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COST BENEFIT RATIO OF IMPORTED AND LOCALLY PRODUCED ANTISERUM AGAINST PAKISTANI ISOLATE OF BANANA BUNCHY TOP VIRUS

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Abstract

An effort was made to estimate the cost benefit ratio of commercially available and locally produced antiserum against Banana Bunchy Top Virus (BBTV) to encourage scientists for indigenous research, a step towards self-reliance in sero-diagnosis. Comparing the cost of both antisera, the locally produced serum was found economical incurring an amount of Rs. 20,000/- that is 2.5 times lower than the imported antiserum kit involving expenditure of Rs. 45,000/-. Beside saving foreign exchange earning, locally produced antiserum is obtained in large quantity (>25 ml), which is strain specific sufficient for testing of approximately 65000 samples as compared to imported one (1 ml volume) enough for 1000 samples only. Furthermore, standardized procedure can be exploited for commercial production creating funds for routine lab-oriented research work and provides opportunity to train manpower. Besides, locally produced antiserum can be made available to provincial agricultural research institutes, extension and entry ports for checking health status of the crop and safe national and international germplasm movement. Thus the indigenous technology makes diagnosis of exact pathogen easier and convenient that will ultimately help in proper management of viral diseases.

Introduction

Banana Bunchy Top Disease (BBTD) is the most serious viral disease affecting banana (*Musaceace spp*.) crop worldwide (Dale, 1987). The history of the disease is very recent as compared to banana cultivation. It has been confirmed in the continents of Asia except Israel, Africa and Australia as well as in Pacific islands but not yet reported from America (Smith *et al*., 1998).

The particles of banana bunchy top virus (BBTV) are isometric, 18-20 nm in diameter and have multi-component genome with nine cssDNA components of 1Kb each, a single coat protein of about M_r 20,500 Da. The virus is transmitted through black aphid (*Pentalonia nigronervosa* Coq.) in a persistent manner as well as through suckers from infected mother plants. The symptoms of the disease are characterized by chlorosis, dark green streaks on leaf petiole, midrib and pseudostem and bunchy appearance also called strangle or cabbage top disease (Thomas *et al.*, 1994; Burns *et al*., 1995; Hu *et al.*, 1996; Smith *et al.*, 1998 and Yasmin, 2001). All BBTV isolates are identical serologically reported so far from different countries. However, two BBTV groups have been categorized worldwide on the basis of DNA component 1 sequence viz., South Pacific (Australia, Hawaii & some Pacific Islands) and Asian group (China, Taiwan and Southeast Asia) (Karan *et al*., 1994; Xie & Hu, 1995).

In Pakistan, banana is cultivated throughout the country which thrive best in the tropical areas (Thatta, Badin, Hyderabad, Mirpurkhas, Sanghar, Nawabshah, Naushero-

feroze and Khairpur districts) of Sindh province occupying 87% of the total area. Since 1989, BBTV appeared in an epidemic form adversely affecting banana plantation which reduced the area from 23,500 ha to 11,300 ha, and production from 209,800 tones to 44,200 tones during 1990-92. After a decade, the disease still persists with more or less the same frequency spreading from seaward to interior of the province yielding 4.8 tonnes/ha (Anon., 2002).

BBTV purification and the production of high titre antiserum always remained a difficult task to tackle through conventional methods of virus purification. Polyclonal antiserum production is a pre-requisite for developing sero-diagnostic kits and biochemical analysis of the pathogen. In the present study, a comparison of cost has been made of locally produced polyclonal antiserum with commercially available imported antiserum purchased in the year 2002 to explore the cost benefit ratio beside additional merits of indigenously produced antiserum.

Materials and Methods

Polyclonal antiserum against BBTV was produced involving the following steps:

Collection and propagation of plant material

Banana plants of cv. *Dwarf Cavendish* showing characteristic symptoms were collected from district Thatta, Sindh during disease monitoring survey and were brought to National Agricultural Research Centre, Islamabad. The plants were grown under glass house and field conditions.

Virus purification

Virus purification was done using conventional procedure following the method of Wu & Su (1990). Infected plant material was pulverized in liquid nitrogen and homogenized in 0.02 M KPO_4 buffer containing 0.2% 2-mercapto-ethanol and 0.1% sodium diethyl-dithio-carbamate and stirred for few minutes at 4°C. The mixture was emulsified with n-butanol/chloroform 10%, stirred for one hour and subjected to two cycles of differential centrifugation (36,000rpm for 2.5 hrs and 7,000rpm for 10 min). Finally the partially purified virus preparation was layered on 10-40% sucrose density gradient and centrifuged at 26,000 rpm for 3.5 hours. The virus containing zone was collected by measuring absorbance through spectrophotometer. Fractions having absorbance values above 0.6 were used further for equilibrium centrifugation in Cesium sulfate (Cs_2SO_4) solution and virus band was finally resuspended in re-suspension buffer.

Electron microscopy (EM): Carbon coated copper grids (300 mesh) were floated on a drop of virus suspension, stained with 2% Uranyl acetate (pH 4.8), rinsed with distilled water, filter dried and observed under EM for particles concentration.

Polyclonal antiserum production

Immunogen: Purified virus preparation obtained from method described above was used as immunogen for the production of polyclonal antiserum against BBTV.

Immunization procedure: Purified BBTV preparation quantified and adjusted to 100-150µg/ml, were injected intramuscularly at multiple-sites in chinchilla rabbit, emulsifying first with an equal amount of Freund's complete adjuvant. Later three multiple-site intramuscular injections were given at 10 day intervals by emulsifying the virus preparation with an equal amount of Freund's incomplete adjuvant.

Serum collection: For serum collection, blood (6-8ml) was taken from rabbit after one month (after 3-4 injections) and left over night for clotting. Next day serum was separated by low speed centrifugation and Sodium azide was added as a preservative. Booster injections were administered to rabbit and similarly blood was taken again and antibody titre was checked finally. The antiserum titre was determined through DAC-ELISA and agar gel precipitation test (described later in the text).

Cross-absorbance of polyclonal antisera: In order to remove the host protein from viral preparation, healthy banana sap was prepared (1:10) by first pulverizing in liquid nitrogen. To this sap, 100ml of acetone was added, mixed and incubated for half an hour at room temperature. Tissue was separated from the acetone and the process was repeated over three times. Finally antiserum was incubated with the host tissue for 1-2 hours at 35ºC and used in ELISA.

Purification of immunoglobulins (IgG): BBTV antibodies (IgG fraction) were separated from serum by ammonium sulfate precipitation. Polyclonal antiserum was diluted 10 times with distilled water. To 1 ml of diluted antiserum, one volume of ammonium sulfate (4M) solution was added drop-wise with constant stirring to precipitate out immunoglobulins. The mixture was left for an hour at 4°C. The precipitates were collected by centrifugation at 10,000 rpm for 10 minutes and dissolved in 0.5M Phosphate Buffer Saline (PBS).

The suspension was further dialyzed against PBS (pH 7.8). After dialysis, the suspension was concentrated at low speed centrifugation. Titre of purified IgG was confirmed through DAC-ELISA. Extinction coefficient of purified IgG was determined by measuring absorbance at 280 nm.

For the determination of antiserum titre following techniques were employed:

Agar gel diffusion test (AGDT): For AGPT, pure agar (7.5 g) was dissolved in 9.0 g of NaCl and 0.4 g of NaN_3 in one liter of distilled water and heated until dissolved. Molten agar was poured into Petri dishes to a depth of 5mm (5-15 ml). Agar was allowed to solidify and equi-distance wells were made in agar using a template. Agar block was removed using a suction pump. Antiserum was diluted 1:100, 1:500, 1:1000, 1:5000 and 1:10000 etc., while antigen was diluted tenfold. Antigen (20 µl) was added in the central well while antiserum dilutions (20 µl) in the peripheral wells. Agar plates were incubated at room temperature overnight in a humid chamber. Precipitation lines were observed using an indirect source of light beneath the agar plate after 24 hours. The level of antibodies was detected through cloudy or milky precipitation lines of antigen and antibodies.

Direct antigen coating (DAC) ELISA: Healthy and BBTV-infected banana tissues (leaves and midribs) were extracted in carbon coating buffer (0.05M carbonate buffer, pH 9.6) using chilled mortar and pestle. The sap extract was centrifuged at 10,000rpm for 5

minutes, supernatant was collected and diluted 1:10, 1:20 and 1:100. Polystyrene ELISA plates (Nunc Maxisorb or Fastech brand) were coated with antigen samples (100 µl/well) along with positive and negative controls according to a known layout plan and left overnight at 4°C. Plates were washed with three quick washes followed by 3 washings with 3 minutes interval using washing buffer (PBS-Tween 20). Antiserum (diluted 1:500, 1:1000, 1:1500, 1:2000, 1:4000, 1:6000, 1:8000 and 1:10,000 etc., in coating buffer) was loaded 100 µl/well and incubated at 37°C for 2-3 hours in a moist chamber. Plates were again washed. Whole molecule conjugate (anti rabbit IgG, Sigma brand) was used at 1:500, 1:1000, 1:1500, 1:2000 and 1:4000 dilutions in PBS-Tween-PVP, added to plates 100µl/well and incubated at 37°C for 2-3 hours followed by washing as before. Substrate (p-nitrophenyl phosphate) @ 0.6 mg/ml dissolved in substrate buffer (10% Diethanolamine, pH=9.8) was added to each well (150 µl/well). Results were observed visually for half an hour at room temperature and reaction strength was rated visually as - = no reaction, + = mild reaction, ++ = moderate reaction and +++ = severe/heavy reaction. Later absorbance was also determined spectrophotometrically at 405nm with a Beckman ELISA reader.

Outlay estimates

The expenditures of the above mentioned steps involved in polyclonal antiserum production were estimated and compared with the cost of imported sero-diagnostic kit that was purchased from Agdia Company through Abbasi Traders, Islamabad.

Results and Discussion

Expenditures and cost benefit ratio

The expenditures estimate of each step of locally produced antiserum was done approximately. The steps involved in antiserum production along with the expenditures are given in Table 1 & 2.

On comparing the expenditures of local antiserum production and cost of the imported antiserum, it was found that commercially available IgG has 2.5 times higher cost than the local production. However, the amount of polyclonal antiserum in a routine kit is supplied for testing of 1000 samples (1 ml vial) while the quantity of locally produced IgG was more than 25ml that is enough for indexing of at least 65000 samples (see Table 1 & 2).

Merits of locally produced polyclonal antiserum

It is evident from cost benefit ratio that locally produced antiserum against a plant virus is more useful than purchasing it commercially. In order to encourage national scientists and motivating them to utilize maximally their own resources through minimum grants as well as to strengthen local research capabilities, additional merits of locally produced polyclonal antiserum are needed to be emphasized. Some of the advantages of indigenous antiserum production are:

Economical: Indigenous antiserum production is of low cost (less than two times) as compared to commercially available or imported one as obvious from Table 2.

Table 1. Stepwise expenditures of locally produced antiserum.

S. No.	Steps involved in locally produced antiserum	Cost in Pak. rupees
1.	**Infected material**	
	Collection	**5,000**
	Propagation and maintenance	**1000**
2.	**Purification**	**000**
	Chemicals/reagents	**8,000**
	Electricity	**2000**
	Consumables	**1000**
3.	**Antiserum production**	**000**
	Animal purchase	**1000**
	Animal house Facility (attendant, feed etc.)	**2000**
	Total	**20,000**

Table 2. Cost benefit ratio.

Antiserum type	Antiserum Quantity	No. of samples that can be tested	Cost/expenditures (in rupees)
Locally produced	25ml	65,000	20,000
Imported/purchased	2ml	1000	45,000
Difference	**23ml**	**64,000**	**25,000**

Strain specific: If antiserum is imported from other countries, it is definitely produced against their local existing virus strain and may not react positively in diagnosis against the virus strain prevalent in our environment, hence local production would overcome this problem, as it would be strain specific. Hence the risk factor involved in purchasing IgG kit from the market would be minimal.

Large quantity: Local production of antiserum is obtained in large amount by minimum investment than purchased one. Once animal is immunized, only 1-2 booster injections are required to get an additional amount (10-15 ml) of antiserum after one month for a period of 2-3 years.

Saving of foreign exchange: Antiserum production within own laboratory resources saves foreign exchange in turn benefiting national economy.

Commercial scale production: Once procedure is standardized and equipment is stream lined, antiserum against various viruses and other pathogens can be produced on a large scale or commercial level within the country, which can further minimize its cost. In addition, income can be generated through foreign earning by marketing it internationally. This in turn would enhance national research finances and resources.

Skilled manpower: A large number of manpower (researchers, extension workers/field assistants etc) can be trained in sero-diagnostics of this new emerging field.

National virus indexing facility: Antiserum (against a specific virus) availability in a national diagnostic laboratory can be utilized for indexing of viral infected plants/samples

from farmers field of various regions of the country for early and prompt disease detection, which is an indispensable element of successful management.

Facility expansion: Polyclonal antiserum production and availability at a national research institute would be helpful to make it available to other national and provincial agricultural research institutes, research stations and entry ports for checking crop health status (Quarantine measures) and improving phyto-sanitary conditions. In this way, certified virus free plants or propagating material can be made available to farmers at their door step. Moreover, personnel of provincial agricultural institutes can also be trained to index plant material by providing antiserum at cheaper rate.

Enhancement of research opportunities: Indigenous IgG production would certainly lead to the development and improvement of research facilities and services within the country and would create new opportunities for technical and non-technical unemployed labor force.

Orientation towards indigenous research: Local production of antiserum within own resources would not only develop interest in scientists and researchers but also change their orientation towards indigenous research and experimentation besides creating curiosity for research innovation.

Self-reliance: Locally antiserum production would definitely pave way towards self-sufficiency and self-reliance through utilization of own resources and means. Government can encourage scientists and researchers by initiating such schemes to promote indigenous research.

Food security and poverty alleviation: Huge crop losses incurred by the plant viral diseases would be protected through timely intervention of highly sensitive technology used for detection and identification of such pathogens. This would in turn lead to food security and poverty alleviation by benefiting national economy especially farming community.

Besides the above-mentioned merits of indigenous IgG production, it was also noted that time period spent on production of antiserum in a laboratory as well as importing it from other countries is same (2-3 months). However, the initial capital investment on strengthening lab capabilities cannot be underestimated.

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