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Production and Characterization of Hepatitis B Recombinant Vaccine in Tobacco (*Nicotiana tabacum* cv. 'Kanchun')

Yogendra Kalenahalli Narasimhamurthy • Paramanahally Hanumanthegowda Ramanjini Gowda* • Sandesh Hakkare Swamidatta • Gunnaiah Raghavendra • Asha Velayudhan Nair • Ningaraju Toresuragondanahalli Malatheshaiah • Deepak Nanjappa

Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore-560065, India

Corresponding author: * **ramanjini@yahoo.com**

ABSTRACT

Immunization with Hepatitis B vaccine is the most effective means of preventing Hepatitis B virus infection and its consequences. Plants are a potential source of Hepatitis B surface Antigen (HBsAg) that is not dependent upon process technology to ensure protein folding and particle assembly. A plant-based HBsAg expression system makes possible the testing of an oral immunization strategy by simply feeding plant samples. The primary means of transformation is *Agrobacterium*-mediated gene transfer which has provided a reliable means of creating transformants in a wide variety of species and also can express important pharmaceutical products. Leaf explants of tobacco were transformed with the Hepatitis B surface antigen gene along with *npt*II as an antibiotic selection marker gene. The presence of the HBsAg gene in putative transformants was confirmed by the presence of a 900-bp band in PCR analysis. The crude protein obtained from the transformed tobacco plants was tested by SDS-PAGE for the presence of a 24 kDa protein. Western-blot and ELISA confirmed the antigen specificity and immunogenic nature of the Hepatitis B surface antigen S-protein. T_1 generation seeds obtained from transgenic tobacco plants were tested for inheritance analysis by germination in the presence of 100 ppm kanamycin. These showed a 3: 1 segregation ratio indicating Mendelian inheritance. Transgenic plants hold promise as low-cost vaccine production systems and this study emphasizes the integration and stability of recombinant protein expressed in tobacco plants.

Keywords: *Agrobacterium*, Hepatitis B vaccine, kanamycin, Mendelian inheritance, surface antigen, surface antigen small protein

Abbreviations: CTAB, N,N,N,N,-cetyl trimethyl ammonium bromide; **ELISA**, enzyme linked immunosorbant assay; **HBsAg**, hepatitis B surface antigen; **kDa**, kilo Dalton; ***npt*II**, neomycin phosphotransferace; **PCR**, polymerase chain reaction; **SDS-PAGE**, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

INTRODUCTION

Hepatitis B virus infection occurs throughout the world and is endemic in Africa, Eastern Europe, the Middle East, Central and Southeast Asia and the Amazon basin of South America. Worldwide Hepatitis B virus causes more than one million deaths per year and about 350-400 million people are persistently infected with this agent. India has been placed into the intermediate zone of prevalence of Hepatitis B (2-7% prevalence rates) by the WHO (Qamer *et al.* 2004). Hepatitis B virus is 42 nm coated DNA virus with a circular, partially double-stranded 3.2 kb genome that preferentially infect hepatocytes and are referred to as hepadnaviridae. Each complete virion consists of an inner core (nucleocapsid or hepatitis core antigen, HBcAg) surrounded by an outer protein coat or envelope (the hepatitis B surface antigen, HBsAg).

Hepatitis B virus infection results from percutaneous or mucosal exposure to the blood or bodily fluids of infected persons. Hepatitis B virus is not transmitted by the fecal–oral route. Young adults (18 to 39 years of age) are at increased risk for hepatitis B virus infection (Gregory *et al.* 2004). Hepatitis B virus vaccine has been recommended as a routine infant vaccination worldwide since 1991 and as a routine adolescent vaccination since 1995. The vaccine is delivered in a series of three intramuscular injections over a six-month period. It requires refrigeration, and injections must be administered by a medical professional, with the total cost ranging between $100 and $150 per person. Hence, plant-based production of vaccine for Hepatitis B may be an economically feasible alternative (Sharma *et al.* 1999).

HBsAg expression has been reported in transgenic tobacco plants (*Nicotiana tabacum* cv. 'Samsun') (Mason *et al.* 1992), tobacco cell lines (*N. tabacum* NT-1) (Sunil Kumar *et al.* 2003), lupin (*Lupinus albus*) and lettuce (*Lactuca sativa*) (Kapusta *et al.* 1999), carrot (*Daucus carota*) (Imani *et al.* 2002) and potato (*Solanum tuberosum*) (Richter *et al.* 2000). He *et al.* (2008) expressed Hepatitis B virus surface antigen middle protein gene (HBV S2+S/HBsAgM) under the control of E8 promoter in tomato and tobacco plants. Qian *et al.* (2008) expressed the SS1 gene that encodes a protein which consists of pre-S1 protein fusion with the truncated C-terminus of HBsAg protein. The expression of the fusion proteinwas controlled by seed specific GluB-4 promoter in rice seeds and highest expression level reported was to 31.5 ng/g dry weight of rice grains. HBsAg derived from transgenic tobacco plants is physically, biochemically and immunologically similar to yeast-derived rHBsAg (Mason *et al.* 1992; Thanavala *et al.* 1995). Looking into all these feasibilities, the present investigation lays emphasis on the study of integration and stability of the HBsAg gene in T_0 (derived directly from tissue culture) and T_1 (first generation of transgenic plants from seeds) generations of tobacco plants by *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Bacterial strain and plasmid vector

The disarmed *Agrobacterium tumefaciens* strain LB4404 (obtained from the National Centre for Biological Sciences, Bangalore, India) was used for transformation. The plant binary vector pHB118 was mobilized into *Agrobacterium* strain by heat shock

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transformation. During this process *Agrobacterium* competent cells were prepared by using 0.1 M $CaCl_2$ and the construct was transferred to competent cells by freezing cells in liquid nitrogen for 5 min. and immediately incubated in 37°C for 5 min. The plasmid pHB118 contains the complete unmodified hepatitis B surface antigen from the hepatitis B virus strain under the control of a modified CaMV 35S promoter. The *npt*II gene is included in the vector as a selectable marker. This construct was provided by Dr. Hugh S. Mason, Arizona Biodesign Institute, USA.

Tobacco plant variety kanchan (*N. tabacum* cv. 'Kanchun') procured from cental tobacco reasearch institute; Rajamundry, India was used as experimental material.

DNA isolation and analysis

Genomic DNA was isolated from second and third leaves from the top of putative transgenic tobacco plants by the CTAB method (2% CTAB, 1.4 M NaCl, 0.02 M EDTA, 0.2% β-mercaptoethanol, 0.1 M Tris-HCl and 1% PVP). Genomic DNA was subjected to polymerase chain reaction (PCR) analysis. 0.025 ml of a PCR mix contained 10 pMol of each primer, 1 U of *Taq* DNA polymerase, 2.5 µM of each dNTP, 1X Taq buffer (Bangalore Genei) and 0.2 µg of genomic DNA as template.

The PCR conditions were 94°C for 2 min of initial denaturetion followed by 30 cycles of amplification with each cycle consisting of following steps: 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec with a final extension of 5 min and a final hold at 4°C after all the cycles were completed (Nagaraju *et al.* 1998). The amplified products were separated on 1.2% agarose (Sigma-Aldrich) gel. The primer sequences used to amplify a 900 bp fragment of the HBsAg gene are: Forward primer (5′-CATTCTACTT CTATTGCAGC-3′) and reverse primer (5′-ACGTGGTAACTTAG ATGTACACCCAAAG-3′) (Zhong *et al.* 2005).

Protein extraction and analysis

Total protein was extracted by using protein extraction buffer (pH 8.0, 0.1 M Tris-Hcl, 0.001 M PMSF, β-mercaptoethanol, sodium sulphate, 2% PVP) from transgenic and control tobacco plants (Nagesha *et al.* 2006). These crude proteins were subjected to ammonium sulphate precipitation and concentrated by dialysis. Electrophoresis through 12% sodium dodecyl sulphate (SDS) gel and visualized through silver staining and Coomasie brilliant blue (CBB) staining.

Protein dot blot

Protein dot blotting is a quick method of confirming the presence of specific proteins in total proteins. This was carried out by spotting 5-10 µL of each transgenic and control plant samples onto a PVDF membrane (Whatman). After the sample was absorbed, the membrane was incubated in blocking solution (5% skim milk powder in 1X phosphate buffer saline) for 1 h at room temperature. This was followed by incubation in primary antibody (mouse anti Hepatitis B surface antigen, Invitrogen) and secondary antibody (goat anti-mouse IgG horse radish peroxidase conjugate, Invitrogen) for 1 h each at room temperature. The signal was detected using DAB (Diamino benzidine) substrate (Bangalore Genei).

Western blotting

The crude protein samples extracted from the transgenic and control tobacco plants were used as antigens for Western blotting. The crude protein samples were run on 12% SDS-PAGE and transfer to a PVDF membrane (Whatman) by electroblotting. This is done by using Mini trans-blot electrophoretic transfer cell (Bio-Rad) by appling 60 V for 2 h. then blocking, incubation with primary and secondary antibodies and detection were similar as described for protein dot blotting.

Enzyme Linked Immunosorbant assay (ELISA)

The crude protein samples of control and transgenic tobacco leaves were used as antigen source for ELISA. They were incubated in blocking solution for 1 h at 37°C. This was followed by incubation with the primary and secondary antibodies for 1 h each at 37°C. Detection involved 0.4 mg/ml of chromogen orthophenylene diamine (Sigma-Aldrich) with 1 µl/ml of hydrogen peroxide as substrate. Absorbance was recorded at 492 nm. The protein extracted from non transgenic tobacco plant was used as negative control and yeast derived HBsAg protein was used as positive control.

Inheritance of the hepatitis B surface antigen by kanamycin assay

Seeds obtained from putative transgenic tobacco plants with hepatitis B surface antigen gene and non-transgenic plants were tested for their germination in the presence of 100 ppm kanamycin (Kan, Sigma-Aldrich) (at this concentration non-transgenic plants cannot survive). The *npt*II gene was used as a selectable marker gene in transformation of tobacco with the HBsAg gene. Expression of the *npt*II gene in transgenic plants confers resistance to Kan and aids in selection of putative transgenics in tissue culture. This trait can also be used to study stable gene expression in transgenics over generations. Observations were made after 10 days of incubation by counting the number of germinated seeds. The ratios were recorded between germinated and non-germinated seeds.

Statistical analysis

The goodness of fit of the observed segregation ratio for the transgene was tested against the Mendelian segregation ratio (3: 1) using the chi-square (χ^2) test. The χ^2 values were calculated using the formula (Greenwood *et al.* 1996):

$$\chi^2 = \sum \frac{(\text{Observed frequencies - Expected frequencies})^2}{\text{Expected frequencies}}$$

RESULTS

PCR

The genomic DNA from putative transgenics, non-transformed tobacco plants from T_0 and T_1 generations and pHB118 plasmid DNA from *Agrobacterium* subjected to PCR, gene-specific primers showed the presence of a 900-bp amplified HBsAg gene fragment in putative transgenics that corresponded to the amplified band of plasmid DNA but it was absent in the untransformed control tobacco when run on a 1.2% agarose gel (**Figs. 1, 2**).

SDS-PAGE

Crude protein from non-transformed control and putative transgenic T_0 and T_1 plants, when subjected to SDS-PAGE, showed the over-expression of HBsAg S-protein by the presence of a 24 kDa band and the absence of the same band in control plants when run on a 12% acrylamide gel (**Figs. 3, 4**).

Protein dot blot and Western blot analysis

The protein product of the HBsAg gene was detected on Western blots using a monoclonal antibody specific for the hepatitis B virus. The successful expression of the HBsAg gene in transgenic tobacco plants showed positive results i.e. a 24 kDa antigenic HBsAg, which was not detected in the non-transformed control plant samples (**Figs. 5, 6**). This indicates that transgenic tobacco plants positively contain the immunogenic HBsAg.

ELISA

ELISA is a rapid and sensitive technique by which the immunogenic nature of a large number of samples can be detected. Enriched mouse anti-HBsAg was used as the primary antibody. The highest absorbance of one of the transgenic samples was 1.10, which was significantly higher than the negative control which gave an absorbance of 0.59

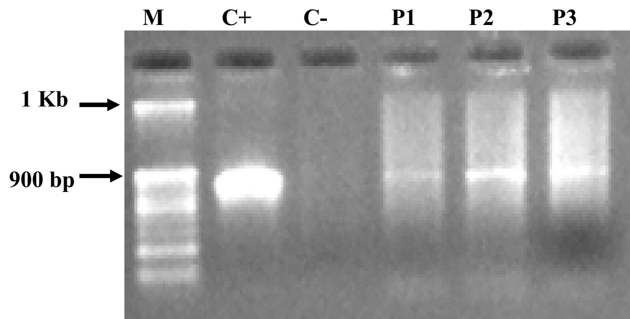

Fig. 1 PCR amplification of Hepatitis B surface antigen in T_0 generation transgenic Tobacco plants with HBsAg gene-specific primers. M, 100 bp DNA marker (Chromus Biotech Pvt. Ltd. Bangalore, India); C+, positive control (plasmid DNA); C-, negative control (non-transgenic tobacco plant); P1- P3: putative transformants.

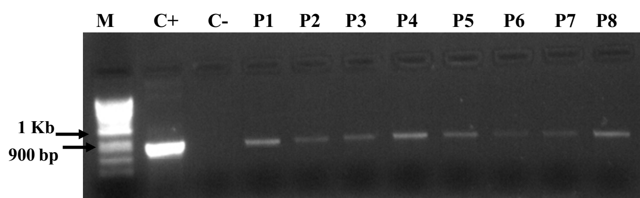

Fig. 2 PCR amplification of Hepatitis B surface antigen in T_1 generation transgenic Tobacco plants with HBsAg gene specific primers. M, 1 Kb DNA marker (Chromus Biotech Pvt. Ltd. Bangalore, India); C+, positive control (plasmid DNA); C-, negative control (non-transgenic tobacco plant); P1- P8: putative transformants.

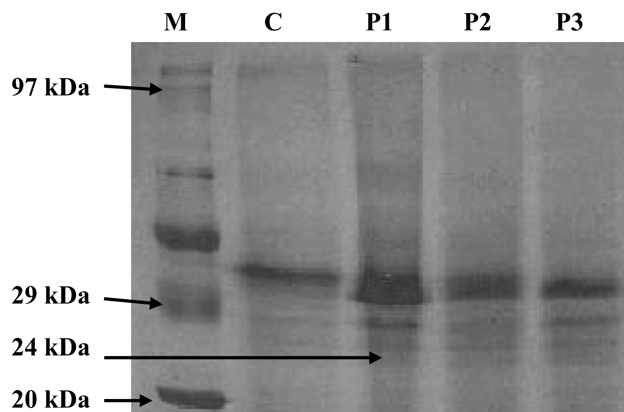

Fig. 3 Confirmation of expressed HBsAg protein in T_0 generation tobacco plants by SDS- PAGE. M, Protein molecular weight marker (Bangalore Genei Pvt. Ltd., Bangalore, India); C, crude protein from control tobacco; P1-P3, crude protein from putative transformants.

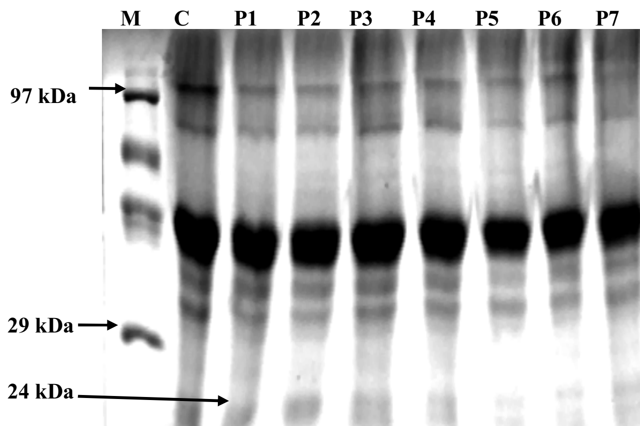

Fig. 4 Confirmation of expressed HBsAg protein in T_1 generation tobacco plants by SDS-PAGE. M, Protein molecular weight marker (Bangalore Genei Pvt. Ltd., Bangalore, India); C, crude protein from control tobacco; P1-P7, crude protein from putative transformants.

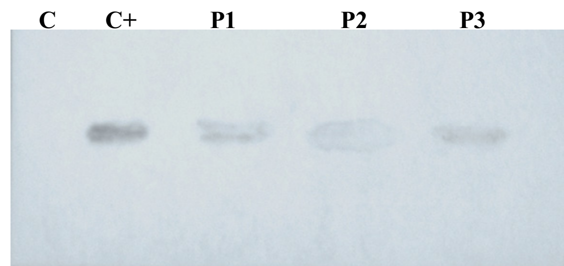

Fig. 5 Western blot analysis showing the expression of the 24 kDa Hepatitis B surface antigen from the T_0 generation transgenic tobacco. C+, positive control (yeast derived HBsAg protein); P1-P3, crude protein from putative transformants; C, crude protein from control tobacco.

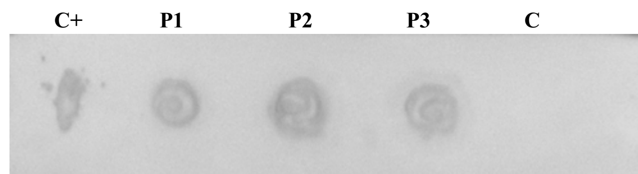

Fig. 6 Protein dot blot analysis showing the expression of the 24 kDa Hepatitis B surface antigen from the T_0 generation transgenic tobacco. C+, positive control (yeast derived HBsAg protein); P1-P3, crude protein from putative transformants, C, crude protein from control tobacco.

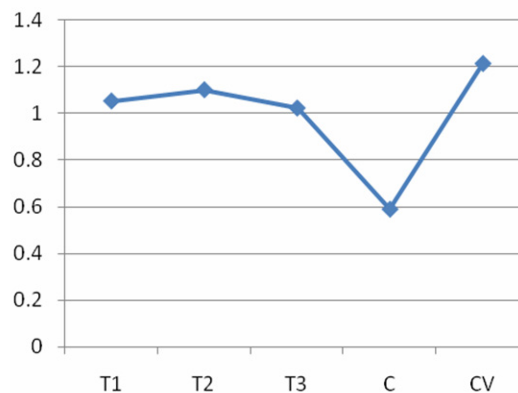

Fig. 7 ELISA absorbance values recorded at 492 nm for T_0 generation of transgenic and control tobacco leaf protein (crude) samples. T1-T3, transgenic tobacco leaf protein samples; C, control tobacco leaf protein sample; CV, commercial vaccine.

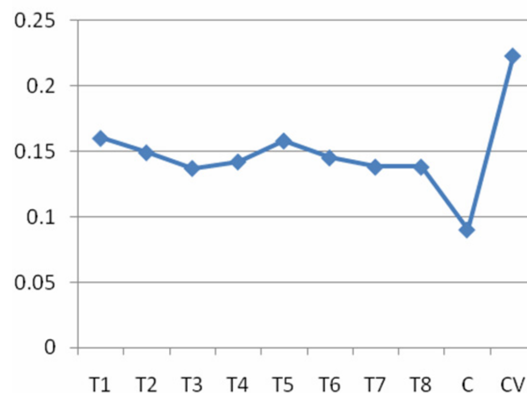

Fig. 8 ELISA absorbance values recorded at 492 nm for T_1 generation of transgenic and control tobacco leaf protein (crude) samples. T1-T8, Transgenic tobacco leaf protein samples; C, control tobacco leaf protein sample; CV, commercial vaccine.

whereas the yeast derived HBsAg protein gave an absorbance of 1.21 (**Fig. 7**). A similar trend was observed in the T_1 generation of transgenic tobacco plants (**Fig. 8**). The higher absorbance values of transgenic plant samples indi-

Table 1 Kanamycin assay for study of transgene expression in transgenic (T_1) and non transgenic tobacco seeds by germinating on 100 ppm of kanamycin.

	№ of seeds sown	Kanamycin (100 ppm)	
		Germinated	Non-germinated
Non Transgenic	32	0	32
T_1	32	23	9
T_2	32	24	8
T_3	32	25	7
Chi-square (χ^2) test			
	Observed	Expected	Chi-square (χ^2) value
Transgenic seeds			
Germinated	72	72	0.0
Non germinated	24	24	0.0
Non transgenic seeds			
Germinated	0	0	0.0
Non germinated	32	32	0.0

cate the immunogenic nature of the sample, which confirms the presence of the recombinant HBsAg. This validates the earlier SDS-PAGE and Western blot results.

Inheritance analysis of HBsAg gene in seeds obtained from transgenic tobacco

The seeds obtained from putative transgenic tobacco plants with the HBsAg gene were tested for their germination in the presence of 100 ppm of Kan. The seeds obtained from the transgenic tobacco plants showed the Hepatitis B surface antigen segregation ratio of 3:1 as expected confirming to Mendelian inheritance, when they were germinated in presence of 100 ppm of kanamycin (**Table 1**).

The chi-square test indicated that the observed data/ ratio fits the Mendelian segregation ratio. We can conclude that there was no difference between the observed and expected ratio, null hypothesis was accepted.

DISCUSSION

Tobacco is readily amenable for genetic transformation. Hence, most of the researchers used tobacco for expression of the recombinant protein. The other advantage being it is a short duration crop produces enormous biomass. The advantage of transgenic tobacco is that it is a non-food crop; hence there is no chance of entering into the food chain. Even though tobacco contains nicotine, a process can be adopted to remove the nicotine content from tobacco extracts and a recombinat protein can be used for therapies. The yield of extractable tobacco protein varied between 155 and 228 kg/ha (Woodleif *et al*. 1981). Mason *et al*. (1992) reported the expression of HBsAg in tobacco (*Nicotiana tobaccum* cv. 'Samsun'). The expression levels were as high as 66 ng/mg of the total soluble protein in leaf.

For studying stable expression, we have transformed tobacco (*Nicotiana tobaccum* cv. 'Kanchun') with pHB118. The presences of transgene in putative transgenic plants were evident by PCR in both T_0 and T_1 generations. The transgenic lines yielded a 0.9 kb band, the same as pHB118 plasmid; the PCR product was absent in non-transgenic plants. The results confirm those of Zhong *et al*. (2005), although these authors transformed *Nicotiana benthamina* plants with pHB117 and pMHB plasmid constructs.

Recombinant pHB118 protein was extracted from mature tobacco leaves by ammonium sulphate precipitation. The total protein was run on a 12% acrylamide gel and a 24 kDa band was observed only in transgenic plants. Western blotting was performed to confirm the expression of the HBsAg protein in transgenic plants. The 24 kDa yeast-derived HBsAg protein was compared with the recombinant protein produced in transgenic tobacco plants. A band at the same position was found in both. ELISA was conducted using transgenic and non-transgenic tobacco plant proteins. The higher absorbance values of transgenic plant samples indicated the immunogenic nature of the sample, which confirms the presence of the recombinant HBsAg.

The seeds obtained from T_0 and T_1 generation transgenic tobacco plants showed the hepatitis B surface antigen, segregated in a 3: 1 ratio, as expected, confirming Mendelian inheritance, when they were germinated in the presence of 100 mg/l kanamycin. This result is in confirmation with the findings of Vasil *et al*. (1993) who transferred the gene coding for phosphinothricin acetyl transferase (PAT) in wheat plants and PAT activity was detected in a 3: 1 ratio in R_1 generation plants following cross or self pollination. Both male and female transmission of the PAT gene and its segregation as a dominant Mendelian ratio in R_1 and R_2 plants. Becker *et al*. (1994) showed contradictory results: histochemically stained pollen grains of a *GUS* gene transformed wheat plants showed a 1:1 segregation of the *uid A* gene in all the plants tested. A 3:1 segregation of the introduced gene was demonstrated by enzyme sensitivity test and southern blot analysis of R_1 generation plants.

CONCLUSIONS

There is a possibility to transform and express tobacco with the HBsAg gene using *Agrobacterium*-mediated transformation. The integration of recombinant HBsAg gene in transgenic tobacco plants was shown by the presence of a 900 bp band with HBsAg gene-specific primers. The expression of 24 kDa Hepatitis B surface antigen in transgenic tobacco plants were shown by SDS-PAGE analysis. The specificity of the crude protein from transgenic tobacco plants were also analyzed by western blotting and dot blot. The stable integration and expression of the gene in T_0 and T_1 generations were confirmed by PCR, SDS-PAGE, western blot, ELISA and kanamycin assays.

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