23 Comparison of Serological and Molecular Methods for Detection of *Xanthomonas oryzae* pv. *oryzae* in Rice Seed

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INTRODUCTION

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae*, is a serious disease in rice-growing areas worldwide. The disease is seedborne and hence poses a problem to international seed trade. However, the extent to which the pathogen, once it becomes established in seed, survives, is transmitted and subsequently infects the next generation of emerging rice seedlings is questioned (Mew *et al.*, 1989). The significance of the seedborne phase is difficult to assess because detection of *X. oryzae* pv. *oryzae* in infested rice seed is masked by numerous faster growing rice seed contaminants.

Improved methodology is needed for detection and identification of *X. oryzae* pv. *oryzae* in rice seed. Serological and DNA-based methods have been developed for detection of many seedborne bacterial pathogens including *X. oryzae* pv. *oryzae*. Serological methods include the enzyme-linked immunosorbent assay (ELISA) using monoclonal and/or polyclonal antibodies, miniplate enrichment/ELISA, immunofluorescence and the immunofluorescence colony-staining technique (IFC) (Benedict *et al.*, 1989; Quimio, 1989; Rehman, 1995; Van Vuurde and Van der Wolf, 1995). Current studies focused on IFC to increase the resolution and enhance the sensitivity of previous immunofluorescence detection methods.

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DEVELOPMENT OF IFC TECHNIQUE FOR *X. ORYZAE* PV. *ORYZAE*

The basic IFC technique (Van Vuurde, 1987; Van Vuurde and Van der Wolf, 1995) was modified to detect and identify *X. oryzae* pv. *oryzae*. Species- and pathovar-specific monoclonal antibodies (mAbs) were generated and characterized using 178 strains assembled from a worldwide collection (Benedict *et al.*, 1989). Most strains reacted with mAb Xco-1 (clone 139-159, IgMκ) but this mAb gave weak immunofluorescence by IFC. In contrast, mAb Xco-2 (clone 138-68, IgG3κ) gave bright immunofluorescence, but it did not react with all strains (Benedict *et al.*, 1989). Additional mAbs were generated to identify nonreactive strains. mAbs of subclass IgG were selected because they diffused through agar and produced brighter colonies than IgM antibodies (Rehman, 1995). A new mAb (Xoo-7, clone 211-G4, IgG3κ) was particularly useful because it reacted with numerous strains from Nepal and some from India that did not react with mAb Xco-2. mAbs Xco-2 and Xoo-7 reacted with 85% and 12%, respectively, of 268 typical strains of *X. oryzae* pv. *oryzae*. The strains that reacted with mAb Xco-2 did not react with mAb Xoo-7 and vice versa. Only eight of the 268 typical strains failed to react with either of these mAbs. A mixture of mAbs Xco-2 and Xoo-7 reacted with 97% of the *X. oryzae* pv. *oryzae* strains that induced typical bacterial blight symptoms in pathogenicity tests.

EVALUATION OF SEMI-SELECTIVE MEDIA

Successful application of IFC depends on rapid growth of target colonies during the enrichment step. Thus, five semi-selective media were evaluated with representative *X. oryzae* pv. *oryzae* cultures from a worldwide collection (Gnanamanickam *et al.*, 1994). Of these, XOS medium, developed by Di *et al.* (1991), was found to be the most suitable. However, typical strains of *X. oryzae* pv. *oryzae* required 6–8 days before colonies could be enumerated under 40× magnification on agar plates. A few strains formed colonies in 5 days, but only atypical fast growing (and often avirulent) strains produced colonies in 3 days. When phosphate was removed from XOS medium, distinct colonies were visible under 40× magnification in 3–4 days and fast-growing strains were visible in 2 days. The efficiency of plating on the modified medium (medium E) was not statistically different from the efficiency on XOS medium. Thus, because of the more rapid colony development, medium E was used for IFC studies.

DETECTION OF *X. ORYZAE* PV. *ORYZAE* IN EXTRACTS OF ARTIFICIALLY INFESTED RICE SEED

Sterile (autoclaved and dried) rice seeds were artificially infested by soaking individual batches of 300 seeds in a 5 ml suspension of *X. oryzae* pv. *oryzae* strain PXO86 (approximately 2×10^8 cfu (colony-forming units) ml^{-1}). Seeds were dried overnight in a laminar flow hood then stored in a dessicator at 4°C. The artificially infested seed was mixed with viable healthy seed at rates of 1, 5, 10 and 100%. Subsamples of 100 infested seed were placed in 200 ml plastic beakers containing 5 ml sterile saline and crushed with a pestle for 3 min. The mixture was incubated for 2 h on a rotary shaker adjusted to 200 rpm. The extract was decanted from the seed and centrifuged at 12,350 *g* for 10 min. The pellet was resuspended in 1 ml saline, ten-fold dilutions were made and 100 µl was added to pour plates. For pour plating, medium E with 1.2% molten agar was cooled to approximately 37°C. A 100 µl aliquot of the appropriate dilutions of the seed extract was placed in the center of a 90 mm sterile Petri plate, and approximately 12 ml of medium E was added per plate. Plates were immediately swirled to spread the suspensions prior to solidification of the agar. Plates were incubated at 28°C and cfu were recorded from day 1 to day 7. The numbers of rice seed contaminants were determined by plating noninfested seed extracts separately on medium E. Dilutions of a pure culture of PXO86 were plated separately on medium E; each sample had four replicates. When pinpoint colonies of *X. oryzae* pv. *oryzae* became visible in medium E under 40× magnification, agar squares (approximately 1 cm^2) were cut from the plate, placed in a microplate (12 wells per plate) and stained with fluorescein isothiocyanate (FITC)-conjugated mAb Xco-2 (1 : 50 dilution) for 12 h. In subsequent assays a mixture of mAb Xco-2 and mAb Xoo-7 (separately conjugated to FITC) were mixed 1 : 1 prior to staining. Colonies showing bright immunofluorescence were lifted with a needle and restreaked on to yeast dextrose calcium carbonate medium (YDC) containing 10 g yeast extract, 20 g dextrose, 20 g $CaCO_3$ and 13 g agar per litre. Colonies were compared with colonies of pure cultures of PXO86, then tested by direct immunofluorescence, and inoculated into leaves of rice cultivar IR-20 for pathogenicity tests using the scissor-clipping method (Kaufman *et al.*, 1973).

Using the IFC technique, pure cultures of *X. oryzae* pv. *oryzae* were detected in approximately 30% of the samples plated at the 10^{-7} dilution (1 cfu per 100 µl sample) and in all samples plated at the 10^{-6} dilution (10 cfu per 100 µl sample). Fluorescent colonies were detected in all replications when healthy seed lots were mixed with infested seed at 1% or greater (Table 23.1). At least one or more colonies showed bright fluorescence even when contaminants outnumbered fluorescent colonies by 37:1. In a repeated experiment, fluorescent colonies were detected in all of five replicate samples with 1% infested rice seeds (as well as all samples with 5% and 100% infested seed). No

Table 23.1. Detection of *Xanthomonas oryzae* pv. *oryzae* (strain PXO86) on medium E (pour plate) from infested rice seed.

Sample no.	Infestation (%)[a]	Cfu ml^{-1}[b]		Reaction in IFC[d]
		Contaminants	*X. oryzae* pv. *oryzae*[c]	
1	0	2.6×10^5	none	–
2	1	3.0×10^5	8.0×10^3	+
3	5	3.3×10^5	2.0×10^4	+
4	10	2.7×10^5	7.4×10^4	+
5	100	none	5.1×10^5	+

[a]Infested seeds per 100 seeds.
[b]Calculated from observed cfu in dilutions of samples 1–5.
[c]Presumptive *Xanthomonas oryzae* pv. *oryzae* based on colony morphology after 3 days' growth.
[d]The immunofluorescence colony staining technique was used to identify *Xanthomonas oryzae* pv. *oryzae* from dried agar pieces (approximately 1 cm^2). A positive reaction is defined as one or more colonies showing bright immunofluorescence.

fluorescent colonies were detected in control samples. Representative fluorescent colonies that were restreaked on to YDC medium appeared identical to colonies of PXO86. They gave bright immunofluorescence when reacted with FITC-conjugated mAb Xco-2 but not mAb Xoo-7. Pathogenicity tests on rice cultivar IR-20 were positive.

Among rice seed contaminants, several species of bacteria that form yellow colonies can be confused with *X. oryzae* pv. *oryzae*. One is a Gram-negative, oxidative bacterium identified by Biolog Microstation System™ as *Pseudomonas paucimobilis*. Another (also Gram-negative, oxidative) resembled *X. campestris* in culture and was identified by Biolog as *X. campestris* pv. *strelitizia*. Although these *Xanthomonas*-like yellow bacteria had other bacteriological characteristics of *X. campestris*, they were nonpathogenic on *Strelitizia reginae* as well as rice. Similar yellow-pigmented, Gram-negative, oxidative bacteria have been reported previously in association with rice seed (Benedict *et al.*, 1989; Di *et al.*, 1991; Gnanamanickam *et al.*, 1994).

ASSESSMENT OF THE IFC TECHNIQUE

The advantages of the IFC technique over previously applied serological methods (ELISA and immunofluorescence) lie in its greater sensitivity (Van Vuurde and Van der Wolf, 1995; Van Vuurde *et al.*, 1995). With IFC lower limits of detection are 1–10 cfu per 100 µl sample (10–100 cfu ml^{-1}) whereas with direct ELISA, strong signals are observed only when bacterial suspensions

exceed 5×10^5 cfu (Alvarez and Lou, 1985; Gnanamanickam *et al.*, 1994). Microcolonies observed with IFC are readily detected in a microscope field; thus, larger sample numbers can be handled with relative ease. Moreover, living and dead cells are clearly distinguished because dead cells do not develop into fluorescing microcolonies. Thus, even if stained with the antibody, single cells are not visible in the range of observation (40–200×).

MOLECULAR METHODS FOR DETECTION AND IDENTIFICATION OF *X. ORYZAE* PV. *ORYZAE*

Molecular methods have been useful for identifying *X. oryzae* pv. *oryzae*, analysing natural populations in the Philippines and comparing them with populations worldwide (Leach *et al.*, 1990, 1995). Recently, Vera Cruz *et al.* (1995, 1996) compared Rep-PCR with RFLPs produced by hybridization with IS*1112*, an insertion element isolated from *X. oryzae* pv. *oryzae*. The genetic groups detected by Rep-PCR were consistent with those found by RFLP analysis. Rep-PCR was selected as the method of choice for further population studies because it is less expensive and simpler to perform. An assay called IS-PCR was then developed using outwardly directed primers that amplify DNA between the endogenous *Xoo* insertion elements, IS*1112* and IS*1113*. In an analysis of 300 different strains of *X. oryzae* pv. *oryzae* collected from Nepal and other rice-growing countries around the world, polymorphisms related to geographical origin of the strains were detected and hence were useful in analysis of population structure (Adhikari and Leach, unpublished data; Vera Cruz and Leach, unpublished data). J1, a single outwardly directed primer based on the sequence of IS*1112*, distinguished *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola*. Primers N1 and N2, outwardly directed from IS*1113*, generated patterns that distinguished *X. oryzae* pathovars from other pathovars of *X. campestris*. Thus patterns generated by IS-PCR are also useful for diagnostic purposes if two sets of primers are used in combination (Adhikari, Vera Cruz and Leach, unpublished data).

A PCR assay using primers which amplified an internal fragment of the *Xoo* element IS*1112* was developed by Cottyn *et al.* (1994) to detect a single band of *X. oryzae* pv. *oryzae* DNA. This assay showed good potential for detecting *X. oryzae* pv. *oryzae* in pure culture and is presently being tested for rice seed assays (Mew, personal communication). A similar assay, based on the amplification of an internal fragment of the element IS*1113*, was developed by Sakthivel, Nelson and Leach (unpublished data). IS*1113* hybridized to over 1000 strains of *X. oryzae* pv. *oryzae* from a worldwide collection, and hybridized to only a few other xanthomonads; thus, it was thought that primers based on this element might be more specific to *X. oryzae* pv. *oryzae*. After screening several combinations of primers, TXT (5′-GTCAAGCCAACTGTGTA-3′) and

TXT4R (5'-CGTTCGCGCCACAGTTG-3') were selected (Sakthivel, unpublished data). These primers consistently amplified a 964 bp fragment from *X. oryzae* pv. *oryzae* but not from *X. oryzae* pv. *oryzicola* or pathovars of *X. campestris*, *Pseudomonas* spp. or saprophytic bacteria (Sakthivel and Leach, unpublished data). It should be noted that these primers were 17-mers that had been used for sequencing. We did not try 20-mers which would have increased the specificity and stability of binding.

The PCR assay used in current studies was based on the method of Sakthivel and Leach using primers that amplify a portion of IS*1113*. The reaction mixture (total 25 µl) contained 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 25 µM KCl, 200 µM each of four dNTPs, 0.1 µM forward primer (TXT), 0.1 µM reverse primer (TX4R), 2.5 U *Taq* polymerase and 20 ng template DNA or 2.0 µl test sample. The program was 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 54°C and 2 min at 72°C. PCR products were resolved by electrophoresis in 1.5% agarose gels at 50 V for 2 h using standard procedures.

SENSITIVITY OF PCR USING TXT/TX4R PRIMERS

A ten-fold dilution series of a suspension of strain PXO86 containing 3.5×10^8 cfu ml^{-1} was plated on CPG agar (1.0 g casein, 10.0 g peptone, 10 g glucose, 16 g agar) and cell counts were made after 5 days' growth. One millilitre aliquots of the whole cell preparations were placed into a 90°C water bath for 2 min, and PCR was performed using 2 µl subsamples per PCR reaction (total 25 µl). A clear band formed with dilutions of 10^{-4} whereas only a faint band formed at the 10^{-5} dilution. Based on the dilution series of live *X. oryzae* pv. *oryzae* cells, this dilution contained 35 cfu per 2 µl subsample or 1.6×10^4 cfu ml^{-1}.

Sensitivity was also determined by comparing bands formed by the PCR product from dilutions of DNA (20 ng μl^{-1}) prepared from PXO86. The 10^{-5} dilution of DNA contained approximately 200 fg DNA. Based on the assumption that the genome size of *X. oryzae* pv. *oryzae* is approximately equivalent to the genome size of *Pseudomonas fluorescens* (7.4×10^3 kb) (Bak *et al.*, 1970) the 200 fg DNA represented approximately 27 cfu per 2 µl added to the PCR reaction mix or 1.3×10^4 cfu ml^{-1} in the original sample. Sakthivel reported that the TXT and TX4R primers detected as little as 55 fg (unpublished data).

An attempt was made to monitor the spread of *X. oryzae* pv. *oryzae* strain PXO86 in rice plants and extracts of seeds harvested from infected plants. Results were inconclusive because bacterial DNA was not detected in inoculated rice leaves or in seed extracts. In preliminary experiments using rice plants inoculated with strain PXO99 Sakthivel (unpublished data) detected bacterial DNA in leaves above inoculated leaves collected at 15, 30

and 45 days after inoculation but he did not detect bacterial DNA in seeds harvested from infected plants. Likewise, no bacterial DNA was detected in seedlings germinated from 200 seeds collected from the infected plants. In a preliminary report of these data it was concluded that although the PCR assay can be used to detect the pathogen in infected leaves and stems, more studies are needed to determine whether or not *X. oryzae* pv. *oryzae* is transmitted through the vascular tissue into developing seed (Gnanamanickam *et al.*, 1994).

COMPARISONS BETWEEN THE IFC AND PCR ASSAYS

In the described protocols, the limits of detection of *X. oryzae* pv. *oryzae* are lower with the IFC assay (10 cfu ml^{-1}) than for the PCR assay (1.3×10^4 cfu ml^{-1}). The greater sensitivity of the IFC assay is due primarily to the larger sample size (100 µl) examined per replicate. Given the ease of plating additional replicate samples, the sample size for IFC can be increased even further. Since the sensitivity of the assay is dependent upon the total volume of sample plated, plating ten replicate samples would easily lower the sensitivity to 1 cfu ml^{-1}. With the added advantage that the identity of a fluorescing colony can be confirmed by reisolation and a pathogenicity test, the IFC test is very reliable. Nevertheless, the 2–4 days needed for development of microcolonies prior to staining them with antibody conjugates is a distinct disadvantage of the IFC method. In contrast, the PCR assay can be completed within 1 day. Additional modifications of the DNA-based protocol, including extraction and condensation of target DNA from larger sample volumes, would improve the sensitivity of the PCR assay. On the other hand, use of an immunocapture assay using a mixture of mAbs Xco-2 and Xoo-7 on the solid phase followed by a washing step and PCR would result in a sensitive assay that could be performed in 1 day. The presence of viable cells could be demonstrated later by plating a subsample of the immunocaptured *X. oryzae* pv. *oryzae* cells. Subsequent pathogenicity tests would confirm the identification.

In the case of bacterial blight of rice additional research is clearly needed before the significance of seed transmission is established. Nevertheless, tools are available to examine this question with precision. Extensive field studies and analysis of population structure of *X. oryzae* pv. *oryzae* have formed an essential foundation for detection methods and have revealed polymorphism among strains isolated from representative geographical areas of rice growing regions throughout Asia (Leach *et al.*, 1990, 1992, 1995; Nelson *et al.*, 1994; Vera Cruz *et al.*, 1995, 1996; Ardales *et al.*, 1996). These detailed characterizations of the *X. oryzae* pv. *oryzae* populations and the numerous cultures available for testing are invaluable for validating the DNA-based methods used for detection of this pathogen.

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