graph 1.***



Graph 1: Antibiotic Sensitivity test of pathogenic bacterial and fungal species.

In the antibiotic sensitivity test five antibiotics such as Oxytetracycline, Rifampicin, Cefotaxime, Tetracycline and Flucanozole used in which Cefotaxime showed highest activity against *Vibrio sps* and *Pseudomonas aeruginosa* with a zone of 29 mm and 28 mm at 0.2 mg/ml concentration and the Oxyteracycline shows the lowest activity against *Pseudomonas aeruginosa* with a zone of inhibition of 19 mm at 0.2 mg/ml concentration. In the fungus sps., Fluconazole shows the highest activity against *Aspergillus sps.* with a zone of inhibition of 29 mm at 0.2 mg/ml.

According to **Gian,** Gram-negative bacilli, especially *Escherichia coli, Klebsiella, Enterobacter, Salmonella, Shigella, Pseudomonas, Streptococcus,* and *Haemophilus influenza*, may become resistant. Amoxicillin, ampicillin, amoxiclav, Amikacin, cefuroxime, cefotaxime, ceftazidime, tetracycline, cefoperazone, chloramphenicol, ciprofloxacin, and gentamicin may cause bacterial resistance. Resistance to bacteria for several pathogens makes complications in the treatment of infections caused by them. Shigella strains may become resistant to ampicillin, cotrimoxazole, chloramphenicol, and streptomycin. Multidrug-resistance of *Streptococcus sps.* due to tetracycline, chloramphenicol. Multidrug-resistance of the *Pseudomonas aeruginosa* may become resistant to β-lactams, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline. The antibacterial activity against Haemophilus strains may occur with trimethoprim-sulfamethoxazole, sulbactam-ampicillin, gentamicin, ampicillin, chloramphenicol, and ciprofloxacin. Multidrug-resistance of Escherichia coli may be caused by ampicillin, cotrimoxazole, chloramphenicol, ceftriaxone, and ceftazidime. Vibrio cholera may become resistant to cotrimoxazole, chloramphenicol, ampicillin, with least resistance to erythromicin, tetracycline, and ciprofloxacin.

4. CONCLUSION

In our work the characterization of pathogenic Bacteria (*Vibrio sps.*, *Pseudomonas aeruginosa*) and *Fungus* (*Aspergillus niger*) through multiplex PCR is an advantageous method providing high output and less time's consuming. Multiplex PCR method also provides the highest supplement to the traditional methods for more accurate monitoring of human pathogenic bacteria and fungus in freshwater and soil.

In the antibiotic sensitivity test, Cefotaxime showed highest activity against *Pseudomonas aeruginosa* and *Vibrio sps* and the Oxyteracycline show the lowest activity against *Pseudomonas aeruginosa*. On the other hand, Fluconazole shows the highest activity against fungal species such as *Aspergillus niger*.

Rapid identification of human pathogenic bacterial and fungal species through multiplex PCR would not only routinely screen the soil and water quality to protect and safe public health but also allow evaluation of water and soil treatment.

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