*Journal of Applied Microbiology* 1999, **87**, 711–717

# An approach to obtain specific polyclonal antisera to *Xanthomonas campestris* pv. cyamopsidis and its potential application in indexing of infected seeds of guar

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7163/04/99: received 7 April 1999, revised 23 June 1999 and accepted 5 July 1999

G.K. VIJAYANAND, M.D. SHYLAJA, M. KRISHNAPPA AND H.S. SHETTY. 1999. Clusterbean seed health testing is warranted since the pathogen (*Xanthomonas campestris* pv. *cyamopsidis* (Xccy)) is seed-borne and seed-transmitted. A polyclonal antibody was developed in rabbit *via* subcutaneous and intramuscular injections and characterized for sensitivity, specificity and its applicability to ELISA which: (i) was sensitive in detecting as few as $10^2$ cells $ml^{-1}$ at a titre of 1: 4000; (ii) was specific, since it reacted only with Xccy and not with other xanthomonads; (iii) reacted both with Xccy cells and culture filtrate, indicating that the antigenic determinant is a secretory component; (iv) was applicable and reliable in seed health testing since it reacted only with infected seeds and plant materials and not with healthy seeds and (v) a purified fraction of antibody was virulent-specific since heat-denatured and avirulent isolates were not detected. The ELISA thus developed is highly reproducible and therefore suitable for the evaluation of the potential disease status of seeds and plant health, which is appropriate for routine seed health testing.

## INTRODUCTION

Bacterial blight of clusterbean or guar incited by *Xanthomonas campestris* pv. *cyamopsidis*. Dye (Xccy) is a devastating disease of guar (Mali *et al.* 1989). Yield loss of up to 68% has been reported in India (Gandhi and Chand 1985). The monsoon season enhances the intensity of the disease (Karwasra *et al.* 1985). The disease is systemic and the pathogen is seed-borne and located in the seed coat (Srivatsava and Rao 1963; Karwasra *et al.* 1983; Parashar and Sharma 1984). There is a lack of a sensitive method for the detection of the pathogen. Over the years, identification has been carried out by detection techniques such as the grow-out test, direct seed plating and liquid assay (Schaad 1980; Parashar and Sharma 1984; Franken and Van Vuurde 1990). These are extremely labour-intensive and time-consuming. Laboratory experiments have the decided advantage of being faster, easier to perform, less expensive and more sensitive (Schaad 1982). Polyclonal antibodies (PAb) and monoclonal antibodies have been developed for *X. campestris* pv. *citri* (Civerolo and Fan 1992), *X. campestris* pv. *undulosa* (Duveiller and Bragard 1992), *X. campestris* pv. *phaseoli* (Malin *et al.* 1985), *X. campestris* pv. *holicola* (Leach *et al.* 1987) and *X. campestris* pv. *campestris* (Thaveechai and Schaad 1984, 1986; Alvarez *et al.* 1985). However, there has been no report on such sensitive serological assays for Xccy. The present investigation was thus aimed at the production of PAb-Xccy and the development of an ELISA protocol for the easy, rapid, sensitive and specific detection of Xccy in plant and seed materials. The study also describes the purification and characterization of PAb-Xccy to develop an ELISA for virulent and avirulent isolates of Xccy. The distinction between possible virulent and avirulent strains, based on variations in antigenic determinants in different isolates, has been described.

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## MATERIALS AND METHODS

### Antigen preparation

The bacterium was isolated from severely infected clusterbean seeds by incubating the surface-sterilized seed samples

on nutrient agar (NA) plates for 48–72 h at 27 °C. Individual colonies appearing yellow and mucoid surrounding the seeds were isolated and repeatedly subcultured for pure colonies and subjected to confirmatory tests such as pathogenicity, hypersensitivity and biochemical tests. The Xccy was cultured on nutrient broth under static conditions for 48 h at 27 °C and harvested by centrifugation at 3000 rev $min^{-1}$ for 10 min; cells were suspended in 20 mmol $l^{-1}$ phosphate-buffered saline (PBS; pH 7·4) and these intact whole cells were washed three times in the same buffer and used as antigen for immunization and for the ELISA.

### Antiserum production

The antibody was raised in a 3-month-old female albino rabbit. Cells ($10^5$ $ml^{-1}$) were mixed, in a proportion of 1 : 1, with Freund's incomplete adjuvant (Robinson *et al.* 1995). Cells ($1 \times 10^4$ $ml^{-1}$) constituting approximately 100 $\mu$g protein were then injected at multiple sites subcutaneously. Prior to immunization, preimmune serum was collected from the same rabbit. Three such injections were given at intermittent intervals of 1 week by increasing the concentration of immunogen (125–150 $\mu$g protein). A booster dose was given at the fourth week and the rabbit was test bled 4 d after the booster dose. The presence of antibody was examined by ELISA as described below, using $10^2$ and $10^5$ cells $ml^{-1}$. The rabbit was further boosted twice up to 6 weeks in a similar way. Since PAb-Xccy with convincing sensitivity was not obtained, antigen at 125 $\mu$g was injected intramuscularly at three sites. A booster injection was given after 1 week and test antisera collected 4 d after injection. After examining the antibody, an additional booster injection was administered, blood collected and allowed to clot at room temperature for 30 min followed by 2–3 h at 4 °C. The separated serum was centrifuged at 1500 rev $min^{-1}$ for 15 min and stored at 4 °C.

### ELISA protocol

The ELISA was carried out in 96-well microtitre plates (Nunc, Denmark) as described previously (Clark and Adams 1977; Hobbs *et al.* 1987; Rajeshwari *et al.* 1998). Results were quantified by measuring the absorbance at $A_{410\,nm}$ using a microtitre plate reader (MR 5000; Dynatech).

### Characterization of PAb-Xccy

*Determination of sensitivity and antibody titre.* Antigen (10–50 $\mu$l) at $10^2$ and $10^5$ cells $ml^{-1}$ was loaded onto the ELISA plate in different rows and tested with 100 $\mu$l developed antibody at various dilutions, 1 : 500, 1 : 1000, 1 : 2000 and 1 : 4000 $well^{-1}$. The ELISA reactivity was examined and the maximum dilution showing significant reactivity was considered as the antibody titre.

*Evaluation of specificity.* Various xanthomonads, such as *X. campestris* pv. *vesicatoria* (Xcv), *X. campestris* pv. *malvacearum* (Xcm), *X. oryzae* pv. *oryzae* (Xoo) and *X. campestris* pv. *oryzicola* (Xco), were tested for cross-reactivity at $10^4$ and $10^2$ cells $ml^{-1}$ with different doses of antigen (10–50 $\mu$l $well^{-1}$). Culture filtrates (100 $\mu$l) of all these organisms at equivalent cell concentration of $10^8$ cells $ml^{-1}$ were also examined by ELISA.

### Validity of pAb-Xccy in detection of the pathogen in seed samples and infected plant materials

*Xanthomonas campestris* pv. *cyamopsidis*-infected clusterbean plants were collected. The brown, streaky stems were dipped in 10 ml sterile PBS for bacterial ooze. Suspected and infected seeds were soaked in sterile saline overnight at 4 °C and crushed in sterile PBS. The supernatant fluid obtained after centrifugation at 1500 rev $min^{-1}$ for 15 min at 4 °C was aliquoted. Aliquots were examined for the total number of colony-forming units (cfu) by plate assay and also for ELISA reactivity.

### Determination of the antigenic determinant by immunoblot assay

As Xccy was positive and specific in ELISA reactivity, an immunoblot assay was performed to identify the antigenic determinant molecule in the pathogen. An equal number of bacterial cells of both the isolates (1 and 2) was loaded in duplicate onto 12% SDS-PAGE in a mini-gel apparatus (Genei Pvt, Bangalore, India). After electrophoresis, the first half with the marker proteins was silver stained for visualization of the protein bands and the second half of the gel was transferred to a nitrocellulose membrane. The nitrocellulose paper was probed with 1 : 500 PAb-Xccy followed by 1 : 1000 alkaline phosphatase-conjugated swine antirabbit IgG. 5-Bromo-4-chloro-3-indolyl phosphate was used as the chromogenic substrate which, on alkaline phosphatase activity, yielded a reduced, stable purple-coloured complex with a band which reacted specifically with PAb-Xccy (Kotani and Mc Garrity 1985).

### Purification of PAb-Xccy by Sephacryl-200-HR column chromatography

The antiserum was subjected to 50% (w/v) ammonium sulphate precipitation and loaded onto a degassed column packed with Sephacryl-200-HR (65 × 1·0 cm); 1-ml fractions were collected at a flow rate of 18 ml $h^{-1}$ and fractions monitored at $A_{280\,nm}$ spectrophotometrically (U-2000; Shimadzu, Japan). Subsequent peak fractions were pooled as peak 1, $P_1$Ab-Xccy, and peak 2, $P_2$Ab-Xccy, and examined for antibody reactivity by ELISA. After lyophilization, they were evaluated for anti-

body titre, sensitivity and detection of cells as well as culture filtrates. Crude antibody was also included in the experiment. Appropriate controls, such as detection with preimmune serum and irrelevant xanthomonads, were prepared. The two peaks showed differential reactivity with virulent and avirulent isolates in ELISA.

### Effect of heat treatment on PAb-Xccy reactivity and virulence

An equal number of cell suspensions ($10^8$ cells $ml^{-1}$) of virulent and avirulent isolates was subjected to treatment at 56 °C for 10 min. Treated cells were examined for $P_1$Ab-Xccy and $P_2$Ab-Xccy reactivity. The results were compared with crude antiserum PAb-Xccy.

## RESULTS

### A potent polyclonal antibody PAb-Xccy: implications for routes of immunization

A PAb against Xccy was obtained after subcutaneous injection. However, it was not sensitive, since antibody could detect only high cell numbers such as $10^5$–$10^8$ $ml^{-1}$ at 1 : 2000 dilution of antibody. Subsequent subcutaneous routes of injection did not improve the sensitivity. However, upon changing the route of injection to intramuscular, the antibody sensitivity and antibody titre were improved (Fig. 1).

### Polyclonal antibody-Xccy is specific to Xccy and does not cross-react with other xanthomonads

Of the plant-pathogenic xanthomonads, such as Xccy, Xcv, Xcm, Xoo and Xco, only Xccy showed dose-dependent reactivity at concentrations of $10^2$ cells $ml^{-1}$; as did its culture filtrate. Higher activity has been found with Xccy relative to Xcm, which showed a negligible cross-reactivity of less than 8%. Other xanthomonads, such as Xoo and Xcv, did not show ELISA reactivity at $10^4$ as well as $10^2$ cells $ml^{-1}$. The culture filtrate of these xanthomonads also showed no reactivity, while significant reactivity was observed for Xccy (Fig. 2).

### Validity of PAb-Xccy reactivity in the diagnostic assay of infected seeds

Different degrees of infected seeds were examined for the level of pathogen on NA plates as cfu and by ELISA reactivity using PAb-Xccy. A correlation was made between PAb-Xccy reactivity and cfu. Infected plant materials with typical symptoms also showed reactivity and the level of reactivity correlated with pathogen levels in the plate assay. Healthy leaves and seeds expressed neither colonies on the plates nor ELISA reactivity (Table 1).

### The antigenic determinant of PAb-Xccy is a virulent-specific protein

Immunoblotting analysis of virulent and avirulent isolates by PAb-Xccy indicated the presence of a predominant band of

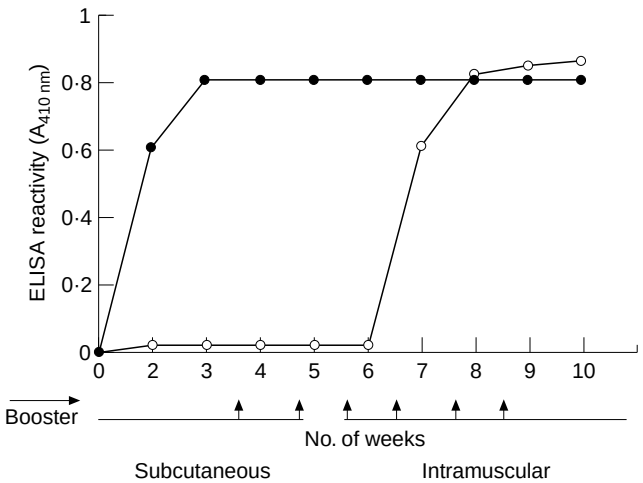


**Fig. 1** Whole *Xanthomonas campestris* pv. *cyamopsidis* cells were injected at multiple sites subcutaneously. After the booster dose, the presence of antibody was examined at 50 $\mu$l $well^{-1}$ of $10^5$ cells $ml^{-1}$ (●) and $10^2$ cells $ml^{-1}$ (○). After 5 weeks, the intramuscular route was selected and examined for antibody reactivity

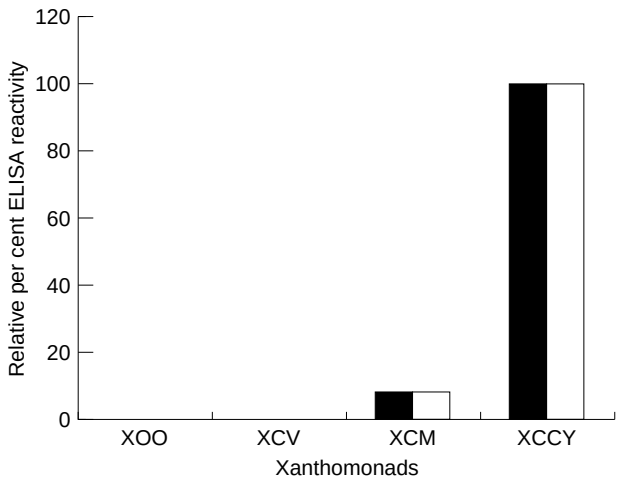


**Fig. 2** Polyclonal antibody-*Xanthomonas campestris* pv. *cyamopsidis* (Xccy) was examined for cross-reactivity with other xanthomonads. $10^2$ cells $ml^{-1}$ (■) and an equivalent culture filtrate (□) were evaluated. Maximum activity was considered as 100% and the relative percentage reactivity is expressed. xoo, *X. oryzae* pv. *oryzae*; xcv, *X. campestris* pv. *vesicatoria*; xcm, *X. campestris* pv. *malvacearum*

**Table 1** Clusterbean seed samples with a varied degree of infection were examined by ELISA as well as by a plating assay on nutrient agar medium

| SI no. | Seed variety | ELISA reactivity | Relative percentage of ELISA reactivity | cfu | Relative percentage of cfu | Conclusions |
|---|---|---|---|---|---|---|
| 1 | PNB 1 | 0 | 0 | 0 | 0 | Healthy |
| 2 | PNB 2 | 0 | 0 | 0 | 0 | |
| 3 | HG 182 | 0 | 0 | 0 | 0 | |
| 4 | FS 277 | 0 | 0 | 0 | 0 | |
| 5 | LOCAL 1 | 0 | 0 | 0 | 0 | |
| 6 | HG 75 | 0·125 | 43·70 | 125 | 20·12 | Infected |
| 7 | PNB 3 | 0·138 | 48·25 | 257 | 41·28 | |
| 8 | PNB 4 | 0·180 | 62·93 | 276 | 44·44 | |
| 9 | LOCAL 2 | 0·246 | 86·01 | 343 | 55·13 | |
| 10 | CP 42 | 0·286 | 100 | 361 | 58·13 | |

cfu formed for each sample were recorded and compared with ELISA reactivity. Correlation was made between ELISA reactivity and the number of cfu.

50 kDa in virulent isolates; this predominant band was absent in avirulent isolates. In contrast, a small-sized minor component of 30 kDa was observed by PAb Xccy only in avirulent and not in virulent isolates (Fig. 3).

### Purification and differential reactivity of fractions of PAb-Xccy

Sephacryl-200-HR column chromatography of PAb-Xccy resulted in fractionation into two peaks, $P_1$Ab-Xccy and $P_2$Ab-Xccy (Fig. 4). They reacted differentially with different isolates. $P_1$Ab-Xccy, at 1 : 4000 dilution, reacted specifically with virulent Xccy cells and not with avirulent cells at doses of $10^2$ cells $ml^{-1}$ (Fig. 5). An increase in concentration of the avirulent cells to $10^4$ cells $ml^{-1}$ also did not show changes in reactivity. However, both crude and $P_2$Ab-Xccy did not show

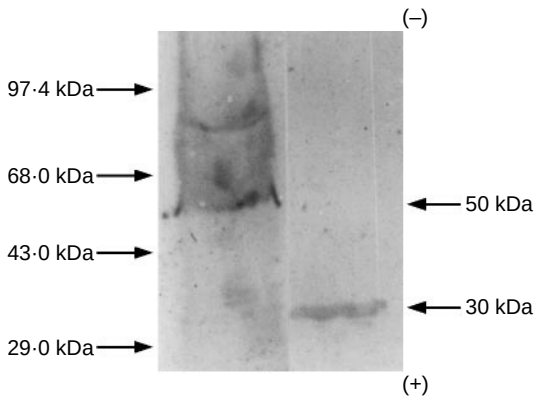


**Fig. 3** Immunoblotting analysis of 48-h-old cultures of avirulent (lane 1) and virulent *Xanthomonas campestris* pv. *cyamopsidis* (Xccy) (lane 2) loaded onto SDS-PAGE (12%). Immunoblotting was performed after the transblot using polyclonal antibody-Xccy. Locations of standard molecular weight markers are indicated by arrows

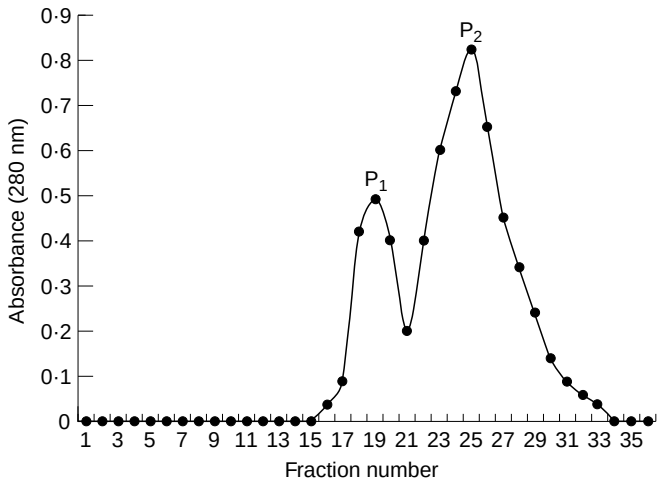


**Fig. 4** Polyclonal antibody-*Xanthomonas campestris* pv. *cyamopsidis* antisera (1·0 ml) was subjected to 50% ammonium sulphate precipitation and loaded onto a Sephacryl-200-HR column (65·0 × 1·0 cm). Fractions (1·0 ml) were collected and the protein profile determined by screening fractions at $A_{280\,nm}$ in a spectrophotometer. Peaks were examined for antibody reactivity by ELISA

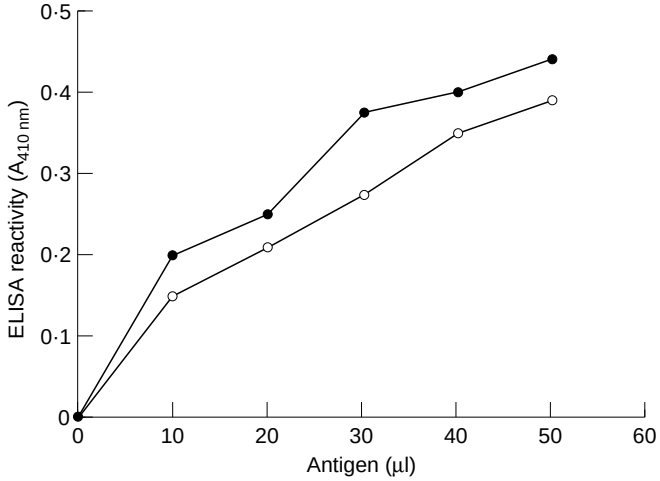


**Fig. 5** Crude polyclonal antibody-*Xanthomonas campestris* pv. *cyamopsidis* (Xccy) reactivity was compared at 10–50 $\mu$l doses of $10^2$ cells $ml^{-1}$ Xccy cells. Virulent (●) and avirulent (○) isolates of Xccy were examined. Equal reactivity was observed

any significant change in reactivity between avirulent and virulent cells (Fig. 6).

### Virulent cell-specific reactivity of $P_1$Ab-Xccy is abolished by heat treatment

Virulent and avirulent cells were subjected to heat treatment at 56 °C for 10 min. This resulted in loss of virulence. Interestingly, $P_1$Ab-Xccy reactivity to virulent cells was abolished after heat treatment (Fig. 7). However, culture filtrates showed little activity (data not shown). Crude (Pab-Xccy) antibody also resulted in abolition of reactivity for Xccy upon heat treatment.

## DISCUSSION

Several methods have been used to detect bacterial pathogens from vegetable crops, including plating on media, pathogenicity studies, hypersensitive reactions, etc. (Duveiller 1992). However, only rapid, sensitive, specific and reliable assays could be employed in routine seed health testing (Franken and Van Vuurde 1990). Diagnostic techniques have proven useful for detecting bacterial plant pathogens on plant tissue and seeds (DeBoer and McCann 1989). The present paper describes the attempts made and strategy employed to obtain specific PAbs against Xccy and to develop a sensitive, rapid and specific ELISA to employ in routine seed health testing. The study also describes the purification and characterization of PAb-Xccy into fractions which can be used to identify virulent strains of the pathogen. *Xanthomonas campestris* pv. *cyamopsidis* appears to contain some virulent factors which, on repeated subculture and storage at 4 °C, showed loss of virulence for long periods and a slow loss at −20 °C, without the loss of viability which failed to induce pathogenicity when injected into the host. There was neither a specific colony morphology (as in *Ralstonia solanacearum*) nor specific probes to distinguish virulent from avirulent colonies (Canale *et al.* 1983). Characterization of host–patho-

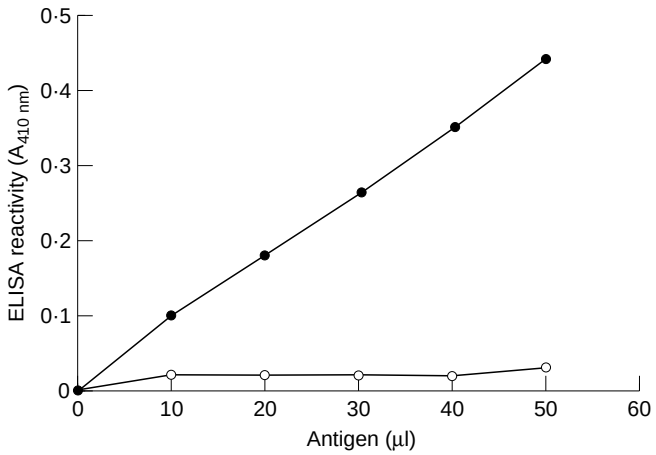


**Fig. 6** $P_1$Ab-*Xanthomonas campestris* pv. *cyamopsidis* (Xccy) on Sephacryl-200-HR column chromatography was examined for antibody reactivity at 10–50 $\mu$l concentrations of antigen at $10^2$ cells $ml^{-1}$. Virulent (●) and avirulent (○) isolates of Xccy were examined. Differential reactivity was observed

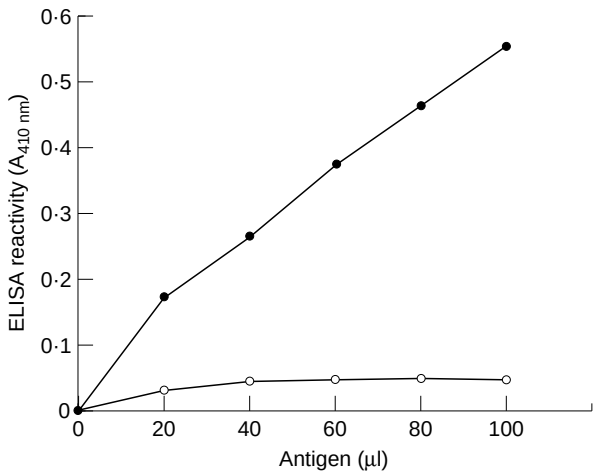


**Fig. 7** A virulent isolate of *Xanthomonas campestris* pv. *cyamopsidis* (Xccy) was subjected to heat treatment at 56 °C for 10 min. 10–50 $\mu$l of $10^2$ cells $ml^{-1}$ of treated (○) as well as untreated cells (●) were examined for ELISA reactivity using $P_1$Ab-Xccy. Antibody reactivity was abolished after treatment

gen interaction studies to understand virulence was therefore affected. The method thus developed has an impact in monitoring virulence and avirulence in Xccy.

The ELISA developed was shown to be very sensitive in detecting as few as $10^2$ cells $ml^{-1}$ of Xccy with a titre of 1 : 4000. The strategies adopted also favoured the production of a non-cross-reactive, specific and sensitive antibody against Xccy. Studies indicated the impact of routes of immunization as well as antigen preparation, i.e. the use of whole bacterial cells as immunogen in obtaining sensitivity, although many investigators have employed glutaraldehyde- or heat-treated cells as immunogens for xanthomonads (Digat and Cambra 1976; Harlow and Lane 1988). The reason for the selection of whole cells was to retain the cell wall components, the state in which they were found in diseased plants. Our previous experience, in this regard, had yielded specific antibody to native bacterial pathogen (Rajeshwari *et al.* 1998). Glutaraldehyde, heat or even formalin treatment has been shown to disrupt the bacterial cell wall structure and, therefore, has been predicted to produce antibodies which are not strain- and virulent-specific. Strain specificity and characteristic virulence are due to specific molecules present at the surface of bacterial pathogens (Allan and Kelman 1977) and their interactions with the host. Change in the route of injection from subcutaneous to intramuscular rapidly yielded sensitive PAb-Xccy. The results can be attributed to the release of different epitope-specific antibodies due to differential presentation of antigen when injected by different routes; most of the dendritic cells may have presented the antigen when subcutaneous injections were given, while intramuscular effects must have allowed macrophages, etc. to present antigens to produce antibodies. The impact of different routes of injection in influencing the production of differential immune responses is not uncommon. In the present study, however, the more abundant cell wall component of Xccy must have been utilized as an antigenic determinant. However, further studies, such as immunization of a new rabbit with an initial intramuscular injection to produce a sensitive antibody without subcutaneous injection, would support the predictions made in this paper.

The results suggested that severely infected seeds contained larger populations of bacterial cells and also showed high ELISA reactivity. Mildly and uninfected seed materials correspondingly showed less and no bacterial cells as evaluated by plate assay, respectively (Table 1). The assay is therefore reliable to employ in routine seed health testing and monitoring of diseased and suspected plants for Xccy infection. Different virulent and avirulent isolates were also reactive to crude antibody.

Immunoblotting analysis was performed to determine the antigenic determinant for different isolates. Despite a similar detection of ELISA, immunoblotting analysis yielded different profiles for different isolates. A 50-kDa band was identified in isolate 1, while a 30-kDa band was located in isolate 2. $P_1$Ab-Xccy reactivity was observed equally in the culture filtrate and the cells, indicating that the antibody is raised against the secretory component which may also adhere onto the surface of Xccy. Further immunofluorescence studies supported these results as fluorescence was observed on the cell surface (results not shown). Results on the characterization of these isolates were intriguing. Isolate 1 was virulent while isolate 2 had lost its virulence during constant storage at 4 °C, as evaluated by pathogenicity studies.

Purification and characterization of PAb-Xccy were further undertaken to explore the possibility of separation of two different epitope-specific antibodies, which might have resulted from changes in the route of injection. Ammonium sulphate (50% (w/v)) precipitation followed by Sephacryl-200-HR column chromatography yielded two peaks, $P_1$ and $P_2$, that had antibody reactivity against Xccy. These fractions were examined for their potential to discriminate between virulent and avirulent cells of Xccy. Results suggested that $P_1$Ab-Xccy had the potential to discriminate between avirulent (isolate 1) and virulent (isolate 2) Xccy, although crude PAb-Xccy did not discriminate this when ELISA was performed.

To confirm these results, virulent cells were subjected to heat treatment at 56 °C for 10 min. In fact, these conditions have been recommended for clusterbean seed treatment, which results in disease-free growth on grow-out tests (Karwasra *et al.* 1984). The preliminary studies conducted in our laboratory showed that loss of pathogenicity was due to heat-labile virulent factors. Exopolysaccharides, proteases and cellulases have been implicated in virulence in xanthomonads (Dow *et al.* 1990). Proteases and cellulases are proteinaceous and therefore heat-labile virulent factors. $P_1$Ab-Xccy may, therefore, be active against one of the virulent components, either protease, cellulase or both. In the present investigation, when $P_1$Ab-Xccy was used to analyse heat-treated and untreated virulent cells, differential reactivity was observed. Similar results were observed by Permar and Gottwald (1989) in a Florida citrus nursery strain of *X. campestris.* The lack of virulence specificity, despite its presence in one of its fractions, $P_1$Ab-Xccy, could be explained by the minor population of $P_1$Ab-Xccy with respect to that of $P_2$Ab-Xccy. Quantitative recovery on fractionation of PAb-Xccy to $P_1$ and $P_2$ suggested that they were present in a ratio of 1 : 4. It is, therefore, necessary to observe reactivity between crude PAb-Xccy and $P_2$Ab-Xccy.

The results of these investigations suggest that the production of a PAb specific to Xccy could be used to develop a sensitive, specific and reliable ELISA test. Fractionation on a Sephacryl-200-HR column resolved a virulent-specific antibody which can be successfully employed to identify virulent from avirulent isolates and to follow up the stability of Xccy during storage.

## ACKNOWLEDGEMENTS

The authors thank Dr S.B. Mathur and Dr C.N. Mortensen, DGISP, Hellerup, Copenhagen, Denmark, for their kind support. M.D.S. acknowledges the financial assistance of the Council of Scientific and Industrial Research, New Delhi, India.

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