



Survey and Molecular Detection of *Melisococcus plutonius*, the Causative Agent of European Foulbrood in Honeybee *Apis mellifera* in the Jammu Region of India

Dharam Pal Abrol* and Yogita Shanni

Dean, Faculty of Agriculture, Sher-e-Kashmir University of Agriculture Sciences and Technology of Jammu (Jammu & Kashmir), India -180009

Abstract

The study was carried out during 2016-17 in different locations of Jammu and Kashmir, India. Infections caused by the European foulbrood disease organism *Melisococcus plutonius* were recorded from a minimum of 6% (Pranu) to a maximum of 16.66% (Sartingal) in colonies of the Doda district and from a minimum of 1.47% (Parnote) to a maximum of 15.18% (Digidol) in the district of Ramban. Incidences of European foulbrood were recorded maximally in September (14.59%) and minimally in March (1.81%). Percentages of European foulbrood infections of experimental colonies of the university apiary were lowest (1.81%) in March and maximal (15.37%) in September. A polymerase chain reaction (PCR) molecular diagnosis for EFB was conducted on 60 larval samples collected from different areas of the four districts for PCR amplification of partial 16S rRNA gene fragments (486 bp). Results showed that out of 60 only 6 samples (10%) were positive for *M. plutonius* in different apiaries. The study documented the occurrence of EFB by PCR assay in apiaries of different regions of Jammu and Kashmir for the first time.

Keywords

European foulbrood, Molecular characterization, *Apis mellifera*

INTRODUCTION

Honeybee colony losses are an increasing problem throughout the world (Biesmeijer *et al.*, 2006; Johnson, 2007; Potts *et al.*, 2010a, b; Neumann and Carreck, 2010; Vanbergen, 2013; Powney *et al.*, 2019). Managed honey bees are the most important commercial pollinators and account for 35% of the global food production (William and Anderson, 1974; Free, 1993; Roubik, 1995; Delaplane and Mayer, 2000, Morandin and Winston, 2006; Klatt *et al.*, 2014). The honeybee diseases usually persist as unapparent infections and cause no overt signs of disease. They can dramatically affect honey bee health and shorten the lives of infected bees under certain conditions (Bailey *et al.*, 1983; Hansen and Brødsgaard, 1999; Forsgren *et al.*, 2009; Okamura, 2016). The pests and pathogens are the single most

important cause of otherwise inexplicable colony losses leading to Colony Collapse Disorder (Paxton, 2010; Dainat *et al.*, 2012; Jeroen *et al.*, 2013; Bojana *et al.*, 2014).

The state of Jammu & Kashmir is blessed with all the four species of honeybees viz., *Apis florea* F., *A. dorsata* F., *A. cerana* F., *A. mellifera* L. and several geographical races/ecotypes are expected to exist (Abrol, 2012). The state offers great potential for both migratory and non-migratory beekeeping (Abrol, 2012). Over the past 20 years, honey bees have been faced with many threats including pests and diseases which has led to the listing of honey bee diseases in OIE (world organization for animal health) Terrestrial Animal Health Code. Honeybees are subject to attack by many viruses (Bailey and Ball, 1991; Allen and Ball, 1996), five of which are associated with *Varroa* and one with tracheal mites. There are two

main bacterial diseases found in Asian honeybees: American Foul Brood disease (AFB) caused by *Paenibacillus* larvae (Oldroyd and Wongsiri, 2006) and European Foul Brood (EFB) caused by *Melissococcus plutonius* (Bailey and Collins, 1982). In India, European foulbrood disease appeared for the first time in *A. cerana* during 1970 and then disappeared after killing 25~30 percent of the colonies in the Karad area of Maharashtra (Diwan *et al.*, 1971; Bailey, 1974). However, in *A. mellifera* the disease was noticed in Himachal Pradesh and the Dharwad area of Karnataka during 1998 killing 25~40 percent of the colonies in some apiaries. 1998 (Anonymous, 1998; Viraktamath, 1998). Then in *A. cerana*, the disease was again noticed in 2002 after an absence of three decades killing about 60 percent of the colonies in Himachal Pradesh. Death of the brood was also recorded at prepupal and pupal stages (Rana *et al.*, 2004).

European foulbrood disease prevalence in *A. mellifera* in northern India is posing a serious threat to the bee keeping industry by affecting colony productivity, colony multiplication and antibiotic residue in honey. There is every chance of this disease spreading to other parts of the country where *A. cerana* and *A. mellifera* exist, and affecting the local beekeeping industry. Some studies have already been done in India on this disease in *A. cerana* (Rana *et al.*, 2004) and its occurrence, symptomatology, cultural, epidemiology and chemical control in *A. mellifera* (Bahman and Rana, 2002).

It is not possible to identify the disease exactly based on symptomology as most of the brood diseases have symptoms more or less similar. Several methods have been used to detect *M. pluton*. These include microscopic examination of carbol fuchsin stained smears of diseased larvae (Hornitzky and Wilson, 1989), culture of bacteria from infected larvae or honey (Hornitzky and Smith, 1998), scanning electron microscopy (Alippi, 1991) and enzyme-linked immunosorbent assays (Pinnock and Featherstone, 1984). However, for isolation of *M. pluton*, specific and sensitive detection methods are required to ensure its absence from bee products for export purposes and for epidemiological studies. Evidently, molecular techniques can help to reliably detect, identify and characterize honeybee pathogens so that a diagnosis of the disease is initiated well in time to save the beekeeping industry before a catastrophe occurs. The prevalence of these and other new patho-

gens and their potential correlation with bee losses in Jammu & Kashmir at present is unknown. No information is available on molecular characterization of *Melissococcus plutonius* of *A. mellifera* in the Jammu region. A hemi-nested polymerase chain reaction (PCR) developed by Djordjevic *et al.* (1998) for the detection of *Melissococcus plutonius* in adult bees and honey bee products could be used for the molecular characterization. Keeping this in view, the study was carried out with the objectives to survey the seasonal incidence of European Foul Brood disease in the Jammu region and its molecular characterization in *A. mellifera* L.

MATERIALS AND METHODS

The present investigations were conducted during August 2016 to April 2017 in *A. mellifera* colonies maintained at the experimental field of Division of Entomology and in apiaries of private beekeepers in several regions of Jammu division (Fig. 1).

1. Incidence of European foulbrood disease

European foulbrood disease was recorded in *A. mellifera* during August 2016 to April 2017 in the university apiary and in apiaries of private beekeepers. Severity of European foulbrood disease was studied with regard to the percent colony infection in the apiary and percent brood infection by using the following formulae:

$$\begin{aligned} &\text{Per cent colony infection in the apiary} \\ &= \frac{\text{Number of diseased colonies}}{\text{Total number of colonies}} \times 100 \end{aligned}$$

$$\begin{aligned} &\text{Per cent brood infection in the colony} \\ &= \frac{\text{Diseased brood area (cm}^2\text{)}}{\text{Total brood area (cm}^2\text{)}} \times 100 \end{aligned}$$

1) Strength of colony

The strength of each colony was estimated by observing the number of combs covered with bees of respective species.

2) Brood area

Brood area was measured with the help of measuring grid having squares, each square measuring 3 cm². The

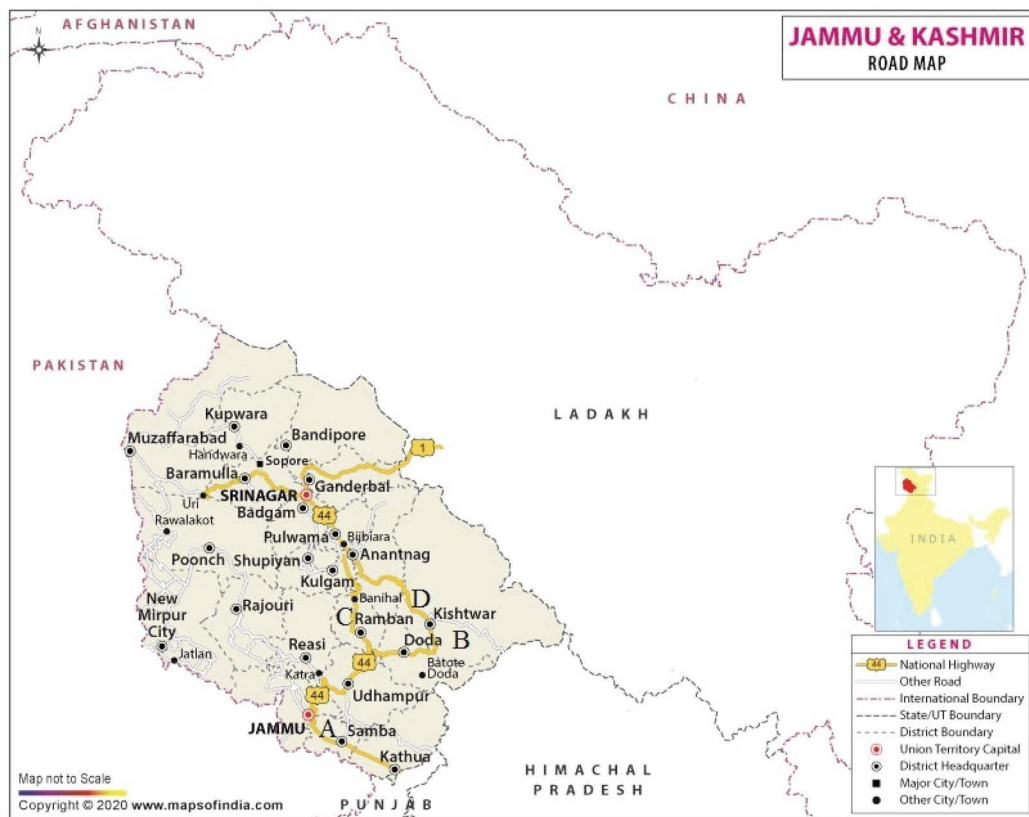


Fig. 1. Map showing EFB inspection areas in Jammu region. EFB has been detected in all the districts. A = Jammu, B = Doda, C = Ramban, D = Banihal.

brood area was converted into cm^2 by multiplying the number of squares with a factor of 3.

2. Symptomology

A. mellifera colonies exhibiting typical symptoms of European foulbrood disease were observed during the period of experimentation. The symptoms were recorded on alternate day with respect to stage, change in colour, position, shape, odour and texture of the brood in the diseased colonies at University Apiary. Five colonies of *A. mellifera* were selected from each apiary in the regions of Jammu, Ramban, Banihal and Doda districts which were expected to have occurrence of diseases. The disease incidence was recorded at an interval of 30 days.

3. Samples

Five 4~5 day old larval samples showing clinical signs of EFB were collected from each apiary for the molecular characterization of the bacterium *M. plu-*

tonius causing EFB. Bee larvae were collected using sterile forceps and the larvae were put in 70% ethanol for further studies.

4. Molecular detection

Molecular level detection of the present isolates was done through polymerase chain reaction (PCR) technique as it is a sensitive, rapid and economical based technique for detecting the disease, European foulbrood microorganism which requires no antiserum as well no monoclonal antibodies. The molecular studies were initiated by the isolation of total DNA from the diseased samples and further confirmed by PCR based gene specific primers.

5. Methods to extract DNA from bee larvae

1) Collection of larva from brood comb

Sixty samples of 4~5 day old larvae showing clinical signs of EFB were collected from apiaries from different areas of the Jammu region, five samples from each api-

ary using sterile forceps and then the larvae were put in 70% ethanol for the further process of molecular characterization of the bacterium *M. plutonius* causing EFB.

2) Isolation of total DNA

(1) Isolation of total DNA

The total DNA extracted from collected larvae by CTAB (cetyl trimethylammonium bromide) based method (Protocol for the isolation of DNA) were prepared from a pool of 5 larvae of *A. mellifera* by adopting the following method:

Extraction buffer was prepared by 2% hexadecyl trimethyl ammonium bromide (CTAB), (1.4 M NaCl, 1% polyvinylpyrrolidone, 0.02 M ethylenediaminetetraacetic acid, 0.1 M Tris-HCl, and incubated for 5 min at 65°C in a dry block heater. 750 µL of the extraction buffer was put into each Eppendorf tube, and then the individual samples were macerated in a sterile pestle mortar into paste and then added to the Eppendorf tubes containing extraction buffer. 20 µL of mercaptoethanol was added in each Eppendorf tube and mix it thoroughly. Then the Eppendorf tubes were incubated for 10 min in a hot air oven at 65°C. After incubation, 500 µL C : I (Chloroform : isopropanol) in the ratio of 24 : 1 is added into each Eppendorf tube and mixed thoroughly. The Eppendorf tubes are then centrifuged at 12000 rpm for 15 min. The transparent liquid was then transferred into fresh Eppendorf tubes and the resultant supernatant was then discarded. The nucleic acids were precipitated with 100 µL of sodium acetate (3 M, pH 5.2) and 500 µL of ice-cold ethanol (100%). The suspension was incubated at -20°C for 20 min and then centrifuged at 15000 rpm for 20 min. The resultant supernatant was discarded from each Eppendorf tube and the pellet was washed twice with 50 µL ethanol (70%), air dried and resuspended in 50 µL of TE buffer (10 mM TrisHCl, pH 8.0, 1 mM EDTA) in each Eppendorf tube. DNA samples were then stored at -20°C.

(2) Checking of DNA

Presence of DNA was checked in 0.8 percent agarose gel, prepared in 1×TBE (Tris borate EDTA) buffer and stained with ethidium bromide (0.4 µL). DNA (3 µL) was loaded with DNA loading dye (bromophenol) and electrophoresed at 115 V for 30~40 minutes and visualized in UV trans illuminator.

(3) Purification of DNA

The DNA visualized in UV trans illuminator had presence of a large amount of RNA. So for the purification of DNA from RNA, the samples were treated with RNase (10 mg/mL). 1 µL of RNase was taken and added to the target sample. Then 500 µL of autoclaved water was added to the target sample and mixed thoroughly. The sample is incubated at 37°C for 1 hour. 500 µL of P : C : I (25 : 24 : 1) was added and mixed thoroughly. The samples were centrifuged at 12000 rpm for 15 minutes. Only the upper layer was taken and transferred to the fresh tubes and the lower layer was discarded. 500 µL of C : I (24 : 1) was added to the fresh Eppendorf tubes. The eppendof tubes are then centrifuged at 12000 rpm for 15 min. The transparent liquid was then transferred into fresh Eppendorf tubes and the resultant supernatant was then discarded. The nucleic acids were precipitated with 100 µL of sodium acetate (3 M, pH 5.2) and 500 µL of ice-cold ethanol (100%). The suspension was incubated at -20°C overnight and then centrifuged at 15000 rpm for 20 min. The resultant supernatant was discarded from each Eppendorf tube and the pellet was washed twice with 50 µL ethanol (70%), air dried and resuspended in 50 µL of TE buffer (10 mM TrisHCl, pH 8.0, 1 mM EDTA). DNA samples were then stored at -20°C and 3 µL was used in the PCR.

(4) Detection of *M. plutonius* by polymerase chain reaction (PCR)

For detection of *M. plutonius*, PCR was used to amplify a portion of the *M. plutonius* 16S rRNA gene which comprised of primer MP1, 5' CTTTGAACG-CCTTAGAGA 3'; and primer MP2, 5' ATCATCT-GTCCCACCTTA 3' (Djordejevic *et al.*, 1998).

Polymerase chain reaction was used to amplify DNA performed in a 25 µL reaction volume containing 5 ng template DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM MgCl₂, 1X PCR buffer and 1 unit *Taq* polymerase. The reactions were carried out under following temperature and time as given below: Initial Denaturation (95°C) for 2 min, Denaturation (95°C) for 30 sec, Annealing (61°C) for 15 sec, Elongation (72°C) for 60 sec and Extension (72°C) for 5 min.

The PCR amplification was performed in Gene AMP PCR system 9700 Thermal cyler (Applied Bio-Sys-

tems, USA). Further, the amplicons so generated were viewed under UV trans illuminator and photographed in Flour-S Multiimager.

6. Statistical analysis

Various statistical tools used in the experiment were mean, standard deviation and 95% confidence interval (Sokal and Rohlf, 2013).

RESULTS

The results of investigation are presented under following headings as under.

1. Symptomology

During the period of investigations, following typical symptoms of the diseases were recorded.

- i) In *A. mellifera*, diseased bee colonies, colour of the infected larvae (brood) was pale yellow while healthy brood appeared glistening shiny white.
- ii) The healthy brood was in coiled position at the bottom of comb cells while 20% of the infected larvae (pale yellow coloured) were found slightly displaced in upward or downward direction from their actual position.
- iii) In general, death of brood was recorded at pupal stage (>70). About 15 percent occurred at larval

stages and rarely in prepupal stage (about 5%), which resulted perforation in sealed brood. However, the colour of the brood did not change which exhibited typical symptoms like those of Thai sac brood virus disease and mite infestation.

- iv) The infected larvae were slightly pulpy or watery. In the advanced stage of the infection, the emitted sour or vinegar like smell.
- v) Remains of the dried brood were noticed in the form of soft scales.

2. Disease incidence

The observations recorded on the incidence of European foulbrood *A. mellifera* in areas of Jammu, Ramban, Banihal and Doda districts of Jammu region are given in Table 1. The colony infection of European foulbrood disease caused by *M. plutonius* was recorded from a minimum of 6% (Pranu) to a maximum of 16.66% (Sartingal) in colonies of district Doda.

1) Brood infection

The present investigations revealed that the percent brood infection of European foulbrood disease in *A. mellifera* in areas of Jammu, Ramban, Banihal and Doda districts of Jammu region are given in Table 2. Brood infection percentages caused by European foulbrood ranged from a minimum of 1.48% (Parnote) to a maximum of 15.25% (Digdol) of district Ramban.

Table 1. Prevalence of EFB disease on the basis of symptoms in different areas

District	Location	Total number of apiaries surveyed	Total number of colonies per apiary	Total number of infected colonies		Percent colony infection	
				EFB	EFB	EFB	EFB
Doda	Attalgarh	1	70	7		10.00	
	Sartingal	1	30	5		16.66	
	Pranu	1	150	19		6.00	
Ramban	Digdol	1	650	73		11.23	
	Dalwah	3	400	235		19.59	
	Parnote	1	300	29		9.66	
Jammu	Miran sahib	1	350	61		7.42	
	Arnia	1	60	20		8.33	
	R. S. Pura	1	80	15		18.75	
Banihal	Shataninallah	3	400	230		19.16	
	Arbal	2	300	81		13.50	
	Zinhall	1	400	45		11.25	

Table 2. Percent brood infection of European foulbrood on the basis of symptoms in different areas

Location	Percent brood infection of EFB in colonies					Mean ± S.D.	95% confidence interval	
	1	2	3	4	5		Lower limit	Upper limit
Attalgarh	9.26	10.40	7.09	11.96	6.06	8.95 ± 2.40	6.85	11.06
Sartingal	12.32	9.90	6.43	12.72	9.95	10.26 ± 2.51	8.06	12.46
Pranu	1.50	2.78	1.91	1.79	1.92	1.98 ± 0.48	1.56	2.40
Digdol	13.50	16.78	11.91	18.79	14.92	15.25 ± 3.11	12.52	17.97
Dalwah	2.10	1.90	1.35	1.28	1.11	1.55 ± 0.43	1.17	1.92
Parnote	1.50	2.05	1.02	1.56	1.26	1.48 ± 0.38	1.14	1.82
Miran sahib	18.20	13.62	18.91	11.4	9.59	14.34 ± 4.11	10.74	17.94
Arnia	12.60	9.35	13.39	10.32	13.83	11.90 ± 1.96	10.18	13.62
R. S. Pura	18.61	6.58	5.12	10.02	9.34	9.93 ± 5.25	5.34	14.53
Shataninallah	1.06	1.44	2.10	1.40	1.66	1.39 ± 0.25	1.17	1.61
Arbal	4.60	2.68	2.44	2.92	3.44	3.22 ± 0.86	2.46	3.97
Zinhall	1.68	1.88	1.32	1.44	2.05	1.67 ± 0.30	1.41	1.94

2) Seasonal incidence of European foulbrood and Sac brood disease in university apiary

Incidence of European foulbrood in university apiary from July, 2016 to April, 2017 was found to be maximum in September (14.59%) and minimum in March (1.88%) (Table 3).

3) Brood infection in university apiary

Percent brood infection of European foulbrood in university apiary was recorded from a minimum of 1.82% (March) to a maximum of 15.38% (September), as given in Table 4.

4) Molecular detection through polymerase chain reaction (PCR) technique

The bacteria present in the larval samples which caused disease in *A. mellifera* was detected and confirmed through polymerase chain reaction assay. A polymerase chain reaction (PCR) molecular diagnosis for EFB was conducted on 60 larval samples collected from different areas of the four districts for PCR amplification of partial 16S rRNA gene fragments (486 bp). Two pairs of *M. plutonius* specific primers (MP1& MP2 and MP1 & MP3 each reported by Djordejevic *et al.*, 1998), were used to amplify the different regions of the present *M. plutonius* genomes. Out of the two primer pairs, 1 primer pair (MP1 & MP3) reported by Djordejevic *et al.* (1998) amplified *M. plutonius* genome of *A. mellifera*. The amplicons (amplified product) were observed as clear distinct bands under UV transilluminator. These

Table 3. Incidence of EFB disease in *Apis mellifera* during July 2016~April 2017 at university apiary

Month	Total number of colonies	Total number of infected colonies	Colony infection in the apiary (%)
		EFB	EFB
July	48	1	2.08
August	48	2	4.16
September	48	7	14.59
October	48	3	6.25
November	48	–	–
December	48	–	–
January	48	–	–
February	48	–	–
March	53	1	1.88
April	53	2	3.77

primers were found specific as resulted in the amplification of genome with amplicons of expected size (486 bp) (Figs. 2 and 3).

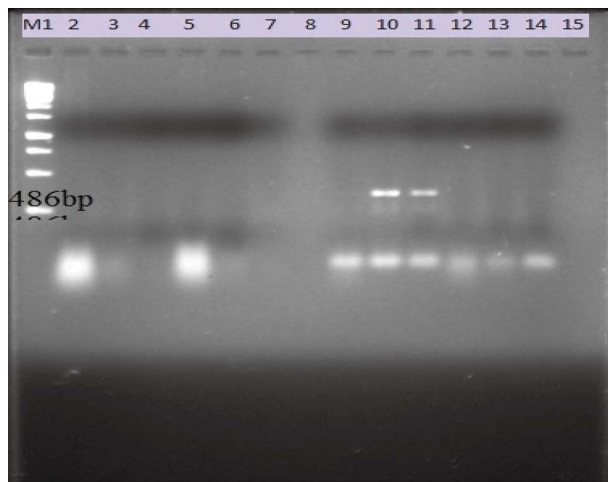
Results showed that out of 60 only 6 samples (10%) were positive for *M. plutonius* in different apiaries. This study document the occurrence of EFB by PCR assay in apiaries of different regions of Jammu (Table 5).

DISCUSSION

The discussion of investigation are presented under suitable headings as under.

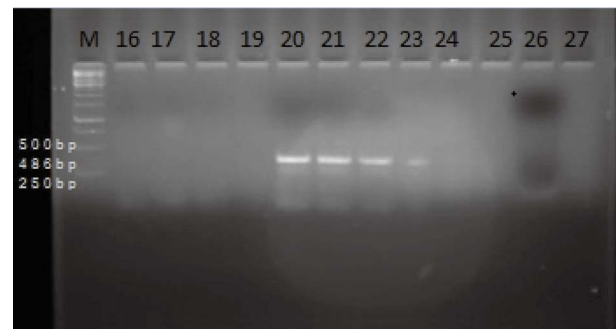
Table 4. Percent brood infection of European foulbrood disease during July 2016~April 2017 in *Apis mellifera* at university apiary

Month	Percent brood infection of EFB in colonies					Mean \pm S.D.	95% confidence interval	
	1	2	3	4	5		Lower limit	Upper limit
July	11.16	12.40	10.90	12.22	19.48	13.23 \pm 3.55	10.12	16.35
August	13.62	15.20	9.43	12.66	16.52	13.48 \pm 2.71	11.11	15.86
September	18.50	12.44	11.51	15.79	18.64	15.38 \pm 3.32	12.46	18.29
October	5.44	6.23	3.44	5.62	2.12	4.57 \pm 1.72	3.06	6.08
November	0	0	0	0	0	0	0.00	0.00
December	0	0	0	0	0	0	0.00	0.00
January	0	0	0	0	0	0	0.00	0.00
February	0	0	0	0	0	0	0.00	0.00
March	2.10	1.66	1.42	2.22	1.68	1.82 \pm 0.33	1.52	2.11
April	2.44	2.38	5.96	1.56	3.82	3.23 \pm 1.73	1.72	4.75

**Fig. 2.** Agarose gel electrophoresis (1.5%) of products of the first generation 16S rRNA PCR using primers MP1 and MP2. The DNA fragment was amplified from DNA extractions of larvae with European foulbrood. Lane 1, 250 bp molecular markers. Lane 2, 3, 4, 5, 6 - pranu colonies, Lane 7, 8, 9, 10, 11 - saringal colonies, Lane 12, 13, 14, 15 - attalgarh colonies, Lane 10, 11 - 2 ng *M. plutonius* DNA.

1. Symptomatology

The present study conducted on the symptoms of European foulbrood disease in *A. mellifera* found that in *A. mellifera* diseased bee colonies, colour of the infected larvae (brood) was pale yellow while healthy brood appeared glistening shiny white. The healthy brood was in coiled position at the bottom of comb cells while some of the infected larvae (pale yellow coloured) were found slightly displaced in upward or downward direction from their actual position. About 70~80 percent

**Fig. 3.** Agarose gel electrophoresis (1.5%) of products of the first generation 16S rRNA PCR using primers MP1 and MP3. The DNA fragment was amplified from DNA extractions of larvae with European foulbrood. Lane 1, 250 bp molecular markers. Lane 16, 17 - Dalwah colonies, Lane 18, 19 - Parnote colonies, Lane 20, 21 - Digdol colonies, Lane 22, 24 - miran sahib Lane 23, 25 - Arbal Lane 26, 27 - Shataninallah, Lane 20, 21, 22, 23 - 2 ng *M. plutonius* DNA.

death of brood was recorded at pupal stage. Sometimes about 15 percent occurred at larval stages and rarely in pre-pupal stage (about 5%), which resulted perforation in sealed brood. However, the colour of the brood did not change which exhibited typical symptoms like those of Thai sac brood virus disease and mite infestation. Also the infected larvae were slightly pulpy or watery. It was also revealed that in the advanced stage of the infection, their emitted sour or vinegar like smell. Remains of the dried brood were noticed in the form of soft scales. Similar results were reported by Forsgen (2013) who conducted study on field diagnosis of EFB based on the visual inspection of brood combs and detection of diseased larvae. He observed that in a colony

Table 5. Hemi-nested PCR results of larvae of *Apis mellifera* from various parts of Jammu

Source of samples	Number of samples	No. of <i>M. pluton</i> PCR positive samples (476 bp fragment)	Percent of <i>M. plutonius</i> positive samples
Attalgarh	5	1	20
Sartingal	5	1	20
Pranu	5	0	0
Digdol	5	2	40
Dalwah	5	0	0
Parnote	5	0	0
Miran sahib	5	1	20
Arnia	5	0	0
R. S. Pura	5	0	0
Shataninallah	5	0	0
Arbal	5	1	20
Zinhall	5	0	0

suffering from EFB there was irregular capping of the brood capped and uncapped cells being found scattered irregularly over the brood frame (known as pepper pot brood). The youngest larvae that die from the infection cover the bottom of the cell and are almost transparent, with visible trachea. Older larvae die mal positioned and flaccid in their cells; twisted around the walls or stretched out lengthways. The colour of affected larvae changes from pearly white to pale yellow, often accompanied by a loss in segmentation. More advanced symptoms can manifest as further colour changes to brown and greyish black, sometimes ultimately leaving a dark scale that is more malleable than those typically found with American foul breed.

2. Disease Occurrence

In the present study, it was observed that the colony infection of European foulbrood disease caused by *Melissococcus plutonius* was recorded from a minimum of 6% (Pranu) to a maximum of 16.66% (Sartingal) in colonies of district Doda. The study got support from Mwale (1992) who examined 343 hived *Apis mellifera* colonies in 36 apiaries at 19 locations in Malawi and found that 8% of the colonies were infected by *Melissococcus plutonius*.

1) Brood infection

It was evident from the findings of the study that

brood infection of European foulbrood was recorded from a minimum of 1.48% (Parnote) to a maximum of 15.25% (Digdol) of district Ramban. Similar study was conducted by Rao *et al.* (2011) who studied on characterization of *Melissococcus plutonius* from European honey bee (*Apis mellifera*) of North-West Himalayas and found that *Melissococcus plutonius*, the causal bacterium of European foulbrood (EFB) disease was isolated from *A. mellifera* L. of North-West Himalayas. The disease affected 2.52 to 18.52% brood in 2.65 to 15.00% honey bee colonies.

3. Seasonal incidence of European foulbrood disease in university apiary

Based on the observation and results of the study, incidence of European foulbrood in university apiary from July, 2016 to April, 2017 was found to be maximum in September (14.59%) and minimum in March (1.88%) and the percent brood infection of European foulbrood in university apiary was recorded from a minimum of 1.82% (March) to a maximum of 15.38% (September). These results got support from the findings of Rao (2009) who conducted study on molecular characterization of *Melissococcus plutonius* of hive honey bees and its control with antibiotics and revealed that maximum incidence of disease was recorded in September 2007 (18.52%) and declined completely by March.

4. Molecular detection through Hemi-nested polymerase chain reaction (PCR) technique

Based on the observation and results of the study, the bacteria present in the larval samples which caused disease in *A. mellifera* was detected and confirmed through polymerase chain reaction assay. A polymerase chain reaction (PCR) molecular diagnosis for EFB was conducted on 60 larval samples collected from different areas of the four districts for PCR amplification of partial 16S rRNA gene fragments (486 bp). Two pairs of *M. pluton* specific primers (MP1 & MP2) and (MP1 & MP3) each reported by Djordejevic *et al.* (1998), were used to amplify the different regions of the present *M. pluton* genomes. Out of the two primer pairs, 1 primer pair (MP1 & MP3) reported by Djordjevic *et al.* (1998) amplified *M. pluton* genome of *A. mellifera*. The amplicons (amplified product) were observed as clear distinct bands under UV transilluminator. These primers were found specific as resulted in the amplification of genome with amplicons of expected size (486 bp). Results showed that of the 60 samples only, 6 samples (10%) were positive for *M. plutonius* in different apiaries. This study documents the occurrence of EFB by PCR assay in apiaries of different regions of Jammu. Similar study was conducted by Djordjevic *et al.* (1998) who conducted study on development of a hemi-nested PCR assay for the specific detection of *Melissococcus plutonius* and in his study a pair of oligonucleotide primers (MP1 & MP2) was used for the polymerase chain reaction (PCR) amplification of a 486 base pair (bp) fragment of the 16S rRNA gene of 26 geographically diverse Australian *M. plutonius* (causative agent of European foulbrood) isolates. PCR primers spanning a region of the 16S rRNA gene from position 893~1377 failed to amplify a product when template DNA from a wide range of pathogenic and saprophytic bacteria were used including *Paenibacillus larvae*, *Enterococcus faecium* and *Spiroplasma melliferum*. The PCR did, however reliably amplify a 486 bp fragment (when the annealing temperature was lowered by 5°C) using template DNA isolated from the phylogenetically-related bacterium *Enterococcus faecalis*. PCR amplicons generated from *E. faecalis* and *M. pluton* were readily distinguished by digestion with the restriction endonuclease *Hinfl* and electropho-

resis in 1.5% agarose or by electrophoresis in 1% agarose containing bisbenzidine/polyethylene glycol. A hemi-nested PCR requiring a combination of primers MP1 and a third primer, MP3, which spanned 25 nucleotides from position 1168~1144 and internal to the 486 bp amplicon generated by primers MP1 and MP2 was developed. The hemi-nested PCR amplified a 276 bp *M. plutonius* specific product that was not amplified with *E. faecalis* DNA. In sensitivity studies, the PCR assay could reliably detect approximately 1~10 organisms/mi. This level of sensitivity was achieved using crude DNA templates (boiled cell lysate) prepared using Insta-gene matrix. The PCR assay could also detect *M. plutonius* in brood with European foulbrood. These findings got support from Khezri *et al.* (2016) who studied on the prevalence of *M. plutonius* by polymerase chain reaction in Kurdistan apiaries, Iran and in his study for multiplex PCR amplification of partial 16S rRNA (=SSU rRNA) gene fragments (486 bp), was conducted on 100 samples. Results showed that of 100 samples only 4 samples (4%) were positive for *M. plutonius* in Kurdistan apiaries.

The colony infection of European foulbrood disease caused by *Melissococcus plutonius* was recorded from a minimum of 6% (Pranu) to a maximum of 16.66% (Sartingal) in colonies of Doda region. Percent brood infection of European foulbrood was also recorded from a minimum of 1.47% (Parnote) to a maximum of 15.18% (Digdol) of Ramban region.

Incidence of European foulbrood in university apiary from July, 2016 to April, 2017 was found to be maximum in September (14.59%) and minimum in March (1.81%). Similarly incidence of sac brood was found maximum in March (9.09%) and minimum in August (2.07%). Percent brood infection of European foulbrood in university apiary was recorded from a minimum of 1.81% (March) to a maximum of 15.37% (September). A polymerase chain reaction (PCR) molecular diagnosis for EFB was conducted on 60 larval samples collected from different areas of the four regions for PCR amplification of partial 16S rRNA gene fragments (486 bp). Results showed that of the 60 samples, only 6 samples (10%) were positive for *M. plutonius* in different apiaries. This study document the occurrence of EFB by PCR assay in apiaries of different regions of Jammu. It is thus concluded that surveys are

required to characterize the distribution and prevalence of *M. plutonius* in different areas of Jammu district.

ACKNOWLEDGEMENTS

The authors thank Dr. Susheel Sharma, Assistant Professor School of Biotechnology for his help during the course of investigation. We also thank anonymous reviewers for their valuable suggestions on earlier draft of this paper.

LITERATURE CITED

- Abrol, D. P. 2009. Bees and beekeeping in India. 2nd Edn Kalyani Publishers, Ludhiana India.
- Abrol, D. P. 2012. Pollination biology: Biodiversity conservation and agricultural production. Springer Dordrecht Heidelberg London, New York.
- Alippi, A. M. 1991. A comparison of laboratory techniques for the detection of significant bacteria of the honey bee, *Apis mellifera*, in Argentina. J. Apic. Res. 30: 75-80.
- Allen, M. and B. Ball. 1996. The incidence and world distribution of honey bee viruses. Bee World 77: 141-162.
- Anonymous. 1998. Studies on control of natural enemies and diseases of Honey bees. Annual Progress Report, Department of Entomology and Apiculture, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, 45-46.
- Bahman, S. and B. S. Rana. 2002. Incidence of the foulbrood disease in *Apis mellifera* L. colonies at Solan, Himachal Pradesh. Pest Manag. & Econ. Zool. 10(1): 87-91.
- Bailey, L. 1974. An unusual type of *Streptococcus pluton* from the Eastern hive bee. J. Invert. Pathol. 23: 246-247.
- Bailey, L. and B. Ball. 1991. Honey Bee Pathology. Academic Press, London.
- Bailey, L., B. V. Ball and J. N. Perry. 1983. Association of viruses with two protozoal pathogens of the honey bee. Ann. Appl. Biol. 103: 13-20.
- Biesmeijer, J. C., S. P. M. Roberts, M. Reemer, R. Oholemuller, M. Edwards, T. Peeters, A. P. Schaffers, S. G. Potts, R. Kleukers, C. D. Thomas, J. Settele and W. E. Kunin. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313: 351-354.
- Bojana, B., J. Marko and S. Jonel. 2014. Honey bee colony collapse disorder (*Apis mellifera* L.) - possible causes. Scientific Papers Series Management, Econ. Engin. Agric. & Rural Dev. 14(2): 13-18.
- Dainat, B., D. Van Engelsdorp and P. Neumann. 2012. Colony Collapse Disorder in Europe. Environ. Microbiol. Rep. 4(1): 123-125.
- Delaplane, K. S. and D. F. Mayer. 2000. Crop Pollination by Bees. CABI Publishing, New York.
- Diwan, V. V., K. K. Kshirsagar, A. V. Ramanrao, D. Raghunath, C. S. Bhambure and S. H. Godbole. 1971. Occurrence of new bacterial disease of Indian honey bee *Apis indica* F. Curr. Sci. 40: 196-297.
- Djordjevic, P., S. L. Noone and M. Hornitzky. 1998. Development of a hemi nested PCR assay for the detection of *Melissococcus pluton*. J. Apicult. Res. 37(3): 165-174.
- Forsgren, E., J. R. de Miranda and M. Isaksson. 2009. Deformed wing virus associated with *Tropilaelaps mercedesae* infesting European honey bees (*Apis mellifera*). Exp. Appl. Acarol. 47: 87-97.
- Free, J. B. 1993. Insect pollination of crops. Academic Press, London-New York.
- Hansen, H. and C. J. Brødsgaard. 1999. American foulbrood: a review of its biology, diagnosis and control. Bee World 80: 5-23.
- Hornitzky, M. A. Z. and L. Smith. 1998. Procedures for the culture of *Melissococcus pluton* from diseased brood and bulk honey samples. J. Apic. Res. 37: 293-294.
- Hornitzky, M. A. Z. and S. Wilson. 1989. A system for the diagnosis of the major bacterial brood diseases. J. Apic. Res. 28: 191-195.
- Johnson, R. 2007. Recent Honey Bee Declines. Congressional Research Service, Washington, DC: 14 pages.
- Klatt, B. K., A. Holzschuh, C. Westphal, Y. Clough, I. Smit, E. Pawelzik and T. Tschamtkke. 2014. Bee pollination improves crop quality, shelf life and commercial value. Proc. R. Soc. B 281: 20132440.
- Morandin, L. A. and M. L. Winston. 2006. Pollinators provide economic incentive to preserve natural land in agroecosystems. Agric. Ecosyst. Environ. 116: 289-292.
- Neumann, P. and C. Carreck. 2010. Honey bee colony losses: a global perspective. J. Apic. Res. 49: 1-6.
- Okamura, B. 2016. Hidden Infections and Changing Environments. Integ. Comp. Biol. 56(4): 620-629.
- Oldroyd, B. P. and S. Wongsiri. 2006. Asian Honey Bees Biology, Conservation and Human Interactions. Harvard University Press, Cambridge.
- Paxton, R. J. 2010. Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)?. J. Apic. Res. 49(1): 80-84.
- Pinnock, D. E. and N.E. Featherstone. 1984. Detection and quantification of *Melissococcus pluton* infection in honeybee colonies by means of enzyme-linked immunosorbent assay. J. Apic. Res. 23: 168-170.
- Potts, S. G., S. P. M. Roberts, D. Robin, G. Marris, M. A. Brown, R. Jones, P. Neumann and J. Settele. 2010. Declines of managed honey bees and beekeepers in Europe. J. Apic. Res. 49(1): 15-22.
- Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger and W. E. Kunin. 2010a. Global pollinator

- declines: trends, impacts and drivers. *Trends Ecol. Evol.* 25: 345-353.
- Powney, G. D., C. Carvell, M. Edwards, K. A. Roger, R. K. A. Morris, H. E. Roy, B. A. Woodcock and N. B. J. Isaac. 2019. Wide spread losses of pollinating insects in Britain. *Nat. Comm.* 10, Article number: 1018.
- Rana, B. S., R. Rana, G. Kumar, H. K. Sharma, J. K. Gupta and K. Dayal. 2004. European foulbrood causing havoc to the native bee, *Apis cerana* F. in Himachal Pradesh. *Pest Manag. & Econ. Zool.* 12(1): 109-111.
- Rao, K. M. 2009. Molecular characterization of *Melissococcus plutonius* of hive honey bees and its control with antibiotics, Ph.D. Thesis. Department of Entomology and Apiculture, Dr. YS Parmar University of Horticulture and Forestry, Solan. 97.
- Rao, K. M., B. S. Rana, S. K. Chakravarty, S. Katna and A. Sharma. 2011. Characterization of *Melissococcus plutonius* from European Honey bee (*Apis mellifera* L.) of North-West Himalayas. *Intl. J. Sci. & Nat.* 2: 632-638.
- Roubik, D. W. 1995. Pollination of cultivated plants in the tropics. *FAO Agricultural Services Bulletin* 118, Rome.
- Sokal, R. R. and F. J. Rohlf. 2013. *Biometry: The principles and practices of statistics in biological research* 4th Edition. W. H. Freeman and Company Ltd., San Francisco, USA.
- Vanbergen, A. J. 2013. Threats to an ecosystem service: pressures on pollinators. *Frontiers Ecol. Environ.* 11(5): 251-259.
- van der Sluijs, J. P., N. Simon - Delso, D. Goulson, L. Maxim, J. M. Bonmatin and L. P. Belzunces. 2013. Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr. Opin. Environ. Sustain.* 5(3-4): 293-305.
- Viraktamath, S. 1998. A bacterial disease of *Apis mellifera* L. in Dharwad. *Insect Env.* 4: 27.
- William, P. N. and L. Anderson. 1974. Insect pollinators frequenting strawberry blossoms and the effect of honey bees on yield and fruit quality. *J. Amer. Soc. Hort. Sci.* 99(1): 40-44.