Research Article

Isolation, Screening and Characterization of the Viral Pathogens from *Apis mellifera* Colonies

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Abstract | Honeybees are the world's most important pollinators of food crops. Honeybee plays an essential role in pollination of commercial crops. Some viral, bacterial, protozoan, and fungal pathogens are attached on the honeybees worldwide. These pathogens affect the honeybee population by creating disease in it, which also effect the economy of country. It destroys the honey production and causes a subsequent reduction in the crop yield. In this present work, we isolated and screened various honeybee pathogens *i.e., Nosema apis, Nosema cerus,* Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Sacbrood virus (SBV), Deformed wing virus (DWV), Kashmir bee virus (KBV), Black queen cell virus (BQCV), chronic bee paralysis virus (CBV) and Israeli acute paralysis virus (IAPV). For the pathogen screening, collection of guts from the diseased honeybee colonies was followed by RNA isolation. The RNAs were quantified on spectrophotometer. Results have shown the occurrence of fungus and/or pathogen infestation which was caused by microsporidium *Nosema apis* with 277bp. It has been observed that the honeybee colonies were badly affected due to Nosemosis disease which was caused by microsporidian fungus *Nosema apis*. This microsporidian fungus causes dysentery in the honeybees, may impede the survival rate of bees, and thus may further cause a subsequent reduction in the honey production and pollination rate which may ultimately cause the reduction in crop yield.

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1. Introduction

Several agricultural crops benefit from the valuable pollination services provided by the western honeybee (*Apis mellifera* L.). This specie of honeybee is the most common pollinator of crops in the world (Geoffery *et al*., 2014; Hung *et al*., 2018). Honeybees aid in the maintenance and sustainability of productive agriculture and non-agricultural ecosystem. Honeybees play a pivotal role in crop pollination and subsequent production of valuable crops for human consumption (Hristov *et al*., 2020). A global concern has been raised in the recent decades due to a drastic reduction in honeybee population (Porto *et al*., 2020;

Van Engelsdorp and Meixner, 2010).

This specie of honeybee *(A. mellifera)* is the first domesticated specie which has been found to produce honey during winter seasons. This specie is widely recognised as the primary specie which can be maintained to produce honey and pollination activities by beekeepers. Human assistance has made this specie currently prevalent in every continent except antarctica (Conte and Navajas, 2008; Han *et al*., 2012). Seven common bee viruses include BQCV, DWV, CBPV, SBV, IAPV, ABPV, and KBV are capable of inflicting serious diseases in A. mellifera. DWV, however, was considered as one of the widely spread virus (Khalifa *et al*., 2021).

Fungi kingdom contains obligate intracellular parasite, termed as microsporidia. Microsporidia, on the other hand, were more recently considered as to be highly derived parasitic protists. The microsporidium *Nosema apis* was the first to be discovered infecting western honeybees (*A. mellifera*), and it has now been discovered on every continent. *Nosema ceranae*, a different microsporidium, was discovered naturally infecting *A. mellifera* colonies in Europe at the start of the twenty-first century. Its global expansion in this and other Hymenopteran hosts was subsequently established (Higes *et al*., 2020).

Honeybees are prone to various diseases due to the attack of different pests which may include protozoan, bacterial, and viral pathogens like varroa *destructor* and *Nosema* spp. (Tantillo *et al*., 2015; Lannutti *et al*., 2022). The interspecific transfer of honeybee pests and pathogens may further contribute to the decline in pollinator populations (Cilia *et al*., 2022). Bee virus outbreaks in Asian apiaries had hardly ever been documented. Current study involves the isolation, determination, and characterisation of seven honeybee viruses by using RT-PCR. This has been done to check the prevalence and spread of viruses through the apiaries in the Asian region (Sanpa and Chantawannakul, 2009; Ali *et al*., 2012; Forsgren *et al*., 2015). Keeping in view the above-mentioned facts, current study was designed to investigate the prevalence, occurrence, detection, and characterization of seven bee-viruses and the two *Nosema spp.* by the isolation and characterization of pathogens from honeybee (*Apis mellifera*) colonies and subsequent RT-PCR assay of the isolated pathogens.

2. Materials and Methods

2.1 Installation of apiaries

Apiaries were installed in bee farm far from the resident areas. They were kept under continuous observation. High level of *varroa* mites infection and infestation was obvious.

2.2 Honeybee samples collection

Sampling of honeybees was carried out from various colonies. Sampling was done on non-windy and sunny days. The average temperature these days was about 19 °C.

2.3 Isolation of gut from the Honeybee

After collection of samples, honeybees were taken in the dissecting tray then legs and wings removed the with the help of fine autoclaved scissors. The gut was removed gently from the abdomen by using the autoclaved forceps. Isolated gut was taken in autoclaved petridish and then stored in -40°C for the further investigation.

2.4 RNA extraction from honeybees

Trizol method (Simms *et al*., 1993) was used for RNA isolation and screening of pathogens from stored honeybees' guts. Then isolated RNA was quantified on the spectrophotometer.

2.5 cDNA synthesis

cDNA synthesis was taken by making dilutions in primer's tube. Distilled water (90µl) was added followed by labelling of primer names on each Eppendorf. This was followed by the addition of 10µl original primer solutions in each respective Eppendorf (Table 1).

Table 1: Composition of reaction mixture for cDNA synthesis and gene amplification.

Components	Amount (µl)
10X Reaction Buffer	2.0
2.5 mMdNTPs	2.0
10pmol Forward primer	1.0
10 pmol Reverse primer	1.0
reverse transcriptase	2.0
RNA	10.0
ddH_2O	2.0
Total volume	20.0

Table 2: Target genes along with their forward and reverse primer sequences and base pairs.

Target	Primer sequence	Size	References
ABPV	F – TCATACCTGCCGATCAAG	197	(De Miranda,2008)
	R- CTGAATAATACTGTGCGTATC		
IAPV	F– CCATGCCTGGCGATTCAC	203	(De Miranda,2008)
	R– CTGAATAATACTGTGCGTATC		
KBV	F – CCATACCTGCTGATAACC	200	(De Miranda,2008)
	R – CTGAATAATACTGTGCGTATC		
Nosema apis	F – CTAGTATATTTGAATATTTGTTTACAATGG	277	(De Miranda,2008)
	R – GCTATGATCGCTTGCC		
BQCV	F – AGTGGCGGAGATGTATGC	294	(De Miranda,2008)
	R - GGAGGTGAAGTGGCTATATC		
SBV	F – TTGGAACTACGCATTCTCTG	335	(De Miranda,2008)
	R – GCTCTAACCTCGCATCAAC		
CBPV	F – CAACCTGCCTCAACACAG	296	(De Miranda,2008)
	R– AATCTGGCAAGGTTGACTGG		
Nosema ceranae	F– TATTGTAGAGAGGTGGGAGATT	315	(De Miranda,2008)
	R – GCTATGATCGCTTGCC		
DWV	F– CAACTACCTGTAATGTCGTCGTGTT	206	(Yang and Cox-foster, 2005)
	R –GACAAAATGACGAGGAGATTGTT		

2.6 Primer sequencing and amplification

Amplification of the desired pathogens were performed by using primers already available in literature (De Miranda, 2008; Yang and Coxfoster, 2005). Primer sequences on each group of genes their match and mismatch sequences or positions of their awaited amplicon sizes along with the amplification of matched positions and their subsequent sequences of eight primers has been shown in Table 2.

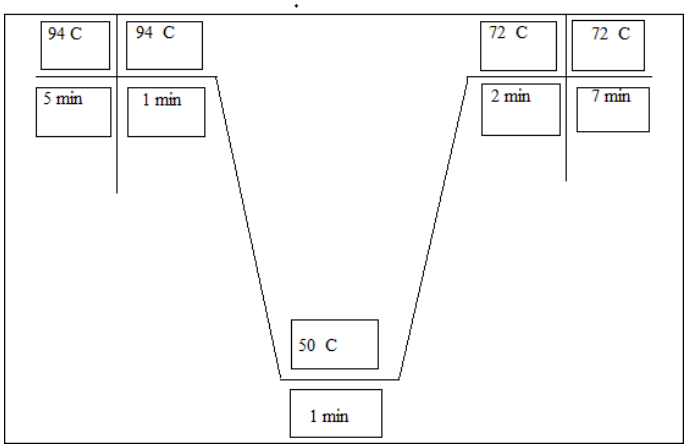

Figure 1: Different steps of PCR.

2.7 Thermo-cycling conditions of PCR

First regular cycling event comprised of heating for 5 minutes at 94°C. This was followed by second regular cycling event of annealing which was comprised of heating for 1 minutes at 94° at 50°C. Third step was extension which was comprised of 7 minutes at 72°C (Figure 1).

2.8 Gel electrophoresis

Gel electrophoresis was used to detect the pathogens affecting the honeybee population by using PCR products. This was followed by photographing under UV light.

3. Results and Discussion

3.1 Primer designing and detection of pathogens

RT-PCR was run by using the thermo-cycling conditions (Sigma-Aldrich). Primers were designed by following the Yang and Cox-Foster (2005) and De Miranda (2008). Primers were cost effective and had good target sites. They were designed to discriminate between the two *Nosema* spp. and the seven most commonly occurring viruses i.e., ABPV, DWV, BQCV, CBPV, KBV, IAPV, and SBV). The detail has been given in Table 2.

3.2 RNA extraction

Isolation of gut and abdomen samples was done from the one hundred and thirty honeybees by Trizol method (Simms *et al.*, 1993). This was followed by extraction of thirty (30) RNAs (Figures 2, 3).

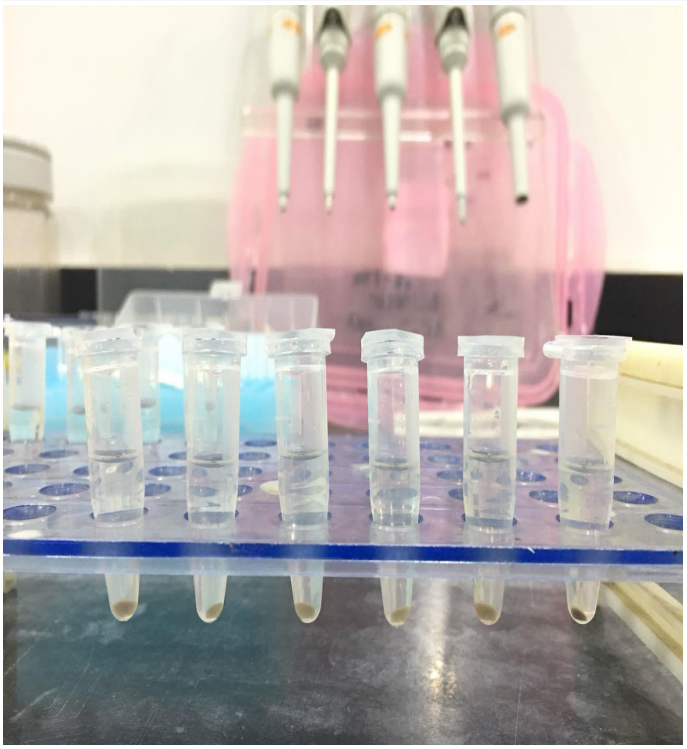

Figure 2: Experiment shown pellets of isolated RNA.

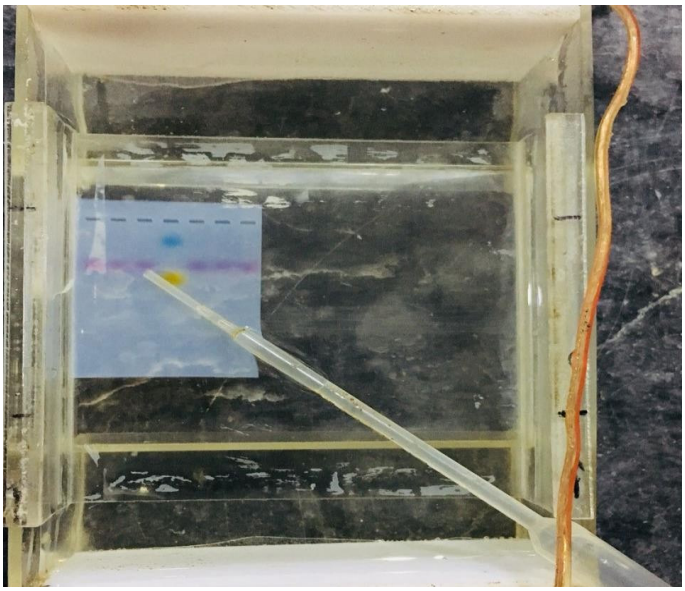

Figure 3: Experiment shown process of gel electrophoresis confirmed RNA isolation.

3.3 Quantification of RNA

The extracted RNAs were quantified on spectrophotometer. This was followed by the synthesis of cDNA by using the PCR. The synthesis of cDNA was done by using the polymerase chain reaction (PCR).

3.4 Gel electrophoresis

The results of PCR reaction are usually visualized (made visible) by using gel electrophoresis. Gel electrophoresis technique is used in which DNA fragments were pulled through a gel matrix by an electric current. It separates DNA fragments as per their sizes. To determine the size of fragments in the PCR samples, a standard and/or DNA ladder was added. The reaction samples were detected by agarose gel electrophoresis using PCR products. This was followed by staining with ethidium bromide. The results were shown by gel electrophoresis in the form of bands by photographing under UV light.

3.5 Pathogen extraction

The results have shown that eight samples of the PCR products were sequenced for each pair of primers. Selected RT-PCR fragments were shown and the pathogen *Nosema apis* (277bp size) were detected (Figure 4).

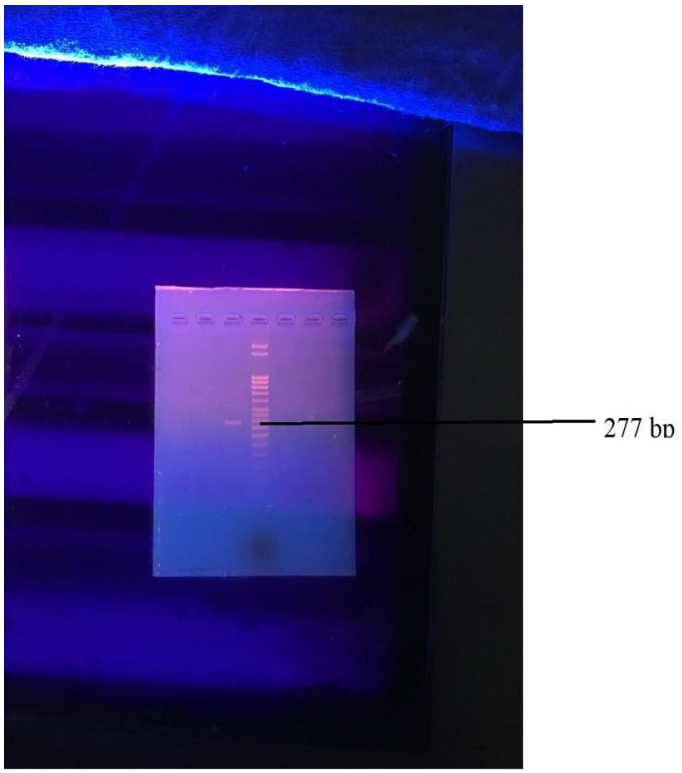

Figure 4: Gel electrophoresis for identification of *Nosema apis*.

The present study showed only microsporidian fungus *Nosema apis* infestation, the predominant species affecting honeybee hives due to availability of hot arid climatic conditions. Other microsporidian fungus *Nosema ceranae* was also not detected due to the non-availability of suitable climatic conditions for their prevalence (Ansari et al., 2017; Hong *et al.*, 2011). Honeybee viral infections of ABPV, DWV, BQCV, CBPV, KBV, IAPV, and SBV were not detected due to nonavailability of proper viral transmission routes

and climatic conditions (Yanez *et al.*, 2020). The RT-PCR technique is used in this study could become a standard method. By RT-PCR describing the screening of pathogens preparations which are used in research. RT-PCR technique is suitable, unique and sensitive. The PCR (polymerase chain reaction) following reverse transcription (RT-PCR) and has been successfully applied for the detection of *Nosema* species in honeybee colonies *Apis mellifera.* RT-PCR technique has been used by other researchers to diagnose various honeybee pathogens.

It was observed that the *Nosema* affected colony having sudden loss of honeybee production as well as honey production (Botias *et al.*, 2013). It can be detected in summer when no suitable climate situation occurs for the fungus growth. However, while in temperate regions *Nosema apis* infections normally show high level in the spring, it reduces in the summer and then boost up again in the fall season before reducing during the early winter months (Higes et al., 2010). This fungus also effects on neural signalling process which reduced learning and memory factors of honeybees. Western honeybees *(Apis mellifera)* are ecologically and economically important pollinators world-wide, with pollination services contributing billions of dollars annually. In summer season, most of honeybee colonies show the infections and infestations of many pathogens which having the outcome on the whole colony. Spores may also keep carry on the combs. However, when the weather changes in autumn, the effect of spores may start an eruption of *Nosema*. Declining of the bee size at this stage may be very heavy. When the bees started defecating inside the hives in the winter season it can also infect the colony population of honeybees with defecation it also secretes the excreta of spores. These conditions cause the effect of declining honeybee population also in the autumn season with producing spores. *Nosema* infection caused sick or creeping bees outside the hive entrance, dead bees lay down on the ground and defecate (dysentery) on hive but may equally be caused by other diseases and unnatural conditions.

The signs and symptoms caused by infection *Nosema apis* are very easy to identify. Their symptoms are like high mortality rate within the colony and another important sign is the stains of diarrhoea at the entrances of hive and these symptoms indicating gastrointestinal disorders (Bourgeois *et al.*, 2010; Araneda *et al.*, 2015).

Negative effects on honeybees are done by *Nosema* infections and are very specific. One week old worker bees normally take food and they do not digest that food very well as well as they are not having the potential to prepare the food digesting secretions. However, at a very young age they become foragers and come out in the rearing phase of life. Up to 78%, their life spans can be decline. Within a month that young queens which ingest *Nosema apis* spores, it become superseded. Our results were related with Kurze *et al.* (2018). They determined *Nosema ceranae* which infects epithelial cells of the honeybee (*Apis mellifera*) midgut. Microsporidian fungus *Nosema* could be change the apoptosis and cell renewal in honeybees 'colonies. This infection occurs in honeybees due to the ingestion of contaminated food and water spores. This infection also happened in bees during the nest cleaning activities and exchange of food. This infection may be due to sexually transmitted to a healthy queen through contact with spore-containing semen during mating (Higes *et al.*, 2020).

Conclusions and Recommendations

It has been concluded that the honeybee colonies were badly affected due to nosemosis disease which was caused by microsporidian fungus *Nosema apis*. This microsporidian fungus causes dysentery in the honeybees, may impede the survival rate of bees, and thus may further cause a subsequent reduction in the honey production. Our results have shown the prevalence and characterization of microsporidian fungus *Nosema apis* with 277bp by using RT-PCR assay. Honeybee colonies with *Nosema apis* infections have shown high risk factors for honeybee population by increasing the mortality rate, declining the honeybee colonies and by negatively affecting the immune system of honeybees *(Apis mellifera)*. Hence, it has become pertinent to find out various ways which may aid in the reduction of microsporidian growth on honeybee colonies. Moreover, the development of multiplex PCR system would play pivotal role in the simultaneous identification of multiple pathogens.

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Novelty Statement

This article showed about the detection of pathogens in honey bees (*Apis mellifera*).

Author's Contribution

All the authors showed its contribution in research conduction and data handling of research work.

Abbreviations

PCR, polymerase chain reaction; ABPV, acute bee paralysis virus; BQCV, black queen cell virus; SBV, sacbrood virus; DWV, deformed wing virus; KBV, Kashmir bee virus; BQCV, black queen cell virus; CBV, chronic bee paralysis virus; IAPV, Israeli acute paralysis virus.

Conflict of interest

The authors have declared no conflict of interest.

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