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RESEARCH

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Characterization and bioassay of indigenous isolates of the fall armyworm, *Spodoptera frugiperda* nucleopolyhedrovirus in India

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Abstract

Background Maize fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a highly migratory polyphagous insect pest that has posed itself as a very threatened insect invaded India since May 2018 and devastated the maize crop. This insect pest caused 62.5% damage on maize plants in Hassan district of Karnataka state, India. Surveys were undertaken in different parts of the country to assess the natural occurrences of all three categories of entomopathogens (bacteria, fungi, and viruses) associated with fall armyworm. Maximum occurrence of infection of entomopathogens (15.13%) was recorded from Chikkaballapura, Karnataka, followed by 12.23% from Hassan, Karnataka.

Results Totally 13 isolates of *S. frugiperda* nucleopolyhedrovirus (SpfrNPV) were collected from 13 locations of the country. Electron microscopy studies clearly showed the tetrahedral shaped occlusion bodies of SpfrNPV with the size of 1.48–1.68 µm. The identity of highly virulent SpfrNPV NBAIR1 (Chikkaballapura isolate) was confirmed using conserved polyhedrin gene-specific primers and NCBI GenBank accession number was obtained (MT422725). Bioassay studies revealed that the SpfrNPV NBAIR1 Chikkaballapura isolate was highly virulent with the highest larval mortality (95.50%) and the lowest LC_{50} value of 2.11×10^3 OBs/ml. SpfrNPV is exclusively host specific and did not infect any other insect species other than *S. frugiperda* tested in this present study.

Conclusions Among 13 isolates of SpfrNPV, SpfrNPV NBAIR1 Chikkaballapura isolate was highly virulent with respect to the larval mortality. Hence SpfrNPV NBAIR1 Chikkaballapura isolate is having a great potential to play in the management of maize fall armyworm *S. frugiperda*.

Keywords *Spodoptera frugiperda*, Nucleopolyhedrovirus, Characterization, Bioassay

Background

Maize fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a highly migratory polyphagous insect pest that has posed itself as a very threatened insect invaded India since May 2018 and devastated the maize crop. This insect pest caused (62.5%) damage on maize plants in Hassan district of Karnataka state, India. FAW is an indigenous to American continent of tropical and subtropical region and invaded to African and Asian countries. Since 2018, the report of FAW from India has been worrying about the maize damage incidence because of their favorable weather conditions and

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crop rotation farming, which spread to different states of the country in a short period of time and also covered all the regions of agricultural lands within 2 years (Shylesha et al. 2018). FAW prefers more than 353 host plants, including maize, millets, sorghum, and sugarcane in India (Visalakshi et al. 2019). The severe damage of FAW occurs in all stages and parts of the plant, like whorls, leaves, cobs and stems. Large numbers of management strategies, including mechanical hand picking, crop rotation with pulses and oil seeds, early sowing, application of sand or wood ash in the whorls and raising early maturing crops, have been followed to reduce the crop damage and loss (Mutyambai et al. 2022). However, the FAW management in maize in India is currently carried out using chemical insecticides. Management of FAW using microbial pesticides is an alternative viable and long-term sustainable management strategy that preserves the environment and human health (Sivakumar et al. 2021). Biopesticides especially the microbial pesticides are environmentally safe, ecologically acceptable, and are highly effective against target insect pest but are absolutely safe to other natural enemies of crop pests (Sagheer et al. 2008). The microbial insecticides can be equally as effective as chemical insecticides to manage the insect pests (Khan et al. 2014). Natural occurrences of native entomopathogens on FAW larvae, *Metarhizium rileyi*, nucleopolyhedrovirus and *Bacillus thuringiensis* (*Bt*) from the maize growing villages of India were reported (Sivakumar et al. 2021). Application of microbial pathogens *Metarhizium rileyi, M. anisopliae*, *Beauveria bassiana*, *Bacillus thuringiensis* and nucleopolyhedrovirus for the management of FAW are in practice in different parts of the globe. *S. frugiperda* nucleopolyhedrovirus (SpfrNPV) belongs to the family Baculoviridae and has been well recognized as an alternative strategy for managing the insect pests effectively and in an environmentally safe manner. Baculoviruses mainly nucleopolyhedroviruses (NPVs) and Granuloviruses (GVs) are microbial pesticides and are widely used as natural enemies of insect pests (Paiva et al. 2021) and have been successfully used since the early 1890s (Khan et al. 2014). Baculoviruses are obligate pathogens of host insects and are widely exploited to manage the insect pests belong to Lepidoptera and Hymenoptera (Khan et al. 2014). They are host specific and replicate in the host cells. Normally, the insect larvae ingest the occlusion bodies of viruses and viral infection is established (Khan et al. 2014). Baculoviruses are highly host specific, virulent and highly safe to non-target organisms such as human beings and other animals (Kannan et al. 2021). Various geographical strains of SpfrNPV with more than 80% efficacy have been identified across the globe and have been utilized as potential biological control agents for the management of FAW (Sivakumar et al. 2020a, b). Several virulent strains of *S. frugiperda* nucleopolyhedrovirus have been characterized and identified in America, Colombia, Brazil and India and have been explored for the management of the pest (Sivakumar et al. 2021). SpfrNPV is very specific to FAW and SpfrNPV infection on its larvae caused 86–100% mortality both in laboratory and field experiments conducted under different conditions. The present study assessed the natural occurrence of SpfrNPV and the bioassay for the virulence of different SpfrNPV isolates was studied.

Methods

Collection of SpfrNPV-infected FAW larvae

Surveys were conducted at 13 maize growing areas of India, viz. Chikballapura (13.4355° N, 77.7315° E), Hassan (13.0033° N, 76.1004° E), Raichur (16.2160° N, 77.3566° E), Gauribidanur (13.6183° N, 77.5146° E); Coimbatore (11.0168° N, 76.9558° E), Krishnagiri (12.5266° N, 78.2150° E), Salem (11.6643° N, 78.1460° E), Jolarpettai (12.5687° N, 78.5749° E); Hyderabad (17.3850° N, 78.4867° E), Anakapalle (17.6896° N, 83.0024° E), Udaipur (24.5854° N, 73.7125° E), Agartala (23.8315° N, 91.2868° E); Pune (18.5204° N, 73.8567° E) during the years 2018, 2019 and 2020 to assess the FAW infestation levels and to record the natural occurrences of entomopathogens associated with *S. frugiperda.* Thirty maize plants (40 days old) at a random from each field in each village were observed to document the natural occurrences of FAW and their entomopathogens. The percentage of FAW infestation and occurrences of FAW entomopathogens were calculated. *S. frugiperda* larvae infected with SpfrNPV were collected from different locations for further characterization and bioassay studies.

Maintaining the host insect

Plastic container with maize leaves covered with nylon mesh served as feed for field-collected 1st instar larvae of *S. frugiperda*. The larvae were reared till the adult stage. The eggs laid by the adult moths were used for maintaining a test insect to carry out the laboratory experiments. Further, the identity of the insect was confirmed through amplification of cytochrome oxidase I gene (COI) marker (Gupta et al. 2016). The nucleotide sequence was submitted to NCBI GenBank data base, and accession number was obtained (accession number MK041922).

Isolation and mass production of nucleopolyhedrovirus

NPV-infected larvae of *S. frugiperda*, showing characteristic viral infection symptoms (Fig. 1), were collected during 2018 to 2020 from the tested locations. The diseased larvae were brought to ICAR-NBAIR, Bangalore, and

Fig. 1 Natural occurrences of infection of *Spodoptera frugiperda* nucleopolyhedrovirus from different maize growing areas of India

were homogenized by distilled water for 3 min and the crude homogenate was filtered twice by muslin and then the larval debris was removed. The filtrate was spun for 1 min at 500 rpm to remove large particles. The supernatant was re-suspended for 20 min at 5000 rpm, and the pellet was collected. Sterile distilled water (100 ml) was used to re-suspend the pellet. The number of occlusion bodies (OBs) in the stock suspension was determined and attained to (3.2×10^4 OBs/ml) using a Neubauer hemocytometer. In vivo mass production of SpfrNPV NBAIR1 was carried out on the 4th instar larvae of *S. frugiperda* using plastic trays. About 150 of 4th instar *S. frugiperda* larvae were reared on maize leaves. Maize leaves were washed thoroughly by distilled water, air-dried and smeared with SpfrNPV NBAIR1 at (3.2×10^4 OBs/ml) using the polished blunt end of a glass rod. Bouquets of these virus-contaminated leaves were placed in plastic trays with a size of (4 cm H×40 cm L×30 cm). Fourth instar larvae of FAW were allowed to feed on these leaves. Once the treated maize leaves were fully consumed by the larvae, fresh maize leaves were provided. All the dead larvae were collected and processed after 6–8 days of inoculation. The OBs were quantified by phase-contrast microscopy, using a double ruled improved Neubauer hemocytometer, and the virus suspension was standardized and stored in the refrigerator.

Laboratory mortality bioassays

Various isolates of SpfrNPV, i.e., SpfrNPV NBAIR1 (Chikkaballapura), SpfrNPV NBAIR2 (Hassan), SpfrNPV NBAIR3 (Raichur), SpfrNPV NBAIR4 (Gauribidanur), SpfrNPV NBAIR5 (Coimbatore), SpfrNPV NBAIR6 (Krishnagiri), SpfrNPV NBAIR7 (Salem), SpfrNPV NBAIR8 (Jolarpettai), SpfrNPV NBAIR9 (Hyderabad), SpfrNPV NBAIR10 (Anakapalle), SpfrNPV NBAIR11 (Udaipur), SpfrNPV NBAIR12 (Agartala) and SpfrNPV NBAIR13 (Pune) were bioassayed on laboratory reared freshly molted second-instar host larvae that were starved for 12 h and then inoculated with three concentrations of SpfrNPV, viz. 1.0×10^4, 1.0×10^3, 1.0×10^2 OBs/ml), using semi-synthetic diet. Serial dilutions were made using sterile distilled water to achieve the desired concentrations. Multiwell trays were used to perform the bioassay experiment. Each tray was having 50 wells, and the diameter of each well was 2 cm. Fresh prepared diet was poured into the wells, and the poured diets were air-dried. Twenty microliter (µl) of each viral concentration from each viral isolate was spread on diet using

a blunt end of glass rod. Diet with sterile distilled water was maintained as control. Each multiwell tray containing 20 wells was maintained as one replication. Twenty larvae were used for each replicate. Six replications were maintained. One starved 2nd instar larvae was placed in each well. The wells were covered with a lid after placing the larva in all wells. Larvae once consumed the entire diet within 24 h, and then, they were transferred to fresh diet and reared at 28 ± 2 °C, 60% RH. Mortality of larvae was recorded starting from first day to seventh day after inoculation. Mortality caused by virus confirmed by microscopic examination through Giemsa staining.

Morphological characterization and molecular confirmation of SpfrNPVs

Morphological characteristics (shape and size) of extracted occlusion bodies (OBs) of all the 13 SpfrNPV isolates were carried out by the use of scanning (SEM Quanta 250, FEI, Netherlands) and transmission (TECNAI120 kV TEM (FEI, Netherlands) electron microscopy as per the standard procedure (Sivakumar et al. 2020a, b). The highly virulent Chikkaballapura SpfrNPV NBAIR1 genomic DNA was extracted by DNeasy blood and tissue kit (Qiagen) and the conserved gene polyhedron (Polh) amplified with gene-specific primer ((F—TCTAGGTTCGGTCATCAA GAAT; R—TTGAACACGAGCGACAGTT)). PCR master mix (Thermo scientific) used for the thermo cycler reaction (Qantarus). The partial Polh gene sequence was obtained and submitted to NCBI (Sivakumar et al. 2020a, b).

Host range studies

Host range of the SpfrNPV NBAIR1 was studied on 1 day old 2nd instar larvae of *Spodoptera litura*, *S. exigua*, *Spilosoma obliqua*, *Helicoverpa armigera*, *Plutella xylostella*, *Amsacta albistriga*, *Maruca vitrata*, *Trichoplusia ni*, *Pieris brassicae*, *Agrotis ipsilon*, and *Bombyx mori* with SpfrNPV NBAIR1 at (3.1×10^4 OBs/ml concentration). The bioassays were monitored and scored 6 days of post treatment to access the viral infection. All the dead larvae were collected from the bioassay trays and examined for the presence of virus OBs by smearing the larval body fluid on a sterile glass slide, stained with Giemsa stain. The stained slides were examined using a phase-contrast light microscope under oil immersion.

Statistical analysis

Larval mortality data were subjected to probit analysis using the software POLO (Leora 1994), and the lethal concentration to kill 50% of the tested larvae (LC_{50}) was calculated. Correct for mortality in the treatments arrived using Abbott's formula is (% test mortality − % control mortality/control mortality × 100).

Results

Survey, collection and characterization of SpfrNPV-infected FAW

The infestation levels of FAW in surveyed maize growing areas (Fig. 1) are presented in Table 1. FAW infestation ranged from 25.25 to 49.01%. Maximum of FAW infestation (49.01%) was recorded from Chikkaballapura, followed by locations from Jolarpettai (48.35%). Natural occurrences of all three categories of entomopathogens (bacteria, fungi, viruses) were recorded from the 13

Table 1 Natural occurrences of nucleopolyhedrovirus infection on maize fall armyworm in India

States	Locations	Strains' name	FAW infestation (%)	Natural occurrence of SpfrNPV infection (%)	Natural occurrences of entomopathogen's infection (%)
Karnataka	Chikkaballapura	NBAIR 1	49.01	4.36	15.13
	Hassan	NBAIR 2	41.12	4.25	12.23
	Raichur	NBAIR 3	45.75	2.11	6.75
	Gauribidanur	NBAIR 4	39.93	3.39	11.21
Tamil Nadu	Coimbatore	NBAIR 5	37.25	2.65	8.25
	Krishnagiri	NBAIR 6	35.25	3.79	11.57
	Salem	NBAIR 7	28.23	2.25	6.25
	Jolarpettai	NBAIR 8	25.25	3.12	8.56
Telangana	Hyderabad	NBAIR 9	42.15	1.39	7.45
Andhra Pradesh	Anakapalle	NBAIR 10	48.35	3.45	10.78
Rajasthan	Udaipur	NBAIR 11	37.55	2.65	9.46
Tripura	Agartala	NBAIR 12	29.15	1.03	5.87
Maharashtra	Pune	NBAIR 13	31.75	2.42	8.95

locations based on the characteristic infection symptoms. Maximum occurrence of infection of entomopathogens (15.13%) was recorded from Chikkaballapura, Karnataka, followed by (12.23%) from Hassan, Karnataka (Table 1). Totally 13 isolates of SpfrNPV were collected from 13 locations of the country. The natural occurrence of SpfrNPV infection ranged from (1.03 to 4.36%) with respect to all locations. Maximum natural occurrence of SpfrNPV infection (4.36%) was recorded from Chikkaballapura, followed by those from Hassan (3.79%) (Table 1). SpfrNPV was extracted from infected cadavers of the 13 locations. Electron microscopy studies revealed the tetrahedral shaped occlusion bodies (Fig. 2) of SpfrNPV with the size of (1.48–1.68 µm) (Table 2). The highly virulent SpfrNPV NBAIR1 (Chikkaballapura isolate) was confirmed through PCR amplification using conserved polyhedrin gene (polh)-specific primers and NCBI GenBank accession number was obtained (MT422725) (Sivakumar et al. 2020a, b).

Table 2 Morphological characterization of various isolates of *Spodoptera frugiperda* nucleopolyhedrovirus

SpfrNPV isolates	Occlusion bodies size (µm)	Occlusion bodies shape
NBAIR 1 (Chikkaballapura)	1.64	Tetrahedral
NBAIR 2 (Hassan)	1.65	Tetrahedral
NBAIR 3 (Raichur)	1.48	Tetrahedral
NBAIR 4 (Gauribidanur)	1.52	Tetrahedral
NBAIR 5 (Coimbatore)	1.55	Tetrahedral
NBAIR 6 (Krishnagiri	1.68	Tetrahedral
NBAIR 7 (Salem)	1.57	Tetrahedral
NBAIR 8 (Jolarpettai)	1.60	Tetrahedral
NBAIR 9 (Hyderabad)	1.58	Tetrahedral
NBAIR 10 (Anakapalle)	1.49	Tetrahedral
NBAIR 11(Udaipur)	1.65	Tetrahedral
NBAIR 12 (Agartala)	1.51	Tetrahedral
NBAIR 13 (Pune)	1.59	Tetrahedral

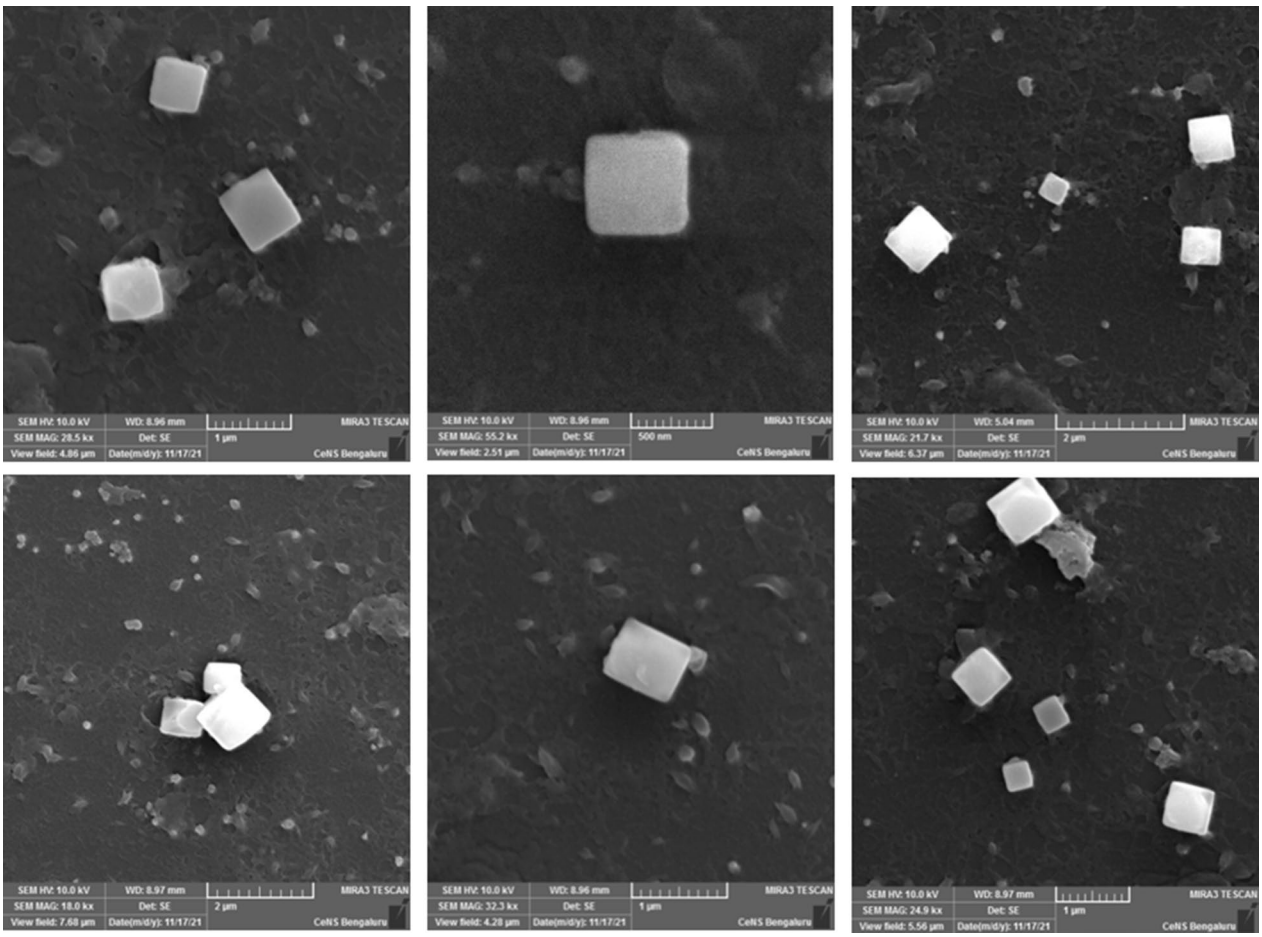

Fig. 2 Scanning electron photomicrographs of tetrahedral shaped occlusion bodies of SpfrNPV

Mortality bioassay study with different strains of SpfrNPV on FAW

Bioassay studies with different isolates of SpfrNPV against 2nd instar larvae of FAW revealed that the highest larval mortality was recorded for all the isolates at the concentration 1×10^4 OBs/ml. The concentration 1×10^4 OBs/ml was the best in suppressing FAW larvae. SpfrNPV NBAIR1 Chikkaballapura isolate was more virulent with the highest larval mortality (95.50%), followed by SpfrNPV NBAIR2 Hassan (89.83%), SpfrNPV NBAIR3 Raichur (83.89%), SpfrNPV NBAIR4 Gauribidanur (80.56%), SpfrNPV NBAIR8 Jolarpettai (70.58%), SpfrNPV NBAIR5 Coimbatore (68.42%), SpfrNPV NBAIR7 Salem (61.73%), SpfrNPV NBAIR10 Anakapalle (61.11%), SpfrNPV NBAIR6 Krishnagiri (57.85%), SpfrNPV NBAIR9 Hyderabad (57.19%), SpfrNPV NBAIR13 Pune (46.30%), SpfrNPV NBAIR12 Agartala (35.14%) and finally SpfrNPV NBAIR11 Udaipur (28.07%) (Table 3). SpfrNPV NBAIR1 Chikkaballapura isolate was more virulent with the lowest LC_{50} value of (2.11×10^3 OBs/ml), followed by SpfrNPV NBAIR2 Hassan ($LC_{50}=3.05\times10^3$ OBs/ml), SpfrNPV NBAIR3 Raichur (3.55×10^3 OBs/ml), SpfrNPV NBAIR4 Gauribidanur (3.79×10^3 OBs/ml), SpfrNPV NBAIR8 Jolarpettai (4.23×10^3 OBs/ml), SpfrNPV NBAIR5 Coimbatore (4.75×10^3 OBs/ml), SpfrNPV NBAIR10 Anakapalle (4.77×10^3 OBs/ml), SpfrNPV NBAIR7 Salem (4.80×10^3 OBs/ml), SpfrNPV NBAIR6 Krishnagiri (4.99×10^3 OBs/ml), SpfrNPV NBAIR9 Hyderabad (5.01×10^3 OBs/ml), SpfrNPV NBAIR13 Pune (5.11×10^3 OBs/ml), SpfrNPV NBAIR12 Agartala (5.77×10^3 OBs/ml) and SpfrNPV NBAIR11 Udaipur (5.91×10^3 OBs/ml) (Table 3). It was clearly understood from the bioassay studies that the SpfrNPV NBAIR1 Chikkaballapura isolate was more virulent and the highest larval mortality was recorded.

Host range studies

Cross infectivity and host range of SpfrNPV NBAIR1 Chikkaballapura isolate on various tested insect species, viz. *S. litura*, *S. exigua*, *S. obliqua*, *H. armigera*, *P. xylostella*, *A. albistriga*, *M. vitrata*, *T. ni*, *P. brassicae*, *A. ipsilon*, and *B. mori*. The results of the experiments clearly revealed that SpfrNPV NBAIR1 did not infect any of the above insect species tested in this present study.

Discussion

The study explored more strains of SpfrNPV from different regions of the country which enable to develop a potential strain against FAW. Baculoviruses are reported to be highly specific, virulent and safe alternative for the management of FAW. Although several SpfrNPV strains from the United States, Nicaragua, Brazil, Argentina, Colombia and China have been biologically or genetically characterized, the selection of indigenous isolates that are suitable for its development as a biological control agent requires the characterization of those isolates present in each geographical region (Figueiredo et al. 2009). The present study clearly brought out the natural occurrences of infection of entomopathogens including nucleopolyhedrovirus associated with FAW across the country. Maximum natural occurrence of SpfrNPV infection was recorded from Chikkaballapura, Karnataka, which could be due to the maximum infestation of FAW and heavy population of maize plants. The natural occurrences of entomopathogen's infection including indigenous

Table 3 Bioassay of *Spodoptera frugiperda* nucleopolyhedrovirus against *Spodoptera frugiperda*

SpfrNPV isolates	LC_{50} values (OBs/ml)	Slope ± SE	Fiducial limits		Heterogeneity	Chi-square (χ^2)	*df*	Correct for mortality (%)
			Lower	Upper				
NBAIR 1 (Chikkaballapura)	2.11×10^3	0.79 ± 0.11	0.035	0.175	0.147	0.34	3	95.50
NBAIR 2 (Hassan)	3.05×10^3	0.81 ± 0.12	0.421	2.105	0.221	0.43	3	89.83
NBAIR 3 (Raichur)	3.55×10^3	0.82 ± 0.11	0.452	2.280	0.351	0.72	3	83.89
NBAIR 4 (Gauribidanur)	3.79×10^3	0.82 ± 0.11	0.765	3.825	0.123	0.77	3	80.56
NBAIR 5 (Coimbatore)	4.75×10^3	0.85 ± 0.11	1.011	5.055	0.342	0.81	3	68.42
NBAIR 6 (Krishnagiri)	4.99×10^3	0.86 ± 0.11	1.532	7.660	0.163	1.21	3	57.85
NBAIR 7 (Salem)	4.80×10^3	0.87 ± 0.11	0.879	4.395	0.561	1.34	3	61.73
NBAIR 8 (Jolarpettai)	4.23×10^3	0.83 ± 0.11	0.911	4.555	0.123	1.39	3	70.58
NBAIR 9 (Hyderabad)	5.01×10^3	0.89 ± 0.11	2.101	10.505	0.431	1.56	3	57.19
NBAIR 10 (Anakapalle)	4.77×10^3	0.85 ± 0.11	0.821	4.105	0.512	1.41	3	61.11
NBAIR 11(Udaipur)	5.91×10^3	0.92 ± 0.11	3.992	13.321	0.213	1.78	3	28.07
NBAIR 12 (Agartala)	5.77×10^3	0.92 ± 0.11	3.207	14.121	0.135	1.91	3	35.14
NBAIR 13 (Pune)	5.11×10^3	0.90 ± 0.11	3.212	11.235	0.402	1.86	3	46.30

SpfrNPV have been well documented, and the laboratory studies have proved that indigenous SpfrNPV isolate had the greatest potential for use in the biological control of FAW (Sivakumar et al. 2021). This present study clearly revealed the tetrahedral shaped occlusion bodies of SpfrNPV, which is in agreement with documented tetrahedral shaped occlusion bodies in nucleopolyhedrovirus described by Sivakumar et al. (2020a, b). The LC_{50} value for all the SpfrNPV isolates ranged from (2.11 to 5.80×10^3 OBs/ml). Earlier reports stated that the LC_{50} value for SfMNPV isolates was constant and very small amount of variation was recorded in LC_{50} values among the tested isolates (Rowley et al. 2010). Among 13 isolates, the SpfrNPV NBAIR1 (Chikkaballapura) was a highly virulent isolate with lower LC_{50} value than other isolates. All the stages of SpfrNPV-infected FAW larvae were collected from the maize field which showed that all the instars of larvae are susceptible to SpfrNPV. Sivakumar et al (2020a, b) also reported that the first, second and third larval instars of FAW were equally susceptible to SpfrNPV. Further the sub-lethal effects of indigenous SpfrNPV NBAIR1 on FAW development and reproduction were reported (Dhanyakumar et al 2023). High rate of larval deaths was recorded with few SpfrNPV strains (Popham et al. 2021). Baculovirus isolates are evaluated for commercial and virus-based insecticide suitability based upon several characteristics like; bioactivity, killing speed and OBs production (Chengfeng et al. 2020). Among different strains tested, the SpfrNPV NBAIR1 Chikkaballapura strain was the best and the same was recorded and characterized by both morphological and molecular tools.

Conclusion

Maximum natural occurrence of SpfrNPV infection on FAW was recorded from Chikkaballapura, Karnataka, which could be due to the most infestation of FAW and the dense crop canopy. Among 13 isolates of SpfrNPV, SpfrNPV NBAIR1 Chikkaballapura isolate was highly virulent with respect to the larval mortality. Hence, SpfrNPV NBAIR1 Chikkaballapura isolate had a great potential to play in the management of maize fall armyworm, *S. frugiperda.*

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Author contributions
RKP, SG and DO designed and executed the experiments and carried out the study. RKP, SG, DO, MM, VT and SSN wrote the manuscript and analyzed the data. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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