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DETECTION OF BIODIVERSITY IN *XANTHOMONAS CAMPESTRIS* PV CITRI

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Abstract

Biodiversity among 25 strains of *Xanthomonas campestris* pv *citri* isolated from different localities of Pakistan was distinguished through Immunodiffusion tests. Two serovars were detected based on the presence of a sharp band or faint band in gel diffusion plates. Antisera XC-91 and XC-93 formed a very strong precipitin band with strain XC 58, XC 91, XC-93, XC-100, XC-101, XC-105, XC-107, XC-114 and XC-125 with a faint band with XC-92, XC-94, XC95, XC-96, XC-102, XC-103, XC-106, XC-109, XC-110, XC-111, XC-113, XC 115 and XC-121. Three strains viz., XC-97, XC-98 and XC-99 did not give any reaction against either of the two antisera and thus appeared to be non specific.

Introduction

Xanthomonas campestris pv. *citri* (Hasse) Dye, the cause of citrus canker, is the most destructive pathogen of citrus world wide (Aubert, 1990). A method of rapid detection and identification of *X. campestris* pv. *citri* would greatly help in development of control measures. Serology is one of the most useful tools available for identification and detection of phytopathogenic bacteria (Otta & English 1971; Schaad, 1979; Yakrus & Schaad, 1979, Thaveechai & Schaad, 1984; Huang *et al.*, 1993; Rott *et al.*, 1994, Alvarez *et al.*, 1996). The antisera in chicken and rabbit was produced to check whether there is any serological variation among the antisera produced in two different animals. Gel diffusion have been used for identification of various bacterial strains and the immunogens that have been used include whole cells treated with heat (Westphal & Jann, 1965; Lazar, 1972; Samson, 1973; Victoria, 1977; Prasad & Sinha, 1978; Cother & Powell, 1983; Mazarei & Kerr , 1990). The objective of the present study was the application of gel diffusion test for the detection of diversity in *Xanthomonas campestris* pv. *citri* strains.

Materials and Methods

Preparation of Antisera: Citrus Bacterial Canker Disease (CBCD) isolates XC-91 and XC-93 (Table 2) were streaked twice on yeast extract-dextrose-calcium carbonate agar (YDCA) medium. Single colonies were selected and grown for 48 h at 28°C. The cells were removed with a spatula and suspended in sterile 0.01 M PBS pH 7.2. Bacterial cells were sedimented by centrifugation at 4°C for 10 min., at 10,000 g (Beckman Modle J 2-21). The pellet was suspended in 5 ml sterile PBS and centrifuged again. The pellets were washed twice or more and after final centrifugation at 12,000 g for 20 min., resuspended in 5 ml sterile PBS and maintained as stocks. Bacterial suspension

was sonicated for 15 min , in a Yamato Bransonic 12 ultrasonic cleaner (U S. patent No. 3, 681, 626) sonicator.Total protein was estimated according to Lowry *et al*., (1951), by preparing the bacterial suspension in 1N NaOH and adding reagent folin phenol. Two local breed rabbits were injected intramuscularly with 500 µl of bacterial suspension emulsified with an equal volume of Freund's incomplete adjuvant once a week for 10 weeks (Akhtar & Clafin, 1986). The rabbits were test bled from the marginal ear vein after the fourth injection and agglutination titer was determined by micro agglutination test. Before, the first injection, pre-immune antisera were obtained. Blood was clotted after incubation for 2-3 h at 37°C and left overnight at 4°C. The sera were recovered as supernatant after centrifugation at 2000 g for 10 minute. After each bleeding, antiserum was titrated by a Ouchterlony gel double-diffusion test (Ouchterlony, 1962).

A similar type of experiment was conducted for the production of antisera in chicken. Bacterial isolates XC-92, XC-96, XC-100, XC-102, XC-105 and XC-106 were grown on YDCA medium. Bacterial suspension was prepared using same techniques as explained above. Two ml of each suspension was mixed thoroughly. Each bird was injected intramuscularly with 250 µl of composite antigen emulsified with 250 µl, Freund's incomplete adjuvant. Composite antigen was used for wider antigenic components determination. The birds were slaughtered after the 12th injection and antiserum recovered from blood as explained above.

Ouchterlony gel double diffusion (ODD): Tests were made in 90 mm Petri dishes containing 15 ml of 7.5g/l purified agar in 0.01 M PBS at pH 7.2 and 0.2g Sodium azide added as preservative. The plates were allowed to dry and then stored in a refrigerator until needed. Circular patterns with 6 wells surrounding a central antiserum well were used in gel diffusion tests. Wells were 3 mm in diameter and 3.5 mm apart. A template was used to cut seven wells of equal diameter and distance. Whole untreated sonicated bacterial cells were used as antigens, 15 µl of antigen was placed in each outer well and 15 ul of undiluted antiserum in the centre well. Petri dishes were sealed with parafilm incubated at 25 °C and observed for precipitin lines after 2-3 days. Double-diffusion (DD) tests were performed with sera having agglutination titers of 1024 or 2048. The 25 isolates were compared in double diffusion tests against antisera XC-91, XC-93 produced in rabbit, pooled antisera (XC-100, XC-92, XC-102, XC-105, XC-106, XC-96) produced in chicken, antisera XC-05188 from France and XC-62 from USA.

Results

Titers of antisera were determined by agglutination method with sonicated bacterial cells. The homologous titers ranged from 512 to 2048; higher titer values were obtained with sera collected after a second course of immunization.

In ODD tests serological patterns similar to each other were obtained from sonicated as well as the whole cell antigen; but treated (autoclaved) antigen gave inconsistent results. In consistent results with autoclaved antigen might be due to some changes in lipopolysaccharide which are responsible for antigenic differences (Rott *et al*, 1994). Among the pathogen strains included in the study 9 antigens appeared to be

common. Thus a high degree of serological relationship was observed among strains that were pathogenically typical of *X. campestirs* pv. *citri*. The sonicated and whole cell antigens of strains gave a sharp band near the antigen wells with the antisera XC-91, XC-93, XC-05188 but faint reaction with XC-62 . The pooled antisera from chicken gave a diffused band with homologous antigen. Generally heated antigen gave a very faint reaction with homologous antisera. There was correlation between the presence of an arc like band close to antigen wells with nonheated antigen and the formation of a very light band near the antiserum well with heated antigen. Both nonheated and heated antigens of isolates were compared in gel-diffusion reactions against antisera of various

Table 1. Ouchterlony double diffusion reaction of antisera prepared to test *Xanthomonas campestris* pv. *citri*.

Antigen	Serovar	XC-91	XC-93	XC-05188	XC-62	Pooled Antisera
XC-58	I	+	+	+	±	0
XC-91	I	+	+	+	±	0
XC-92	II	±	±	±	±	+
XC-93	I	+	+	+	±	0
XC-94	II	±	±	±	±	0
XC-95	II	±	±	±	±	0
XC-96	II	±	±	±	±	±
XC-97	-	-	-	-	±	0
XC-98	-	-	-	-	-	0
XC-99	-	-	-	-	-	0
XC-100	I	+	+	+	±	+
XC-101	I	+	+	+	±	0
XC-102	II	±	±	±	±	+
XC-103	II	±	±	±	±	0
XC 105	I	+	+	+	±	+
XC-106	II	±	±	±	±	+
XC-107	I	+	+	+	±	0
XC-109	II	±	±	±	±	0
XC-110	II	±	±	±	±	0
XC-111	II	±	±	±	±	0
XC-113	II	±	±	±	±	0
XC-114	I	+	+	+	±	0
XC-115	II	±	±	±	±	0
XC-121	II	±	±	±	±	0
XC-125	I	+	+	+	±	0

Y = + = Reaction of identity, ± = Faint reaction.

- = No rection, 0 = Not tested

Antisera XC-91 and XC-93 produced in rabbits, Pooled antisera produced in chickesn, XC-05188 from Fracne and XC-62 from USA.

isolates. On the basis of the resulting two types of reaction the strains were grouped into two types and designated as serovar I and II. In the first pattern, serovar I type strains XC-58, XC-91, XC-93, XC-100, XC-101, XC-105, XC-107, XC-114 and XC-125 gave a sharp arc like band with antisera XC-91, XC-93, and XC 05188 (Table 1). In the first pattern, majority of the strains showed a correlation between the presence of arc like bands of nonheated antigens near the antigen well and heated antigen near the antiserum well. Type I strains formed very sharp arc like bands (Table-2). Type II strains did not form an arc like band, rather gave only a faint reaction with both heated and nonheated antigen. A strong relationship of serovar and host of origin of strain was noticed. Either serovar I or II was detected from one host, except in sour orange where both serovar I and II were detected.

Discussion

Preliminary results obtained from studies through agglutination and ouchterlony gel double diffusion (ODD) tests on serological relation among 25 strains indicated that the

Table 2. Relationship of host of origin and serovar of *Xanthomonas campestris* pv. citri.

Host of origin	No of strains in serovar I	II
Jullundri Khatti	XC-58	
Sour orange	XC-91, DC-114	XC-92
Kinnow/Rough lemon	XC-93	
Feuterell's early/Rough lemon		XC-94
Feuterell's early/sour orange		XC-95
Jamberi		XC-96
Rossette	XC-100	
Fair child	XC-101	
Frost Marsh		XC-102
Kinnow		XC-103
Musambi	XC-105	
Rough lemon		XC-106
		XC-115
Seville kimb	XC-107	
Reed		XC-109
Kinnow/sour orange		XC-110
Mitha		XC-111
Hamlin		XC-113
Frost Valencia		XC-121
Malta	XC-125	

Serovar I formed sharp band, While II faint band.

two antisera produced by injecting strain XC-91 and XC-93 into rabbit elicited in the agglutination test early a faint reaction against some of the strains and were thus not specific. In agar gel diffusion test, anti-XC-91 and XC-93 formed a very strong precipitin band with strain XC-58, XC-91, XC-93, XC-100, XC-101, XC-105, XC-107, XC-114 and XC-125 and faint band with XC-92, XC-94, XC-95, XC-96, XC-102, XC-103, XC-106, XC-109, XC-110, XC-111, XC-113, XC-115 and XC-121. Thus these strains appeared to belong to two distinct serovars of which serovar I, strains predominantly formed a strong precipitin band and serovar II formed only a faint band. Antisera 65188 from France formed very strong reaction like the antisera XC-91 and XC-93 produced locally in our laboratory, while the antisera XC-062 from U.S.A. showed weak reaction. It may be due to immunogenic response of different antigens. It indicates that serovar in U.S.A. might be different than France and Pakistan. Three strains XC-97, XC-98 and XC-99 did not give any reaction against either of the two antisera appeared to be non-specific. The pooled antisera from chicken also gave a diffused band with homologous antigen which is a specific method required to clear the IgG in the antiserum. Lovrekovich & Klement (1965); O'Brien *et al.*, (1967); Otta & English. (1971); Chrudattan *et al.*, (1973); Schaad, (1974); Pastushenko *et al* (1976), Schaad, (1979); Yakrus & Schaad , (1979); Thaveechai & Schaad (1984),Mazarei & Kerr (1990) and Huang *at al.*, (1993) differentiated between strains of *X. vesicatoria* using ODD test. In the present studies ODD was found to be a powerful tool for effectively distinguishing between serovars of *X. campestris* pv. *citri*. The serological similarity between any two strains, however, did not appear to be related to the strain's pathogenic ability.

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