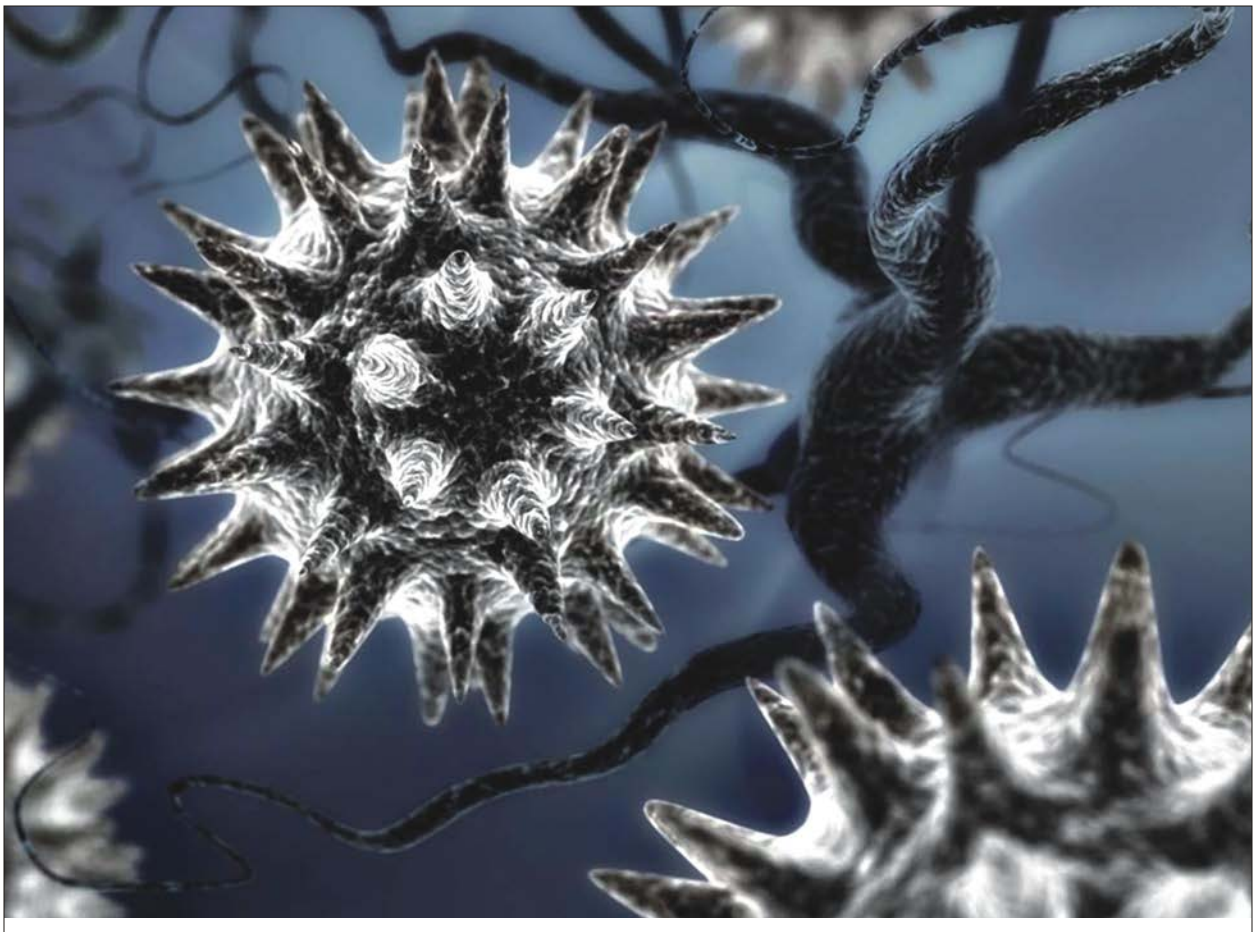

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology 7 (1): 33-38, 2011
ISSN 1816-4900 / DOI: 10.3923/ijv.2011.33.38

Attenuation of *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus (AmCPV) and its Potential as an Oral Vaccine Against Virus Diseases in Tasar Silkworm, *Antheraea mylitta* D

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ABSTRACT

The aim of the present investigation was to attenuate the *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus (AmCPV) and to test their efficacy against the AmCPV infection in tasar silkworm. AmCPV was attenuated by treatment of Na_2CO_3 and formalin. The attenuated AmCPV were tested for its potential as oral vaccine in tasar silkworm, *Antheraea mylitta* D. against the infection of cytoplasmic polyhedrosis virus. The vaccination with attenuated AmCPV 24 h prior to challenge inoculation was effective to protect tasar silkworm from infection of cytoplasmic polyhedrosis virus. It was effective at higher dosages of 30 and 40 μL mL^{-1}. Triple vaccination protected silkworm for comparatively a longer period and reduced the mortality 80.78% due to cytoplasmic polyhedrosis virus infection.

Key words: *Antherae mylitta*, cytoplasmic polyhedrosis virus, attenuation, vaccination, challenge inoculation

INTRODUCTION

Silkworm diseases are major constraint in silk cocoon production. Among diseases in tasar silkworm, *Antheraea mylitta* D. cytoplasmic polyhedrosis commonly known as virosis caused by Cytoplasmic Polyhedrosis Virus (CPV), a reovirus is highly contagious and prevails all through the year in tasar culture regions. In India, virosis accounts for considerable loss to cocoon production (25-30%) and is most common during rainy and autumn season i.e., July to October (Sahay *et al*., 2000). At present, the disease is managed following preventive measures using chemical disinfectants which have limitations to be effective in open and out door rearing and hazardous to the environment and users (Balavenkatasubbaiah *et al*., 1994, 1999). The tolerance to the disease among the silkworm breeds is variable. In the biological field, in the event of non-availability of resistance in the host to a particular virus infection, vaccination of the host is one of the most sought after approach to prevent the infection (Ourth and Parker, 2006). Some efforts have been made by few workers to induce tolerance to virus diseases in mulberry silkworm, *Bombyx mori* by feeding inactivated viruses (Sivaprakasham and Rabindra, 1995;

Nataraju *et al.*, 2000). Even though some work has been done on the breeding aspects of Tasar silkworm, not much study has been published so far on pathological aspects of tasar silkworm (Reddy *et al.*, 2010a,b). Some study has been carried out on biochemical changes in tasar silkworm related to stress (Pandey *et al.*, 2010; Kumar *et al.*, 2011). To date reports on attenuation of AmCPV and its use as an oral vaccine to protect the tasar silkworm from virus infection are scanty. Hence, in the present investigation an attempt was made to attenuate the AmCPV and to test their efficacy against the AmCPV infection in tasar silkworm.

MATERIALS AND METHODS

This study was carried out during the period July, 2009 to October, 2010 in Silk worm Pathology section, Central Tasar Research and Training Institute, Piska Nagri, Ranchi, Jharkhand, India.

Cytoplasmic polyhedrosis virus (AmCPV) inoculum: Fresh cytoplasmic polyhedrosis virus inoculum's was prepared from diseased silkworm. Completely whitened mid-gut obtained from cytoplasmic polyhedrosised silkworm at an advanced stage of infection were homogenized in sterile distilled water. The polyhedral suspension was filtered through a cheese-cloth and the filtrate was centrifuged at 3000 rpm for 15 min and the polyhedra were purified following Aizawa (1971) by repeated and differential centrifugation. The resultant pellet suspended in distilled water was examined by light microscope for purity. The polyhedral suspension in sterile distilled water was prepared to contain 1×10^5 polyhedra mL^{-1}.

Attenuation of cytoplasmic polyhedrosis virus (AmCPV): The AmCPV polyhedra was treated by 0.114 M Na_2CO_3 (pH 10.35) at 27°C for 24 h, then by 0.02% formaline at 27°C for 36 h. This attenuated AmCPV was used as vaccine against AmCPV infection in tasar silkworm. The attenuated AmCPV was tested by bioassay to confirm their no-infectivity to silkworm.

Bioassay of attenuated cytoplasmic polyhedrosis virus (AmCPV): The attenuated AmCPV was tested by bioassay to confirm their no-infectivity to silkworm. Then the attenuated AmCPV was tested for their efficacy as vaccine by oral vaccination to tasar silkworm inoculated with different doses of Polyhedra Occlusion Bodies (POB) of AmCPV (1×10^1, 1×10^2, 1×10^3 and 1×10^4 POB mL^{-1}), 24 h prior or post vaccination. The AmCPV POB/attenuated AmCPV were smeared on to the *Terminalia tomentosa* (assan) leaves and fed to Daba bivoltine silkworm breed. The silkworms were inoculated with AmCPV POB/vaccine on second day of first instar. To determine the effectiveness of multiple vaccination, one time vaccination on second day of first instar (single vaccination) and immediately after I moult (double vaccination) and II moult (Triple vaccination), were given. The effectiveness and functional doses were determined by bioassay for AmCPV infection and mortality due to disease. The treated and controlled larvae reared in indoor rearing condition and the cumulative mortality due to AmCPV was recorded, every day and microscopic examination. Each treatment had three replications of 50 larvae each.

Data analysis: For data analysis the statistical computer application package SPSS 10.0 was employed. The data generated were average of three independent experiments. Data were subjected to analysis of variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; $p = 0.05$) (Duncan, 1955).

RESULTS

Effect vaccination on *Antheraea mylitta* cytoplasmic polyhedrosis virus (AmCPV) infection in tasar silkworm: The results of effect of vaccination on mortality in AmCPV inoculated tasar silkworm presented in Table 1. It is observed that the vaccination of attenuated AmCPV provides protection against development of virosis disease for a short period (6 to 8 days). The vaccination 24 h prior to AmCPV inoculation was more effective to delay and reduce the mortality due to AmCPV infection. The cumulative mortality was 0.00% on 6th day which increased to 16.67% on 16th day. The mortality in vaccinated silkworms was reduced significantly as compared to non vaccinated inoculated control (15.33% on 6th day which increased to 52.33% on 16th day). The vaccination after inoculation was comparatively ineffective as the mortality was higher (8.33% on 6th day which increased to 47.33% on 16th day.

Dosage effect of vaccination on infection of AmCPV in tasar silkworm: The results presented in Table 2 depicts that the higher doses of 30 and 40 μL mL^{-1} were observed to be effective to provide protection against AmCPV infection in tasar silkworm. Cumulative mortality was significantly reduced (17.67 and 16.00%, respectively on 16th day) than the lower doses of 10 and 20 μL mL^{-1} (43.67 and 32.33%, respectively on 16th day) and non vaccinated inoculated control (50.67% on 16th day).

Table 1: Effect of vaccination (50 μL mL^{-1}) on mortality in AmCPV inoculated (1×102 POB mL^{-1}) tasar silkworm, *A. mylitta* D

	Cumulative mortality (%) due to AmCPV infection					
	Days after inoculation of AmCPV					
Treatment	6	8	10	12	14	16
Vaccination prior to inoculation	0.00	0.00	1.67±0.35^{c}	3.00±0.34^{c}	7.33±0.37^{c}	16.67±0.32^{c}
Vaccination after inoculation	8.33±0.23^{b}	16.33±0.23^{b}	20.00±0.34^{b}	24.33±0.33^{b}	30.67±0.32^{b}	47.33±0.37^{b}
Inoculated control	15.33±0.31^{a}	21.33±0.34^{a}	25.67±0.31^{a}	35.67±0.31^{a}	41.33±0.31^{a}	52.33±0.23^{a}
Vaccinated control	0.00	0.00	0.00	0.00	0.00	0.00
Normal control	0.00	0.00	0.00	0.00	0.00	0.00

Every value represents the Mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Table 2: Effect of vaccine dosage on cytoplasmic polyhedrosis in tasar silkworm, *A. mylitta*

	Cumulative mortality (%) due to AmCPV infection					
	Days after inoculation of AmCPV					
Vaccine dosage (μL mL^{-1})	6	8	10	12	14	16
10	2.33±0.31^{b}	5.67±0.33^{b}	9.67±0.13^{b}	15.33±0.34^{a}	22.67±0.33^{b}	43.67±0.37^{b}
20	1.67±0.37^{c}	3.33±0.31^{c}	7.67±0.43^{c}	11.67±0.31^{a}	18.33±0.43^{c}	32.33±0.31^{c}
30	0.00	0.66±0.31^{d}	2.33±0.43^{d}	6.66±0.23^{a}	12.33±0.23^{d}	17.67±0.27^{d}
40	0.00	0.00	1.33±0.33^{e}	6.33±0.34^{a}	12.00±0.31^{d}	16.00±0.13^{e}
Innoculated control	10.67±0.31^{a}	13.33±0.23^{a}	18.67±0.43^{a}	25.67±0.37^{a}	35.33±0.37^{a}	50.67±0.23^{a}
Vaccinated control	0.00	0.00	0.00	0.00	0.00	0.00
Normal control	0.00	0.00	0.00	0.00	0.00	0.00

Every value represents the Mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Table 3: Effect of vaccination against different dosage of AmCPV and development of development of disease in tasar silkworm

	Cumulative mortality (%) due to AmCPV infection					
	Days after inoculation of AmCPV					
AmCPV (POB mL^{-1})	6	8	10	12	14	16
1×10^{1}	0.00	0.00	2.33±0.34^{e}	5.67±0.37^{e}	8.67±0.31^{e}	10.67±0.13^{e}
1×10^{2}	0.00	1.67±0.43^{d}	3.67±0.32^{d}	7.33±0.34^{d}	11.67±0.37^{d}	15.67±0.33^{d}
1×10^{3}	3.33±0.32^{c}	5.67±0.53^{c}	9.67±0.31^{c}	15.33±0.27^{c}	18.67±0.43^{c}	20.33±0.37^{c}
1×10^{4}	4.33±0.37^{b}	6.67±0.63^{b}	12.33±0.37^{b}	20.67±0.29^{b}	26.67±0.32^{b}	32.33±0.35^{b}
Innoculated control	10.67±0.23^{a}	13.33±0.23^{a}	18.67±0.31^{a}	25.67±0.32^{a}	35.33±0.37^{a}	49.67±0.23^{a}
Vaccinated control	0.00	0.00	0.00	0.00	0.00	0.00
Normal control	0.00	0.00	0.00	0.00	0.00	0.00

Every value represents the Mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Table 4: Effect of multiple vaccination in prevention of AmCPV infection tasar silkworm

	Cumulative mortality (%) due to AmCPV infection							
	Days after inoculation of AmCPV							% reduction
Vaccination stage	6	8	10	12	14	16	18	in mortality
1st instar	0	1.67±0.37^{b}	2.67±0.42^{b}	5.67±0.34^{b}	10.33±0.31^{b}	15.67±0.33^{b}	18.67±0.37^{b}	62.90
1st and 2nd instar	0	0	0	3.33±0.37^{c}	5.67±0.34^{c}	10.67±0.31^{c}	13.33±0.31^{c}	73.51
1st, 2nd and 3rd instar	0	0	0	1.67±0.33^{d}	4.67±0.37^{d}	7.33±0.27^{d}	9.67±0.37^{d}	80.78
Vaccinated control	0	0	0	0	0	0	0	
Innoculated control	12.67±0.23^{a}	14.67±0.33^{a}	17.33±0.73^{a}	26.67±0.23^{a}	37.33±0.23^{a}	46.67±0.23^{a}	50.33±0.23^{a}	

Every value represents the Mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Effect of vaccination on different doses of AmCPV inoculum: The vaccine was most effective against the low dosage of AmCPV inoculation (1×10^{1} and 1×10^{2} POB mL^{-1}) where cumulative mortality was low (10.67 and 15.67% on 16th day). Its effectiveness at higher dosage of AmCPV inoculum (1×10^{3} and 1×10^{4} POB mL^{-1} was reduced (Table 3).

Effect of multiple vaccination on infection of AmCPV in tasar silkworm: The multiple vaccination during 1st, 2nd and 3rd instar delayed and reduced the mortality due the AmCPV infection in tasar silkworm (Table 4). With single vaccination during 1st instar the mortality was noted on 8th day after inoculation while in double (once in 1st instar and again once in 2nd instar) and triple (once in 1st instar and once again in 2nd and 3rd instar) vaccination the mortality was observed on 12th day after inoculation. In case of single vaccination the mortality was 18.67% on 18th day after inoculation whereas it was 13.33 and 9.67% in case of double and triple vaccination. The mortality was reduced 80.78% by triple vaccination when compared with control.

DISCUSSION

Results indicated that the attenuated AmCPV and its use as oral vaccine prior to inoculation was effective which protects tasar silkworm from AmCPV infection up to some extent for a short period. Single vaccination delayed the development of cytoplasmic polyhedrosis for a short period

as indicated by delayed mortality in silkworm. Triple vaccination further delayed the development of the disease and reduced the mortality significantly than control.

The results of present investigation are in agreement with the earlier report of Aizawa (1954) who observed that the vaccination of mulberry silkworm pupae was effective in preventing some of the pupae dying due to viral infection. Similar observations have also been made by Liu and Zhong (1989) who have also reported reduction of loss in *Bombyx mori* due to the infection of cytoplasmic polyhedrosis virus by 30-60 and 40-60% by oral vaccination with attenuated BmCPV at laboratory and farmers level respectively. The study of Nataraju *et al.* (2000) supports the present results of oral vaccination in tasar silkworm. They tested the potential of attenuated BmNPV as oral vaccine in mulberry silkworm, *B. mori* against BmNPV which resulted reduction of infection by 85%. Reports of Tanada and Kaya (1993) are more or less similar to the present study in which they observed that in insects, in general the induced immunity is rapidly acquired with single inoculation of killed antigen and the immunity is enhanced with repeated inoculations. Such acquired immunity generally remains for a brief period, which may last up to two weeks.

A virus inactivation principle was detected in the haemolymph of silkworm, *Bombyx mori* infected with NPV (Aizawa, 1970). Interferon inducers such as poly-IC and 2.5A have been known to increase antiviral response against Cytoplasmic Polyhedrosis Virus (CPV) (Zong *et al.*, 1988). The defense response reported in *Bombyx mor*i are in the form of resistance (Watanabe and Maeda, 1981; Eguchi *et al.*, 1986), production of antiviral substance in the gut and/or viral inhibitory factors in the haemolymph (Hayashiya *et al.*, 1968).

There are several reports of antiviral activity resulting from vaccination of an insect with inactivated virus preparations (Raheja and Brooks, 1971). Antiviral immunity was also reported in greater wax moth against densonucleosis virus.

CONCLUSION

The attenuated AmCPV showed positive results towards the control of cytoplasmic polyhedrosis virus. Triple vaccination protected silkworm for comparatively a longer period and reduced the mortality 80.78% due to cytoplasmic polyhedrosis virus infection. Attenuated pathogen can be used to control the severe strains of pathogen infecting tasar silkworm.

ACKNOWLEDGMENT

The authors are grateful to Central Silk Board, Bangalore, Research Council, Research Advisory committee of CTR and TI, Ranchi for extending facility and encouragement in pursuing this study. Thanks are also due to Mr. Suresh Rai and Mr. Ram Nagina, Assistant Directors, Statistics (Statistics Section), CTR and TI, Ranchi for statistical analysis of the scientific data.

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