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Full Length Research Paper

Identification of some *Penicillium* species by traditional approach of morphological observation and culture

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Ninety six samples of water collected from Dal Lake, Kashmir at eight different sites for a period of one year from April 2010 to March 2011 were tested for the presence of *Penicillium* species. Five different species of the said genus were isolated from the samples by serial dilution technique. Out of total 122 fungal colonies isolated, five species of *Penicillium* viz, *Penicillium olivicolor*, *Penicillium commune*, *Penicillium chrysogenum*, *Penicillium funiculosum* and *Penicillium dimorphosporum* were obtained. Among the isolated species *P. funiculosum* was the most abundant (28.7%) followed by *P. chrysogenum* (27.04%), *P. dimorphosporum* (23.77%), *P. olivicolor* (16.40%) and *P. commune* (4.09%). Maximum percentage of fungal colonies (27.7%) was observed at site PKB followed by TBN (21.13%), DLG and GB (13.15%) each, NL (8.45%), BHN (7.04%), HB (5.63%) and BD (3.76%).

Key words: Macroscopic, microscopic, Dal Lake, serial dilution, water, *Penicillium.*

INTRODUCTION

Many molecular identification approaches have been evaluated to date for fungi. Of these, polymerase chain reaction (PCR) amplification of genomic DNA followed by sequencing of resulting amplicons has shown the most promise, at least for the more unusual isolates from human infections. The identification, taxonomy and epidemiological analyses of fungal pathogens are increasingly dependent on modern molecular techniques, based on PCR amplification of conserved regions of the genome and sequencing of the resulting PCR products (Borman et al., 2006; Haynes et al., 1996; Makimura et al., 1994; Sandhu et al., 1995).

However, such approaches have long been hindered by the presence of potent PCR inhibitors in fungal cultures, and difficulties inherent in breaking fungal cell walls.

Generally identification of the fungal species is based on the morphological characteristics of the colony and microscopic examinations. Although molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used and essential tools for identification of the fungal species like *Penicillium* and *Aspergillus*. The identification of microfungi by morphology is generally regarded to be very difficult, as traits used for delimitation of species within a genus often show minute differences that can only be reliably evaluated by experienced mycologists. Different strains of one species can slightly vary in their morphology. This is often used as an argument by investigators with rather limited experience that identification of a morphological basis is not reliable and leads to inconsistent results, but still a survey by the American society for microbiology (American Society for Microbiology, 2004) documented that 89% of laboratories were performing mycological examinations (morphology based), 16% of them use serologic tests and fewer than 5% use molecular tests for identification of microbial pathogens. Only 3% of reporting laboratories use 'home-brew' molecular testing for microbial pathogens.

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Abbreviations: DNA, Deoxyribose nucleic acid; **PCR,** polymerase chain reaction; **HB,** Hazratbal; **NL,** Nagin Lake; **GB,** Gagribal; **BD,** Bod Dal; **TBN,** Tailbal Nallah; **BHN,** Boathall Nallah; **DLG,** Dal Lock Gate; **PKB,** Pokhribal Nallah; **PDA,** potato dextrose agar; **MEA,** malt extract agar; **CYA,** Czepek yeast agar; **DW,** distilled water; **CZA,** Czapekdox Agar.

Table 1. Site wise colony count and percentage occurrence of *Penicillium* spp.

S. No	Name of fungi	TC									
		HB	NL	GB	BD	TBN	BHN	DLG	PKB	GT	(%) age
1.	*P. olivicolor*	2	1	1	0	3	4	2	7	20	16.40
2.	*P. commune*	1	0	1	0	1	0	1	1	5	4.09
3.	*P. chrysogenum*	1	2	3	1	10	1	2	13	33	27.04
4.	*P. funiculosum*	1	2	6	1	10	1	3	11	35	28.70
5.	*P. dimorphosporum*	1	0	2	1	2	7	2	14	29	23.77
Total		6 (4.91%)	5 (4.1%)	13 (10.7%)	3 (2.45%)	26 (21.31%)	13 (10.7%)	10 (8.2%)	46 (37.70%)	122	100

HB = Hazratbal; NL = Nageen Lake; GB = Gagribal; BD = Bod Dal; TBN = Tailbal Nallah; BHN = Boathall Nallah; DLG = Dal Lock Gate; PKB = Pokhribal Nallah; GT= Grand Total.

If determinative taxonomy of *Penicillium* is to become easier and more certain, it seems essential that hitherto neglected characteristics or new physiological tests be put to use. Ideally, such characters or tests should be stable and consistent among related strains; and procedures for their assessment should be simple and relatively rapid. Given the continued reliance on microscopy and culture, the diagnostic value of these methods must be improved by procedural changes and adequate training of laboratory personnel.

MATERIALS AND METHODS

Location and site description

The Dal Lake, located at 34° 07′ N, 74° 52′ E, 1584 m a.s.l in Srinagar, Jammu and Kashmir, India, is a multi-basined lake with Hazratbal, Bod Dal, Gagribal and Nagin as its four basins, having two main inlets as Boathall Nallah and Tailbal Nallah and two main outlets as Dal Lock Gate and Pokhribal Nallah, which were taken up for the current study. Eight sites were selected for the present study with four sites in the four basins, two sites in the two inlets and two more sites in the two outlets. The selected sites were HB (Hazratbal), NL (Nagin Lake), GB (Gagribal), BD (Bod Dal), TBN (Tailbal Nallah), BHN (Boathall Nallah), DLG (Dal Lock Gate) and PKB (Pokhribal Nallah).

Collection of water samples

The water samples were collected on seasonal basis for a period of 12 months between April 2010 to March 2011, at eight different sites of the lake in white plastic containers, which were previously sterilized with 70% alcohol and rinsed with distilled water. At the lake, the containers were rinsed thrice with the lake water before being used to collect the samples.

Isolation of fungi

Water samples obtained from different sites were serially diluted five folds and then spread plate technique was followed for isolation of *Penicillium* species in the study, spreading 0.1 ml inoculum from the serial dilution tubes on the Petri dishes containing Rose-Bengal Streptomycin Agar medium.

Inoculation and incubation

A three-point inoculation on an 80 mm Petri dish, which is an accepted standard technique for the cultivation and morphological identification of *Penicillium*, *Aspergillus* and other related genera, was followed in the present study for the morphological features of *Penicillium*. The three-point inoculation was done by using glass Petri dishes inoculated with very low quantities of conidia using glass needles. Petri dishes were incubated at different temperatures upside down for 7 days to prevent spread of conidia all over the plate and growth of the colonies.

Culture and identification

Penicillium isolates were identified in the level of genus on Potato Dextrose Agar (PDA). To improve the sensitivity and specificity of routine culture approach for identification of *Penicilli* in the level of species, we used some differential media including, Czapekdox Agar (CZ) {czapek concentration 10.0 ml, K_2HPO_4 1g, sucrose 30 g, agar 17.5 g, distilled water (DW) 1 L, Czapek Yeast Agar (CYA) (czapek concentration 10.0 ml, K_2HPO_4 1 g, powdered yeast extract 5 g, sucrose 30 g, Agar 15 g and DW 1 L) and Malt Extract Agar (MEA) {powdered malt extract 20 g, Peptone 10 g, Glucose 20 g, Agar 20 g, DW 1L. Morphological features of *Penicillium* cultures were studied, the major and remarkable macroscopic features in species identification were the colony diameter and color (conidia and reverse). We used Riddle's classic slide culture method (Riddle, 1950) for microscopic study of our isolates. Microscopic characteristics for the identification were conidia length, conidia width, conidia shape, conidia ornamentation, stipe length, stipe width, stipe ornamentation, phialide shape and branching pattern.

RESULTS

During a period of 12 months a total of122 isolates (Table 1) of *Penicillium* species were obtained from the water samples collected from Dal Lake, Kashmir with a maximum percentage (28.7%) of *Penicillium funiculosum* followed by

Table 2. Macroscopic features of different species of *Penicillium*.

Name of fungi	Macroscopic features					
	On CYA			On MEA		
	Colour of conidium	Reverse colour	Diameter (mm)	Colour of conidium	Reverse colour	Diameter (mm)
P. olivicolor	White to cream, yellow	White to cream, yellow	28	Green	White to cream, yellow	33
P. commune	White to cream, yellow	Brown	19	Green	Brown	23
P. chrysogenum	Yellow orange	Pale brown	44	Green	Yellow	50
P. funiculosum	Green	White to cream	31	Green	Brown	39
P. dimorphosporum	Green	yellow	20	White to cream	White to cream	22

Table 3. Microscopic features of different species of *Penicillium.*

Name of fungi	Microscopic feature								
	Conidia length (µm)	Conidia width (µm)	Conidia shape	Conidia ornamentation	Stipe length (µm)	Stipe width (µm)	Stipe ornamentation	Phialide shape	Branching pattern
P. olivicolor	3	2.8	Globose, Ellipsoid	Coarsely Roughened	295	3	Coarsely roughened Warted	Flask-shaped, (ampulliform, with constriction)	Bi-verticillate
P. commune	3.5	3	Ellipsoid, Pyriform	Smooth	350	3.5	Smooth	Phialide shape	Mono-verticillate,
P. chrysogenum	2.8	2.5	Subglobose	Smooth, Finely roughned	258	3.4	Smooth	Flask-shaped, (ampulliform, with constriction)	Quarte-verticillate,
P. funiculosum	2.5	2.2	Globose, Subglobose	Coarsely roughened	35	2.7	Coarsely roughened	Cylindrical	Ter-verticillate
P. dimorphosporum	3.2	2.5	Ellipsoid	Smooth	28	2	Smooth, Finely roughned	Flask-shaped, (ampulliform, with constriction)	Bi-verticillate

Penicillium chrysogenum (27.04%), *Penicillium dimorphosporum* (23.77%), *Penicillium olivicolor* (16.40%) and *Penicillium commune* (4.09%). The maximum percentage of fungal colonies (27.7%) was observed at site PKB followed by TBN (21.13%), DLG and GB (13.15%) each, NL (8.45%), BHN (7.04%), HB (5.63%) and BD (3.76%). We identified five species of *Penicillium* by using differential culture media for their growth. Besides this various macroscopic and microscopic features as shown in Tables 2 and 3, were studied for their identification. The macroscopic characteristics presented in Table 2 depict that the most common colour shown by these fungal species was white to cream and yellow. However, some more colours like green, brown and orange were also shown by the fungal isolates.

DISCUSSION

The overwhelming presence of these terrestrial moulds in water supports the paradigm that their deposition is attributable to contamination of the

water body due to the entry of sewage from the catchment areas, as they survive conventional treatment strategies and enter the distribution through the sewage coming out from the sewage treatment plants (Neimi et al., 1982).The results recorded for different species of genus *Penicillium* in the present study corroborate with that of Suhail et al. (2006) who reported that the percentage occurrence of *P. funiculosum* was maximum (28.36%) followed by *P. chrysogenum* (14.74%) and *P. notatum* (12.52%) in their study. The highest total percentage occurrence of the *Penicillium* species at site PKB followed by TBN, BHN and GB, DLG, HB, NL and BD can be attributed to the entry of sewage from the drains into the lake, as this genera has been reported frequently from the drain waters with maximum densities during higher pollution (Khulbe and Drugapal, 1994). It can therefore be inferred that these species are good indicators of pollution.

In this study 5 *Penicillium* species were identified by using the differential media, we demonstrated that the use of differential media like CYA, MEA and CZA was a simple and reliable method for identification of *Penicillium* species. Although some researches conducted on *Aspergillus* species with a similar design was reported by some studies (Curtis and Baker, 2005; Klich, 2002; McClenny, 2005), this study is the first of its kind in Kashmir that identified some *Penicillium* species from Dal Lake. A recent similar Douche study identified seven species of *Aspergillus* isolated from the water, air and surface samples (Anaissie et al., 2003). Another recent Spanish study identified *Aspergillus* spp. isolated from damp walls paper and the other surfaces (Leenders and Belkum, 1999). Our study showed that single medium PDA is only useful for identification of *Penicilli* in genus level. In this study we used morphological method with four differential culture media for identification of 5 important *Penicillium* species. A culture time of 5 days or more is generally required for identification of these fungal species. In our study using differential media like CYA and MEA with macroscopic and microscopic characteristics of fungal growth on these culture media enabled us to discriminate 5 *Penicillium* species. We recommend this sensitive and reliable method for identification of *Penicillium* species. Further studies would be helpful in clarifying the media and conditions that are most effective for the recovery and identification of *Penicilli*.

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