

## Comparison of DAC-ELISA and Tissue Blot Immunoassay for the Detection of *Acidovorax avenae* subsp. *avenae*, causal agent of Red Stripe of Sugarcane

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### Abstract

Direct Antibody Coating Enzyme Linked Immunoassay (DAC-ELISA) and Tissue Blot Immunoassay (TBIA) were compared for detection of *Acidovorax avenae* subsp. *avenae* (Aaa) in sugarcane samples surveyed in 2010 and 2011. A total of 27 sugarcane clones contained two samples of symptomatic plants, which were tested simultaneously by both serological tests. The results obtained from DAC-ELISA tests showed that 11 of 27 tested samples of sugarcane clones were infected with Aaa and 16 were bacteria free; whereas, in duplicate samples only 7 samples were positive for Aaa using the TBIA. The TBIA membranes showed dark blue stain colour in the 7 positive and 20 were negative. It was concluded that the DAC-ELISA had false positives in the 4 clones (SPF-238, CP77-400, SPF-213 and GT-11) that were negative using TBIA. Assay time was also minimized in performing TBIA test as equivalence to DAC-ELISA. Related to the test performing time at least 25-30 hours were consumed for the completion of DAC-ELISA assay, while only 6-9 hours was required for TBIA assay with reliable results. Both assays gave Aaa positive for clones HoSG-315, CP5G-437, NSG-49, CP5G-2453, CP-NIA-82-223, CSSG-2402 and US-114. TBIA tests were distinct with clear positive and negative results having minimum diagnostic error. On the other hand quantitative assay of bacteria is a significant feature of DAC-ELISA but low optical density readings above background were inconsistent with the TBIA and were considered false positive results. Amongst the variety of immunological tests available in Pakistan, tissue blot immunoassay (TBIA) is most useful in detecting latent infections in the plant materials.

**Keywords:** Sugarcane; Varieties; DAC-ELISA; TBIA; Symptomatic plants

### Introduction

Sugarcane (a complex hybrid of *Saccharum* spp.) is the oldest energy source (sucrose) for human beings and more recently a source of fuel for motor vehicles. It is a high value cash crop and plays vital role in the enhancement of socio-economic conditions of farming community as well as in industry and trade. It's by-products, molasses and bagasse, are the cheapest source of feed stock for animals and industries [1].

Pakistan ranks fifth in area of sugarcane production in the world with 1.00 million hectares of area. Average cane yield in Pakistan is 50 tons/ha and sugar recovery is 8.0-8.6%, which are lower than the world average of 63 tons/ha and 10.6%, respectively [2]. Per capita demand of sugar in Pakistan is the 25 kg per annum [3]. Low sugarcane yields are mainly attributed to different biotic and abiotic factors: insect pests, diseases caused by different pathogens (fungal, bacterial, viral and nematodes), poor nutrition and management, salinity, frost, unfavorable environmental conditions and low yielding varieties.

Pathological problems alone are responsible for colossal losses in production, 15-20% under ordinary and more than 50% under severe conditions. Losses of 15-20% due to diseases are common, sometimes complete crop failure occurs [4]. Red stripe disease has emerged as a serious bacterial disease in sugarcane, infecting commercial varieties and promising clones showing stalk rot symptoms [5]. Red stripe is particularly a problem when the pathogen invades the stalk where it can cause high yield losses. When the pathogen is only present in leaf tissue the disease is minor. The pathogen associated with red stripe of sugarcane also infects oats [6], corn [7] and rice [8].

Red stripe was first observed on sugarcane in Hawaii [9]. The bacterial pathogen was identified as *Pseudomonas rubrilineans*; now

named *Acidovorax avenae* ssp. *avenae* (Aaa). Bacterium is gram negative, rod 0.4-0.6  $\mu\text{m}$   $\times$  0.8-1.6  $\mu\text{m}$ . The pathogen is now established and reported from 50 sugarcane growing countries of the world [10] and is present in several locations in Pakistan [11].

The disease is initially characterized by the appearance of green stripes near the midrib that later on turn reddish to dark red stripes. The stripes initially develop at the base of the leaves in June and July in Pakistan. Symptoms spread quickly to more leaves. Disease development is favored by high doses of nitrogen application, high temperature and humidity [12].

The pathogen was initially identified on the basis of symptomatology, growth behavior of the bacterium, grown out test and morphological characteristic under light microscope. These methods of identification were uneconomical, laborious, time consuming and non specific. The pathogen was specifically identified by serological methods mainly ELISA. Electron microscopy and PCR were also employed, which have been hardly attempted in Pakistan.

Because of the spread and potential yield losses of red stripe in Pakistan, an accurate and sensitive diagnostic method(s) are required

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to identify bacterial free planting material at the early stage. Thus, a stalk diagnostic assay procedure is required guarantee disease-free seed cane. The objective of the study to specifically confirm the identity of the pathogen through antigen-antibody reaction through tissue blot immunoassay (TBIA) and direct antibody coating enzyme linked immunoassay (DAC-ELISA) for the identification of resistance source to be used in Integrated Disease Management (IDM).

## Materials and Methods

### Antisera

*Acidovorax avenae* subsp. *avenae* was isolated from highly susceptible sugarcane variety CSSG-2402 on Yeast extract Dextrose Chalk-agar (YDC) medium. Bacterium was grown in 150 mL YDC broth in 350 ml flasks. Bacterial culture was centrifuged at 10,000 rpm for 30 minutes at 4°C. Supernatant was discarded and pellets were washed twice with 100 ml membrane filtered sterilized saline (0.85%).

Concentrated suspension was prepared using spectrophotometer to get  $10^9$  cell/ml at 600 nm. The suspension was steamed for one hour to get somatic antigen in sonicator (yamato bran sonic-12 ultrasonic cleaner, US PATENT NO 3, 6.81, 6.26) and then cooled down. One ml specific antigen of *Aaa* was injected intravenously into albino rabbit 6-12 months old. Five such injections were given at weekly interval. Test bleed of rabbit was taken using bleeding rack and obtain approximately 1 ml blood from the ear after one week of the last injection. Blood was relocated into a sterile screw-cap test tube of 50-ml capacity and allowed to clot at room temperature for 2 hours. The clot was separated from the wall of tube using sterilized glass. The serum was kept overnight at 4s. Bacterial culture was centrifuged at 10,000 rpm for 30 minutes at 4°C. Purification was done by centrifugation at 5000 rpm for 10 min. Merthiolate was added in the antiserum and kept in refrigerator at 4s. Bacterial culture was centrifuged at 10,000 rpm for 30 minutes at 4°C.

The antiserum titer was determined by agglutinations in microtiter trays. The first bleed (approximately 10 ml) was taken at two weeks after the last injection and antiserum titer was determined [13]. Preparation of antigen and antiserum against *Aaa* was completed for the first time at National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan. The second antibody used in the TBIA and DAC-ELISA tests were goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-USA).

### Plant materials

The sugarcane producing areas of Punjab Province, Pakistan including Jhang and Faisalabad regions were surveyed in 2010 and 2011. Samples (i.e., one stalk of each clone) were obtained from mature plants 9-10 month old of sugarcane varieties: HoSG-315, CPSG-437, NSG-555, SPF-238, CP77-400, SPSG-79, CPF-237, HSF-240, SPF-213, CSSG-668, CSSG-676, NSG-311, GT-11, CPSG-3481, NSG-59, CPSG-2923, CSSG-212, CPSG-104, HoSG-1257, CPSG-25, CSSG-239, CPSG-2713, NSG-49, CPSG-2453, US-114 and CP-NIA-82-223 showing naturally infected diseased symptoms of red stripe [11]. Sugarcane variety CSSG-2402 was used as standard check because of its susceptibility to the red stripe of sugarcane. All samples were collected in polyethylene bags and stored in a refrigerator at 4s. Bacterial culture was centrifuged at 10,000 rpm for 30 minutes at 4°C until processed. All collected stalk samples were tested by two different serological assays TBIA and DAC-ELISA.

### Detection of *Acidovorax avenae* subsp. *avenae* (*Aaa*) by direct antibody coating enzyme linked immunoassay (DAC-ELISA)

Vascular sap of infected samples was used as antigen. Stalk sections

were centrifuged at 8500 rpm for 10 minutes to collect vascular sap. One hundred  $\mu$ l of vascular sap were loaded into wells of microtiter assay plate. Positive control well had 50  $\mu$ l of cultured *Aaa* cells of unknown titer. Negative control well had uninfected xylem sap sample. ELISA plate was incubated overnight at 4°C in a refrigerator. Wash the sample wells with 150  $\mu$ l PBST buffer 3 times for 3 minutes each. 100  $\mu$ l of primary antibody at 1:1000 was loaded in each well of the ELISA plate [14] and incubated at 37°C for 2-3 hours. Then, the plates were washed with washing buffer 3 times with 3 minutes interval. Conjugate (Goat anti-rabbit immunoglobulin IgG with alkaline phosphatase (ALP) enzyme) 100  $\mu$ l was loaded in each well of the ELISA plate. The plates were incubated at 37°C for 2-3 hours. 5 mg p- nitrophenyl phosphate diluted, in 10 ml of substrate buffer. 150  $\mu$ l of substrate buffer was loaded in each well of the ELISA plate. Results were observed visually for half an hour at room temperature. Later absorbance was also determined spectrophotometrically at 405 nm in an ELISA reader (ASYS Hitech GmbH. Model. Expert Plus. Type. G020150 [15]. A cut off/threshold  $OD_{405}$  (Optical density) of 0.066 was calculated to indicate the presence of *Aaa* [16].

### Detection of *Acidovorax avenae* subsp. *avenae* (*Aaa*) by tissue blot immunoassay (TBIA)

The tissue blot immunoassay (TBIA) procedures were performed for detecting the red stripe disease pathogen of sugarcane. 1cm core of tissue from each internode was cut in 1-cm lengths and the cut end was placed on a nitrocellulose membrane having 2-cm diameter and centrifuged at 1,700 rpm for 15 min. Membranes were blocked with blocking solution (Tris buffer saline 100 ml and dried milk powder 2 g) and incubated for one hour at room temperature. After washing for 1 min in 75 ml Tris buffered saline (TBS), each membrane was immersed in 10 ml primary antibody solution. Incubated and washed 3 times for 5 min in 75 ml TBS. Each membrane (single layer) was exposed with 10 ml anti-rabbit antibody diluted solution and Incubated for 1 hour. Washed 2 times for 15 min in 75 ml TBS. Immersed membranes in working substrate (Tris-24 g, Naphthol-AS-phosphate-3 g, Dimethylformamide-5 ml and distilled water-1000 ml) with 0.075 g of fast blue and 0.375 ml of  $MgCl_2$  (in 75 ml working substrate) in dark for 15-30 min. Membranes were washed and treated with 20% bleach solution for 20 minutes to remove brownish discoloration. After washing and drying, color of stained/processed nitrocellulose membranes were visually observed [15].

## Results and Discussions

### Evaluation by DAC-ELISA

A total of 27 samples of sugarcane varieties were collected from the field surveys during 2010-2011. A summary of the results of the samples tested with DAC-ELISA is shown in table 1. In DAC-ELISA, *Aaa* was detected from the infected or suspected to be infected samples showing positive reactions (yellow color) of the bacterium (Figure 1). Results showed that two representatives, US-114 and CSSG-2402 have the higher bacterial optical density values (1.379 and 1.390 respectively) were detected and showed highly positive reaction by DAC-ELISA. Five sugarcane varieties HoSG-315 (0.560), CPSG-437 (0.555), NSG-49 (0.564), CPSG-2453 (0.556) and CP-NIA-82-223 (0.564) had higher level of absorbance values were positive in the ELISA plate wells [14]. From the representative stalks of 04 varieties like CP77-400, SPF-238, SPF-213 and GT-11 had moderate level of the values (0.101, 0.099, 0.098 and 0.104) respectively, showed the light yellow color (Positive reaction). All of the remaining 16 representative were showed negative reaction by the DAC-ELISA test [17,18].

Sr. No.	Varieties	Visible reaction	(OD <sub>405 nm</sub> )	Sr. No.	Varieties	Visible reaction	(OD <sub>405 nm</sub> )
1	NSG-555	-	0.091	17	CP77-400	+	0.101
2	SPSG-79	-	0.090	18	SPF-238	+	0.099
3	CPF-237	-	0.093	19	SPF-213	+	0.098
4	CSSG-212	-	0.095	20	GT-11	+	0.104
5	HSF-240	-	0.090	21	HoSG-315	++	0.560
6	CSSG-668	-	0.089	22	CPSG-437	++	0.555
7	CSSG-676	-	0.092	23	NSG-49	++	0.564
8	NSG-311	-	0.090	24	CPSG-2453	++	0.556
9	CPSG-3481	-	0.090	25	CP-NIA-82-223	++	0.564
10	NSG-59	-	0.091	26	US-114	+++	1.379
11	CPSG-2923	-	0.095	27	CSSG-2402	+++	1.390
12	CPSG-104	-	0.091	28	+ve control	++	0.561
13	HOSG-1257	-	0.094	29	-ve control	-	0.003
14	CPSG-25	-	0.090	30	Extraction buffer	-	0.004
15	CSSG-239	-	0.091	31	Threshold (OD <sub>405</sub> )	-	0.066
16	CPSG-2713	-	0.093				

Table 1: Visual and serological (DAC-ELISA) determination of Aaa.

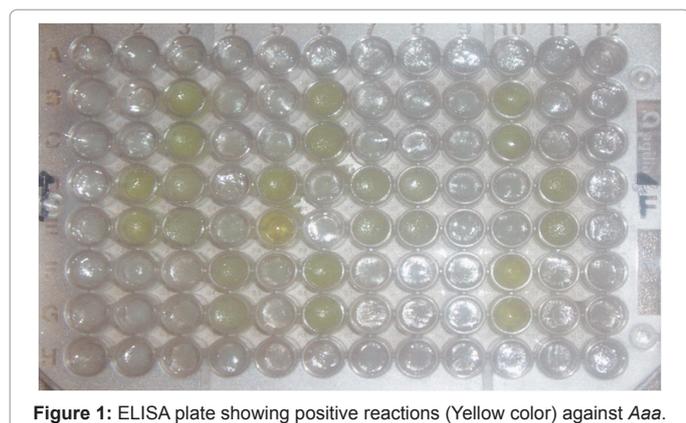


Figure 1: ELISA plate showing positive reactions (Yellow color) against Aaa.

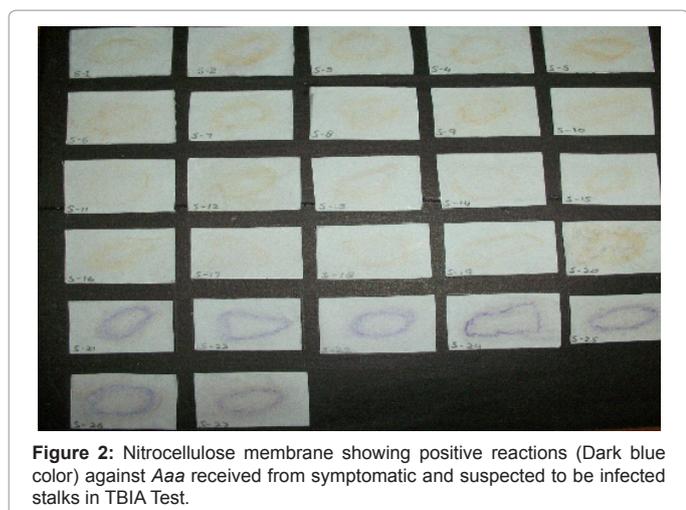


Figure 2: Nitrocellulose membrane showing positive reactions (Dark blue color) against Aaa received from symptomatic and suspected to be infected stalks in TBIA Test.

### Detection of *Acidovorax avenae* subsp. *avenae* (Aaa) by TBIA

Tests were made by making blots of Aaa infected or suspected to be infected tissue on nitrocellulose membranes using standard procedures [15]. Of the twenty seven promising clones of sugarcane,

only seven clones like HoSG-315, CPSG-437, NSG-49, CPSG-2453, CP-NIA-82-223, CSSG-2402 and US-114 were positive for Aaa using TBIA and showed dark blue stain colour where the vascular bundles were imprinted on the nitrocellulose membranes (Figure 2). Representative samples of twenty clones were negative by TBIA and no colour appearance was observed on the membranes [15,19].

### Comparison between DAC-ELISA and TBIA

Both serological tests were performed on duplicate samples of the 27 sugarcane clones. According to the results of the tests, Aaa was not detected in 16 clones viz: NSG-555, SPSG-79, CPF-237, HSF-240, CSSG-668, CSSG-676, NSG-311, CPSG-3481, NSG-59, CPSG-2923, CSSG-212, CPSG-104, HoSG-1257, CPSG-25, CSSG-239 and CPSG-2713 using both DCA-ELISA and TBIA tests. DAC-ELISA showed false positive due to low optical density readings above background were inconsistent results in the 4 clones (SPF-238, CP77-400, SPF-213 and GT-11) based on slight yellow coloration in its test [20]. While, in sugarcane samples viz: HoSG-315, CPSG-437, NSG-49, CPSG-2453, CP-NIA-82-223, CSSG-2402 and US-114 were showed positive detection of *Acidovorax avenae* subsp. *avenae*, by both serological tests [15]. In DAC-ELISA test highly positive values were noted from the samples of CSSG-2402 and US-114 but in TBIA test positive reaction was observed of both clones (Table 2).

As per results, TBIA test was used to confirm results positive or negative. In DAC-ELISA test because of the vascular coloration corresponds to where Aaa is located. Since clones (SPF-238, CP77-400, SPF-213 & GT-11) did not test positive using TBIA the DAC-ELISA results are considered false [21].

Among 27 randomly collected sugarcane samples, TBIA showed 26% positive and 74% negative result. On the other hand DAC-ELISA was showed 41% positive (36% false+64% true) and 59% negative results, when compared with TBIA test. Assay time was also minimized in performing TBIA test as equivalence to DAC-ELISA. Related to the test performing time at least 25-30 hours were consumed for the completion of DAC-ELISA assay, while only 6-9 hours was required for TBIA assay with reliable results [22,23].

diagnostic tests have made a significant contribution in diseases control and eradication. Among the variety of immunological tests, TBIA proved to be extremely helpful for large scale testing at a very low cost without much compromise on sensitivity or specificity [14].

Sample	Varieties	DAC-ELISA	TBIA	Sample	Varieties	DAC-ELISA	TBIA
S-1	NSG-555	-	-	S-15	CSSG-239	-	-
S-2	SPSG-79	-	-	S-16	CPSG-2713	-	-
S-3	CPF-237	-	-	S-17	CP77-400	f	-
S-4	CSSG-212	-	-	S-18	SPF-238	f	-
S-5	HSF-240	-	-	S-19	SPF-213	f	-
S-6	CSSG-668	-	-	S-20	GT-11	f	-
S-7	CSSG-676	-	-	S-21	HoSG-315	+	+
S-8	NSG-311	-	-	S-22	CPSG-437	+	+
S-9	CPSG-3481	-	-	S-23	NSG-49	+	+
S-10	NSG-59	-	-	S-24	CPSG-2453	+	+
S-11	CPSG-2923	-	-	S-25	CPNIA82-223	+	+
S-12	CPSG-104	-	-	S-26	US-114	h	+
S-13	HOSG-1257	-	-	S-27	CSSG-2402	h	+
S-14	CPSG-25	-	-				

-- true negative, +=true positive, f=false positive, h=highly positive

Table 2: Comparison of observation between TBIA and DAC-ELISA.

It is an essential test to be used in developing countries where hi-tech laboratories for diagnoses of the pathogens are not available and cost per sample is a limiting factor for any assay to be adopted for large scale testing [15,19].

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