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Identification of Pakistani Isolates of *Xanthomonas oryzae* pv. *oryzae* by Insertion Sequence based polymerase chain reaction (IS-PCR)

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Abstract
Xanthomonas oryzae pv. oryzae is the causal agent of bacterial blight disease in rice. The pathogen was isolated from infected samples collected after extensive survey of rice growing areas of Pakistan. Afterwards, Insertion sequence based Polymerase chain reaction was applied using the high copy insertion sequence IS1113 based primer for molecular identification of the isolates as well as to differentiate them from *Xanthomonas oryzae pv. Oryzicola*

Keywords: Xanthomonas oryzae pv. Oryzae, Pakistan, IS-PCR

Bacterial blight disease is one of the most destructive diseases of rice in Asia (Mew, 1987). It is said to have been first observed by farmers in Fukuoka prefecture, Kyushu Island, Japan, in 1884-1885 (Tagami and Mizukami, 1962). In Pakistan the disease was first recorded in 1977. Its occurrence was confirmed from all the provinces in a later study by Akhtar and Akram in 1987. It has been observed during recent years that BLB incidence is increasing in Pakistan especially in "Kaller" belt which is famous for rice cultivation (Khan *et al.,* 2000). In order to isolate and characterize Pakistani strains of *Xanthomonas oryzae* pv. *oryzae* which is the pathogen causing the disease, surveys of all rice growing areas of Pakistan were conducted during the years 2010 to 2011. Infected leaf samples with symptoms of bacterial blight disease were collected and bacteria were isolated from them after surface sterilization. One hundred and twenty three bacterial colonies with typical characteristics of *X. oryzae* pv. *oryzae* were picked and purified. However, *X. oryzae* pv. *oryzae* cannot be easily differentiated by colony characteristics from other pathovars specially *Xanthomonas oryzae* pv. *oryzicola*. In order to confirm the bacterial isolates to be *X. oryzae* pv. *oryzae,* polymerase chain reaction was carried out using the high-copy insertion element IS1113 based primer J1. The primer J1 [5′-CGAGTCCAGTCCAGCGGACC-3′, Tm 63.6°C] (Gene Link) was designed by Adhikari and his coworkers in 1999 (Adhikari *et al.,* 1999). The primer J1 (5′-CGAGTCCAGTCCAGCGGACC-3′) had been previously reported for identification of *X. oryzae* pv. *oryzae* by amplifying intergenic regions that have specificity. The outwardly directed primers from the sequence amplified intergenic regions that exhibited some specificity.

For these studies saturated cultures of all bacterial isolates were prepared in Luria broth medium. DNA was isolated from bacterial isolates using specific bacterial extraction kit (Bactozol™ Kit, Cat. No: BA 154, Molecular Research Center). DNA pellet was dissolved in 200µl of TE buffer. DNA of each isolate was quantified using UV spectrophotometer at 260 nm and concentrations were made to about 100 ng μl^{-1}. The primer J1 was synthesized by Gene Link. The reaction mixture contained in a total volume of 25 µl, PCR buffer [180mM Tris-HCl (pH 8.8), 50mM $(NH_4)_2 SO_4$], 2.5 mM $MgCl_2$, 160µM of deoxy nucleoside triphosphate, 50 nmol of primer and 0.5U Taq polymerase. The reaction cycle comprised initial denaturation at 95°C for 7 min, annealing at 55°C for 1 min, extension at 72°C for 3 min and was followed by 35 cycles of 1 min at 94°C, 1 min at 57°C and 1:30 min at 72°C and final extension for 7 min at 72°C followed by incubation at 4°C.

PCR products obtained with J1 primer were separated by electrophoresis on 1.0 % agarose gel containing 2 µl of ethedium bromide (10mg/ml) in 1X Tris-borate EDTA buffer. 10µl of PCR products were mixed with 2µl of 6X loading dye solution. 1kb DNA ladder was run as marker along the PCR products. Samples in the gel were electrophoresed at 100 V until the bromophenol blue had migrated approximately two third of the length of the gel. The DNA fragments in the gel were visualized by ultraviolet illuminator.

It was observed that 105 bacterial isolates produced an intensely stained 500 bp amplification band and very weak bands at ~340bp, ~430 bp, ~800bp, ~1kb and ~1.2kb. This confirmed them to be *Xanthomonas oryzae* pv. *oryzae.* Amplification patterns of representative strains of *X.oryzae* pv. *oryzae* from Punjab , North West Frontier Province (NWFP) and Sindh province are shown in figure (1). However 13 bacterial isolates produced intense bands at ~430 bp and ~340 bp and faint bands at 500bp, ~800 bp, ~1kb, 1.2kb, ~2kb and ~2.8kb. This confirmed them to be *Xanthomonas oryzae.* pv. *oryzicola.* While 5 isolates produced banding patterns different from *X.oryzae* pv *oryzae* and *X.oryzae* pv. *oryzicola* figure (2). The protocol was found to be easy and sensitive. It rapidly detected and identified the strains of *X. oryzae* pv. *oryzae* and differentiated them from other pathovars. The 105 isolates of *X. oryzae* pv. *oryzae* were preserved for further studies. IS-PCR protocol was found to be a reliable molecular tool in the diagnosis of bacterial blight pathogen in rice during these studies.

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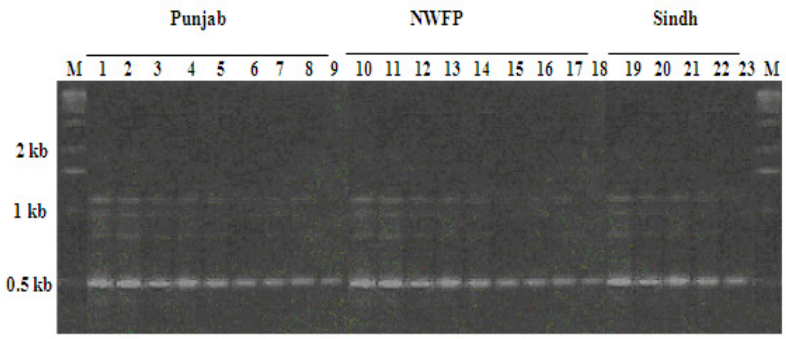

Fig 1. Polymerase chain reaction products patterns obtained by J1 primer with strains of *X. oryzae* pv. *oryzae* isolated from different agroecological rice zones of Pakistan including Punjab, NWFP and Sindh province.

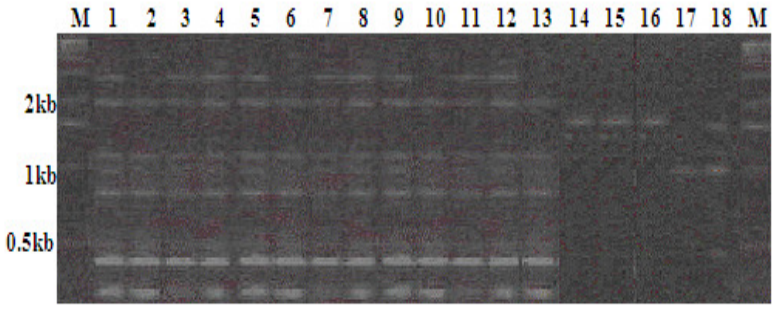

Fig 2. Polymerase chain reaction products patterns obtained by J1 primer with strains of *X. oryzae* pv. *oryzicola* (1 to 13) and other Xanthomonas strains (14 to 18) isolated from different agroecological rice zones of Pakistan

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