A VERO CELL-ATTENUATED GOATPOX VIRUS PROVIDES PROTECTION AGAINST VIRULENT VIRUS CHALLENGE

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Summary. – An Indian isolate of Goatpox virus (GTPV) was adapted and propagated in Vero cells for development of an attenuated virus. The virus was initially passaged in primary lamb testes cells and subsequently in Vero cells. At the 55th passage, the virus showed evidence of attenuation when tested for safety in sero-negative goats. At this stage, the virus was found to be completely non-pathogenic. The virus was passaged further and the 60th passage was usedfor testing its immunogenicity in goats. The latter were inoculated with 10, 100 and 1000 $TCID_{50}$ of the attenuated virus by intradermal (i.d.) route and challenged after 28 days with virulent GTPV. The attenuated virus produced no adverse reaction even at the highest dose and conferred complete protection even at the lowest dose against challenge with a high dose (2×10^6 of 50% skin-reactive dose SRD_{50}) of virulent virus. Increased levels of virus-specific serum antibodies could be demonstrated by both indirect enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test in all the immunized goats. No horizontal transmission of the virus from the immunized to in-contact animals took place. Our results suggest that this attenuated virus could be a safe, immunogenic and potent candidate for developing a vaccine against goatpox.

Key words: attenuated Goatpox virus; immunogenicity; Vero cells

Introduction

Goats of over 120 million population constitute an important component of Indian livestock industry from which benefits a large rural poor population. Amongst a number of infections that adversely affect caprine productivity, goatpox is one of the important diseases with a serious economic impact in terms of lost productivity, mortality, hide damage and losses due to international trade restrictions. Goatpox is a highly contagious viral disease of goats characterized by fever, oculo-nasal discharges, pock lesions on the skin and mucosae of respiratory and gastro-intestinal tract. The disease caused by GTPV is listed in the Group A diseases of the OIE (Office International des Epizooties) and the virus is classified in the species *Goatpox* virus, the genus *Capripoxvirus,* the family *Poxviridae*. The other species of the genus are *Sheeppox virus* (SPPV) and *Lumpy skin disease virus* (LSDV) (Van Regenmortel *et al*., 2000).

Goatpox is endemic in Southwest and Central Asia, Northern and Central Africa and Indian subcontinent (Tulman *et al*., 2002). Control of the disease is possible through an effective immunization with a potent vaccine; preferably with a live attenuated one (Kitching, 1983; Carn, 1993). Considering the low level of immunity and the drawbacks associated with inactivated vaccines, live attenuated vaccines are the best choice as they provide long lasting immunity. Furthermore, results of the studies on cross-protection of sheep and goats against goatpox and sheeppox and other related diseases such as contagious

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Abbreviations: CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; EMEM = Eagle's Minimum Essential Medium; GTPV = Goatpox virus; HBSS = Hank's Balanced Salt Solution; HRPO = horseradish peroxidase; i.d. = intradermal; LT = lamb testes; NI = neutralization index; OPD = ortho-phenylene diamine; p.c. = post challenge; p.i. = post immunization; p.inf. = post infection; SRD_{50} = 50% skin reactive dose; VN = virus neutralization

pustular dermatitis are often contradictory and inconclusive (Ergin *et al*., 1988; Wang and Jiang, 1988). The attempts to protect either goats with SPPV vaccines or sheep with GTPV vaccines have been unsuccessful (Prasad and Datt, 1973). It is usually recommended that for effective protection of goats against goatpox homologous vaccines should be used (Rao and Bandyopadhyay, 2000). In India, a live attenuated vaccine derived from lamb testes cells and prepared from Rumanian Fanar strain of SPPV is widely being used to control sheeppox. However, at present no live attenuated vaccine for goatpox is available. In this study we report the development of an attenuated GTPV by serial passaging of an Indian isolate in Vero cell and its safety, potency and protective immune response in goats.

Materials and Methods

Virus. The Uttarkashi isolate of GTPV (Das *et al*., 1978) was initially isolated in the Naugaon block of the Uttarkashi district, India from a natural outbreak. The virus was propagated in primary lamb testes (LT) cell cultures (34 passages) and subsequently adapted to Vero cells (further 26 passages). At the 55th passage level (34 LT cell and 21 Vero cell passages), the virus was found to have lost its virulence when tested in sero-negative goats. The virus was further passaged in Vero cells and the 60th passage was used in the experiments. The virus produced cytopathic effect (CPE) in Vero cells characteristic by clumping, rounding and ballooning of cells. Like in lamb testes cells, CPE appeared on days 3–4 post infection (p.inf.) and progressed further till 80–90% of the confluent layer on day 7 p.inf. The titer of the virus grown in Vero cells as determined by the method of Reed and Muench (1938) was found to be $10^{7.0}$ $TCID_{50}$/ml. The Mukteswar isolate of virulent GTPV, (Haddow and Idnani, 1946) was used for challenge as this virus has been found to be more virulent than the Uttarkashi isolate. The challenge virus has been maintained by regular passaging in goats. Titration of this virus was carried out by i.d. inoculation of serial 10-fold dilutions of virus in triplicate on the abdomen of goats. The virus titer was 2 x 10^6 SRD_{50}/ml (Reed and Muench, 1938).

Animals. Healthy goats of either sex, aged between six months and one year were used in the experiment. They were screened for GTPV antibodies using an indirect ELISA and then randomly grouped prior to immunization.

Cell cultures. Primary lamb testes cell cultures were prepared from young lambs (<6 months in age) and maintained in Glasgow's Modified Minimum Essential Medium (GMEM, Sigma USA) supplemented with 10% of newborn calf serum (Hyclone, USA). Vero cells (ATCC, USA) were grown in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% of newborn calf serum.

Immunization of animals was done in four groups, namely A (n = 4), B (n = 6), C (n = 6), and D (n = 7) with 10^1, 10^2 and 10^5 $TCID_{50}$ of attenuated virus (the 60th passage), respectively. The virus (0.2 ml) was inoculated i.d. on the caudal skin fold (underneath tail). Group D (n = 7) served as unimmunized control in which each animal received 0.2 ml of Hank's Balanced Salt Solution (HBSS) by the same route. Three goats from this group were kept in contact (group D1) with the immunized animals while others served as non-contact control (group D2) animals. Sera were collected from both the immunized and in-contact animals on days 0, 14 and 28 p.i. The goats were challenged on day 28 p.i. by inoculating 2 x 10^6 SRD_{50} of virulent GTPV i.d. on ventral abdomen. Serum samples were collected from all the animals on day 7 post challenge (p.c), i.e. on day 35 p.i. Animals were examined daily for thermal reaction, development of local skin reaction at the site of inoculation or secondary skin lesions and other symptoms following immunization and challenge.

ELISA. Serum antibody response in animals was monitored by an indirect ELISA using the method described by Sharma *et al*. (1988) with a little modification. Antigen was prepared by sonication of GTPV-infected Vero cells and the supernatant containing viral proteins was coated onto a flat-bottom 96-well plate (Maxisorp, Nunc, Denmark) at optimized dilution by incubation at 37°C for 1 hr. The unbound sites in the wells were blocked by addition of a blocking solution (5% skim milk powder + 3% gelatin in PBS) for 1 hr at 37° C. Diluted sera (1:150) were added in duplicate and incubated for 1 hr at 37°C. A goat anti-HRPO conjugate (Sigma, USA) was added in appropriate dilution to each well and incubated at 37°C for 1 hr. The plates were washed 3 times with PBS (prewarmed to 37°C) containing 0.05% Tween-20 between each incubation step. Color was developed by addition of the substrate (Sigma) and A_{492} was measured in an ELISA reader (TECAN A5082, Austria).

VN test of serum samples collected on day 27 p.i was done by the procedure described earlier (Kitching, 1986). A modification consisted of use of ten-fold dilutions of attenuated GTPV (the 60th passage) against a constant serum dilution. Heat-inactivated sera were diluted 1:5 in EMEM and used in the test. Virus neutralization index (NI) was expressed as a log difference in the titer of virus in the presence of normal serum and in that in the presence of immune serum. The final reading was taken on day 8 p.i.

PCR with the primers B68 and B69 (Heine *et al*., 1999) for detection of GTPV in the scab or skin biopsy of the immunized and control goats after challenge was employed. Skin biopsies were collected on day 13 p.c. from the challenge site of an immunized animal (group B) and from a secondary lesion of a control animal. DNA was isolated from the biopsy and PCR was carried out. DNA from the Mukteswar isolate of GTPV was used as a positive control. The PCR products were electrophoresed in agarose gel and visualized in a standard way.

Results

Clinical monitoring of immunized animals

There was a local skin reaction ("take") at the site of virus inoculation and this reaction was observed in all the immunized animals on day 5 p.i. The size of the "take" (erythema) varied between 0.5 and 1.5 cm in diameter and found to be dose-dependent. Two of the group C animals that received 10^5 $TCID_{50}$ had a marked local reaction leading to formation of scar, which subsided gradually. Animals

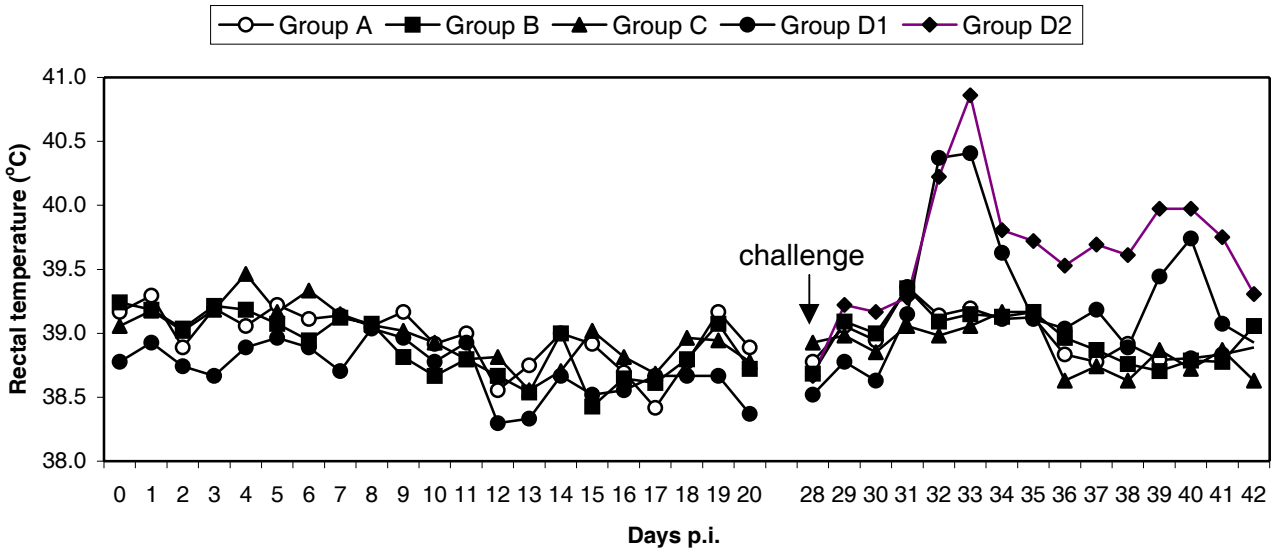

Fig. 1

Thermal response of different groups of goats after immunization and subsequent challenge on day 28 p.i.

The values indicate the mean rectal temperature in each group.

immunized with 10 and 100 $TCID_{50}$ showed mild erythema and/or swelling. Although, there was no pyrexia in any of the immunized animals, the group C animals (10^5 $TCID_{50}$) showed marginal rise in body temperature (39.46 ± 0.40°C) on day 4 p.i (Fig. 1). The size of the "take" was also bigger in these animals. All the animals appeared healthy showing no apparent signs of the disease after immunization.

Experimental protection

Following challenge with the virulent virus, the goats were critically examined for clinical manifestation of the disease. There was no pyrexia in the immunized animals, however, a few animals in the group B showed marginal rise in rectal temperature (the group B average temperature was 39.35 ± 0.35°C) with a maximum of 39.8°C temperature in one of the goats on day 3 p.c. No systemic or local reactions were observed in the immunized animals, except two goats, in which there was mild local reaction at the site of the challenge inoculation. All the immunized animals withstood challenge and remained healthy.

The unimmunized control animals (the group D) developed papulae at the site of i.d. challenge, which gradually acquired pustular and necrotic appearance, followed by scab formation. Diffuse edematous swelling was also observed in the areas adjacent to inoculation sites. There was high pyrexia (up to 41.4°C in some animals) with signs of dullness and anorexia on day 5 p.c. and the symptoms became pronounced between days 6 and 7 p.c. The temperature declined gradually followed by secondary rise between days 11 and 13 p.c. (Fig. 1). Six out of seven goats developed generalized papules on the abdomen, neck, thigh, udder/testes. In some animals, lesions were confined to lateral abdomen only. No animal died of goatpox after the challenge.

Serum antibodies

Serum antibodies were measured by indirect ELISA. The attenuated virus elicited high level of serum antibodies in all the immunized animals. The presence of antibodies that could possibly be directed against Vero cells was ruled out by reacting the sera with various dilutions of the uninfected Vero cell antigen. There was no significant difference in the antibody response among the animals from different immunized groups. High antibody level was noticed on day 14 p.i., which did not change until day 28 p.i. with a further increase on day 7 p.c. (Fig. 2). Animals from the control group showed a rise in antibody level after the challenge; however, this rise was considerably lower as compared to immunized animals.

All the immunized animals showed a high titer of neutralizing antibodies (Fig. 3), while control animals showed no rise in the neutralizing antibody level. NI ranged from 0.5 to 1.75 $TCID_{50}$ in the immune serum samples. The group C animals exhibited a slightly higher NI as compared to other two groups. The ratio between VN titers and ELISA values was also determined and was found to be 0.8.

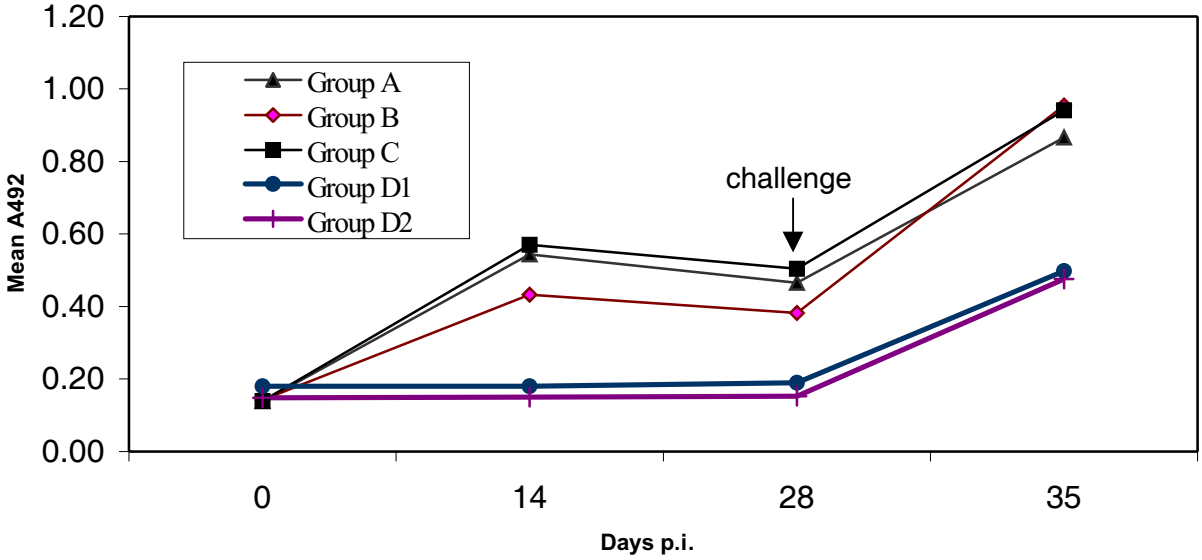

Fig. 2

Serum antibody response measured by ELISA in different groups of animals at weekly intervals

The curve was derived by averaging duplicate A_{492} values of pre-diluted serum samples. No sero-conversion was found in in-contact unimmunized animals.

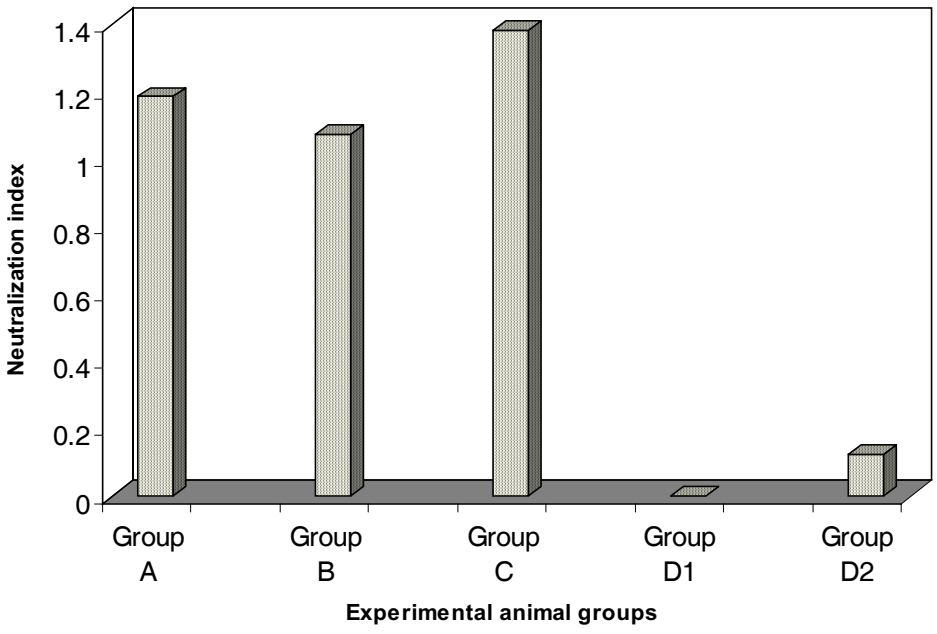

Fig. 3

NI of sera collected on day 27 p.i.

NI are expressed as log differences in the titers of GTPV in the presence of normal serum and in those in the presence of immune sera. The VN test was done on day 8 p.inf.

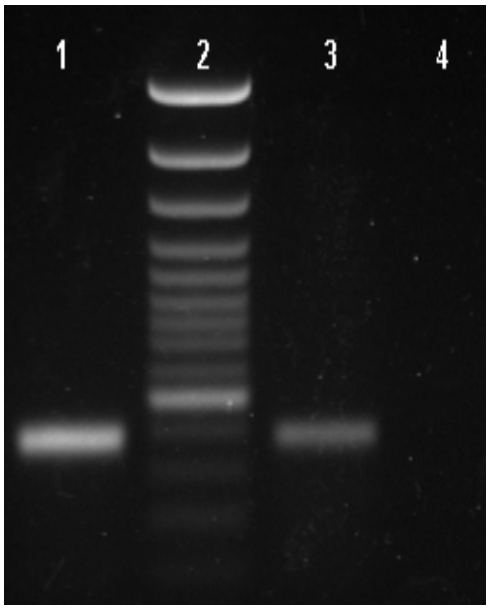

Fig. 4

Agarose gel electrophoresis of GPV DNA amplified by PCR

The 390 bp PCR product, positive control (lane 1); the 100 bp ladder, DNA size standard (lane 2); the 390 bp PCR amplicon from a secondary lesion of a control goat (lane 3); no PCR product from a skin biopsy from an immunized goat (lane 4).

Replication of challenge virus in immunized animals

In order to examine replication of the challenge virus in the immunized animals, a capripox-specific PCR was employed. A 390 bp region of viral DNA was amplified from a secondary lesion from the forelimb of an unimmunized control animal on challenge (Fig. 4), which indicated that the challenge virus actively multiplied in control animals. No amplification was observed in the skin biopsy from the challenge site from the immunized animals, which obviated the assumption that the challenge virus did not replicate locally.

Discussion

Capripox infections can be effectively controlled because of stable nature of the virus. In endemic situation, control of the disease is possible through immunization with a potent vaccine. In the present work, an Indian isolate (Uttarkashi) of GTPV was attenuated by serial passaging (34 passages) in primary lamb testes cell cultures and subsequently in Vero cell cultures (26 passages) and its immunogenicity was tested in goats. The pathogenicity of the virus was tested in goats at the 55th passage (34 LT cell + 21 Vero cell passages) and the virus was found to have lost its virulence. However, the virus was passaged further up to the 60th passage in order to stabilize the attenuation. Heterologous cell systems like bovine fetal muscle cells (Carn, 1993) and baby hamster kidney cells (Kitching *et al.,* 1986) have been used for development of attenuated capripox vaccines, which were safe, stable and potent in animals. Since Vero cells is a heterologous host system as compared to goat- or sheep-derived cells in primary cultures, the attenuation is considered more effective. More over, virus production in Vero cells offers advantages such as cost-effective production and freedom from adventitious agents.

In this work, Vero cell-attenuated GTPV provided complete protection against virulent challenge. It produced no adverse reaction at high challenge virus dose (10^5 $TCID_{50}$), and at the same time, it protected goats at even 10 $TCID_{50}$ against challenge with a high dose (2 x 10^6 SRD_{50}) of virulent virus. Such an exposure to virus is unlikely under natural conditions.

Following immunization, the virus caused slight rise in body temperature and formation of "take" or local hypersensitivity at the site of inoculation. Slight rise in body temperature is a physiological phenomenon associated with secretion of interleukin-1, provided the immune response is triggered by any antigen. Local reaction is considered one of the disadvantages with live pox vaccines (Rao and Bandyopadhyay, 2000); nevertheless, it is a good indicator of viability of the vaccine virus. Since the size of "take" formation was dose dependent, the hypersensitivity may not be of concern at the field dose (10^3 $TCID_{50}$) of vaccine. Mild local reaction, but no secondary lesions were also noticed at the site of challenge in two immunized animals, but they waned off gradually. This may be explained by the fact that local spread of infection proceeds from cell-to-cell and circulating antibodies induced by infection or vaccination limit the spread of the virus in the animal, but it does not prevent virus replication at the site of infection (Boulter and Appleyard, 1973).

Replication of the challenge virus was investigated by detection of viral nucleic acid in skin biopsies by PCR. There was no development of secondary skin lesion in any of the immunized animals. Hence, skin biopsies were used from the site of challenge virus inoculation for amplification of viral DNA. No viral DNA was detected in biopsy samples by PCR. This indicated neutralization of the challenge virus in the immunized animals. In contrast, viral nucleic acid was detected in secondary lesions on control animals. This implies replication of the challenge virus due to lack of humoral/cell-mediated immunity.

Immunity to poxvirus is considered predominantly cell-mediated and the immune status of animals does not correspond to serum neutralizing antibody titers (Carn,

1993). However, high level of serum antibodies was detected in immunized animals by ELISA, which showed anamnestic response to the challenge. Humoral response could also have a role in protecting animals. Animals of the group B that received 100 $TCID_{50}$ of the virus exhibited a marginally lower NI compared to that of the group A animals (10 $TCID_{50}$ of the virus), which could be hardly explained. Mild local reaction observed in two of the immunized goats incidentally showed lower antibody response in ELISA but not in VN titers. Both VN and ELISA showed a good correlation, the correlation coefficient was 0.80. However, since ELISA is more sensitive than VN and neutralizing antibody titers are usually low in poxvirus infection, ELISA can successfully be used in monitoring the immune response of immunized animals. The antibody response was similar in animals immunized with various doses of the virus and no such a response was evident in in-contact animals, suggesting that the vaccine virus did not spread horizontally.

SPPV and GTPV are generally considered host-specific though some strains are reported to affect sheep and goats to a different extent (Carn, 1993; Munz and Dumbell, 1994). Cross-protection within the *Capripoxvirus* genus was first shown by Capstic and Coackley (1961). Since then there has been interest to develop a single vaccine for protecting both sheep and goats. A single vaccine has later been developed from the 0240 strain of Kenyan sheep and goat pox, which protected both sheep and goat against virulent strains of capripoxvirus (Kitching *et al*., 1986) that was equally pathogenic for sheep and goats. This vaccine has been used in many countries in the Middle East and Africa with satisfactory results. However, no single strain of poxvirus has been reported from India, where it caused the disease both in sheep and goats in a mixed flock. Also the host specificity of Indian strains of sheep and goat poxvirus has not yet been clarified. Therefore, vaccination with homologous strain appears to be a better measure to control capripox in India. We have not tested our attenuated GTPV for protection against sheeppox, though cross-protection is likely to occur because of antigenic relationship amongst the capripox viruses (Kitching and Taylor, 1985; Subbarao *et al*., 1984).

The results described here showed that the Vero cell-attenuated GTPV gave complete protection against virulent challenge and could be used to develop a potent live attenuated vaccine. Further work is in progress (i) to study the cross-protection ability of the attenuated virus and (ii) to evaluate the attenuated virus using a larger number of animals.

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