INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

www.scienceandnature.org

CHARACTERIZATION OF *Melissococcus pluton* FROM EUROPEAN HONEY BEE (*Apis mellifera* L.) OF NORTH - WEST HIMALAYAS

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ABSTRACT
Melissococcus pluton, the causal bacterium of European foul brood (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 in different months (except winter) during October 2006 to September 2007. *M. pluton* showed growth on specific basal medium having pH 6.6 under anaerobic (about 10% added CO_2) conditions at 34°C in 3-4 days. Its cells were Gram positive, lanceolate in shape with 0.5-0.9 µm size, arranged in long chains and pathogenic. Antiserum and ELISA product with 1:200 and 1:1000 homologous titres, respectively were produced against the isolate. It was identified through positive immunodiffusion gel and DAC-ELISA tests. PCR studies through 92 DF / 93 DR and BCF_1 / BCF_2 (ITS) primers developed amplicons with 812 and 550 bp sizes, respectively. On sequencing, the respective amplicons yielded nucleotide sequences of 395 and 436 bp. The latter was submitted to EMBL- EBI, UK and got accession no. FN 297846. Phylogenetically found closer to German isolate (99%). Feeding of ciprofloxacin (98%, a.i.) @ 10, 20 or 40 mg in sugar solution per colony controlled cent percent EFB in 9 days.

KEYWORDS: European foul brood, *Melissococcus pluton*, *Apis mellifera*, anaerobic, antiserum, ELISA, ITS primers, nucleotides, ciprofloxacin.

INTRODUCTION

European foul brood disease in *A. mellifera* is distributed throughout the world in about 100 countries (Ellis and Munn, 2005). In India, it is prevalent throughout the country with variable severity (Anonymous, 2009). From the North India, *A.mellifera* colonies were supplied to other parts of the country during late eighties and resulted in spread of the disease (Viraktamath, 1998). Lot of work has been done on various aspects of EFB and its causal organism in the different parts of the world (White, 1912; Bailey, 1960; Bailey and Gibbs, 1962; Buza & Kovacs, 1969; Knox *et al*., 1971; Bailey, 1974, 1977) A large number of causal bacterial, *M. pluton* (= *M. plutonius*) isolates have been reported to occur in the world (Bailey and Collins, 1982a, b; Allen and Ball, 1993). These differed in biochemical, cultural and serological characteristics. Molecular level characterization of some of the isolates has also been done (Dancer and Barnes, 1995; Djordejevic *et al*., 1998; Daffonchio *et al*., 2003). In present studies, attempt has been made to study the seasonal incidence of EFB in *A.mellifera* and to characterize *M. pluton* on the basis of various parameters.

MATERIALS AND METHODS

The present investigations were conducted on various parameters of European foul brood disease causing bacterium *Melissococcus pluton* during 2006 to 2009. *Apis mellifera* L. colonies maintained by the Department of Entomology and Apiculture, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, North-West Himalayas, India situated at 33.3N° latitude, 70.70°E longitude and 1256 m above mean sea level (amsl), were used.

Incidence of European foul brood disease

Incidence of the disease similar to typical European foul brood was recorded in *A. mellifera* during October 2006 to September 2007 at Nauni. Data on healthy brood and diseased brood were recorded with the help of grid at an interval of 21 days. Severity of the European foul brood disease was studied with regard to the per cent colony infection in the apiary and per cent brood infection in the colony as per the formulae given below:

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

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

Isolation of bacteria- The isolation of bacteria from diseased brood (larvae, prepupae and pupae) was done as per the method of Allen & Ball (1993).

Pathogenicity- Pathogenicity of causal bacterial isolate was carried out in healthy *A. mellifera* colonies having equal strength, brood and age of queens. The area under

eggs of each colony was marked and measured. The pure culture (1.9 x 10^7 organisms /ml bacterial suspensions) of the isolate M_1 was fed to the colonies in 500 ml sugar syrup (50%). The control colonies were fed with sugar syrup containing crude extract of healthy brood. Observations were recorded daily on the appearance of any change in brood. Re-isolation of bacterium was also done from the infected brood for further confirmation of the disease.

Characterization of bacterial isolates- Characterization of bacterial isolates was done with regard to morphological, cultural and pathogenic aspects as per the procedure of Buchanan and Gibbons (1974).

Morphological characters- Isolated bacterial cells was studied by differential Gram staining. The bacterial colonies were observed with regard to colour, configuration (shape), margin, elevations and surface texture (density).

Cultural characteristics - Growth of each isolate was observed on specific basal medium by following the method of Allen and Ball (1993), on bacteriological medium, specific basal medium plus 6.0 per cent sodium chloride.

Effect of carbon dioxide- Incubations on different media were done under aerobic and anaerobic (created the vacuum inside the desiccators and added carbon dioxide) conditions at 34°C for 3-6 days. Observations were recorded every 24 h after incubation.

Effect of pH- The optimum growth of the isolates was also recorded on specific basal medium having different pH levels 6.0, 6.6, 7.0, 7.2, 8.0, 9.0, 10.0 and 11.0.

Antiserum Production- Antiserum (Polyclonal antibodies) was produced through traditional method in healthy rabbits (New Zealand White) at Central Potato Research Institute, Shimla, Himachal Pradesh to characterize and identify the isolate. Each rabbit was injected intra-muscularly in each hind leg with 1.0 ml. of *M. pluton* (dead) bacterial suspension (1.9 x 10^7 organisms) in normal saline that had been emulsified with an equal volume of Freund's incomplete adjuvant. Four injections were given to each rabbit at weekly interval. One week after the final injection, the rabbits were bled from ear veins. The blood samples were collected and kept at room temperature overnight for clotting. The samples were then centrifuged for 5 minutes at 10,000 g and the clear straw coloured serum was collected and a pinch of sodium azide was added to inhibit the microbial growth. The titre of the antiserum was estimated through agglutination test on slide.

ELISA Production- ELISA product was prepared from the first harvest of the antiserum by following a standard method given by Mackenzie (1990). Then its efficacy was tested through Direct Antigen Coating – Enzyme Linked Immunosorbent Assay (DAC- ELISA).

Serology- Immunodiffusion gel test and ELISA (Enzyme Linked Immunosorbent Assay) were conducted to identify the causal organisms of present disease.

Immunodiffusion gel test- Immunodiffusion gel tests were conducted in 9 cm plastic petridishes containing 5 mm gel layer prepared from purified agar (0.6 %), sodium azide (0.02%), sodium chloride (1.0 %) in 100 ml 0.1M phosphate buffer (pH 7.0). The presently prepared antiserum was used. The isolate was sonicated (Brasons sonifier 450, at 80 duty cycles, out put 3, time 5 minutes) before loading. The petriplates were incubated at 34°C and 65 % RH.

ELISA Test- Identification of the bacterium isolated from *A. mellifera* was done through DAC-ELISA as per standard method by using the presently prepared ELISA product.

Molecular identification- Molecular level detection of the present isolate was done through polymerase chain reaction (PCR) technique as per the details given below:

Isolation of total DNA- The total DNA was extracted from the pure culture of *M. pluton* by Genei™ spin genomic DNA prep kit.

Checking of DNA- Presence of DNA was checked in 1.0 per cent agarose gel, prepared in 1x TAE (Tris acetate EDTA) buffer and stained with ethidium bromide (0.4 µg/ml). DNA (5 µl) was loaded with DNA loading dye (xylene cyanol) and electrophoresed at 120 V for 30-40 minutes and visualized in UV trans illuminator (TFX-20M France).

Identification through PCR - PCR involves three basic steps which constitute a single cycle, denaturation of target DNA, annealing of primer to the template DNA and primer extension by addition of nucleotides to the 3' end of the primers.

Primer sequences used in detection of *M. pluton* through PCR were:

Primer	Sequences 5' to 3'	References
92 DF	GAAGAGGAAGTTAAAAGGCGC	Govan *et al*. (1998)
93 DR	TTATCTCTAAGGCGTTCAAAGG	

Synthesis of DNA from genomic DNA - PCR studies were carried out by using Banglore Genei chemicals: Buffer (10x) 5.00µl, dNTP mix (200 µM) 4.00µl, DNA sample (5 ng) 2.00µl, Forward primer (100 ng) 3.00 µl, Reverse primer (100 ng) 3.00µl, $MgCl_2$ (4mM) 5.00µl, Taq polymerase (2-5µl) 1.00µl and Sterile distilled water 27.00 µl. The mixture was then mixed by pippetting up and down. The reactions were: initial denaturation at 95°C for 2 min, denaturation at 95°C (30 sec), annealing at 61°C (15 sec), elongation and extension at 72°C for 60 sec and 5 min, respectively. In the above reactions, steps 2-4 were repeated 40 times and final extension at 72°C for 5 minutes. The PCR amplification was performed in Gene AMP PCR system 9700 Thermal cycler (Applied Bio-Systems, USA).

Synthesis of DNA from genomic DNA - Banglore Genei chemicals were used. The PCR reaction mixture comprised of buffer with 2.5 mM $MgCl_2$ (10x) 2.00µl, d NTP mix (2 mM) 1.60µl, primer forward (100 ng) 0.15µl, primer reverse 0.15µl, 0.30µl Taq DNA Polymerase, DNA (5 ng) 4.00µl and sterile distilled water11.80 µl. The mixture was pipetted up and down. The reactions were: initial denaturation at 94°C for 4 min, denaturation at 94°C (1 min), annealing at 58°C (1 min), elongation and

extension at 72°C for 1 and 10 min respectively. In the above reactions, steps 2-4 were repeated 40 times and final extension was carried out at 72°C for 10 minutes.

Gel electrophoresis- PCR product (5 µl) was mixed with 4 µl of gel loading dye (ethidium bromide), and loaded on 1.0 per cent agarose gel and electrophoresed in 1xTAE buffer at 120V for 30-45 minutes. DNA marker Gen ruler-SM 0311 (Fermentas Life Sciences,USA) was used for comparison. The gel was viewed under UV trans illuminator (TFX-20 M, France) and photographed in Flour-S Multiimager Bio-Rad Laboratories, Herculas CA, USA.

Intergeneic Transcribed Spacers (ITS) primers used in PCR studies were:

Primer	Sequences 5^1 to 3^1	References
BCF_1	CGGGAGGCAGCAGTAGGGAAT	Daffonchio *et al*. 2003
BCR_2	CTCCCAGGCGGAGTGCTTAAT	

Gel extraction- Amplified PCR product was extracted from gel for DNA sequencing by Qia™ quick gel extraction kit by following the protocol supplied by Qiagen™ . The efficiency of the eluted DNA was checked in 1.0% agarose gel.

DNA sequencing- The DNA sequencing was done by following Dye Ex 2.00 spin protocol for Dye terminator removal. The reactions were carried out in Big Dye CM PCR cycle. Sequencing was done in Genetic Analyzer (ABI PRISM™ 310 Applied Biosystems, USA).

DNA alignments were done by CLUSTALW multiple sequence alignment programme (www.ebi.ac.uk/tools/clustalw$_2$/indexhtml).

Field evaluation of ciprofloxacin - The efficacy of ciprofloxacin (98% a.i.) was assessed in EFB infected *A. mellifera* colonies by feeding a single dose of ciprofloxacin @ 10, 20 and 40 mg/ dose in 500 ml sugar syrup (50 %) / colony during August 2008. Each dose was fed to 5 colonies having equal strength and almost similar level of brood infection. Data on effect of the treatment on per cent brood infection were recorded daily till the infection reduces to negligible level.

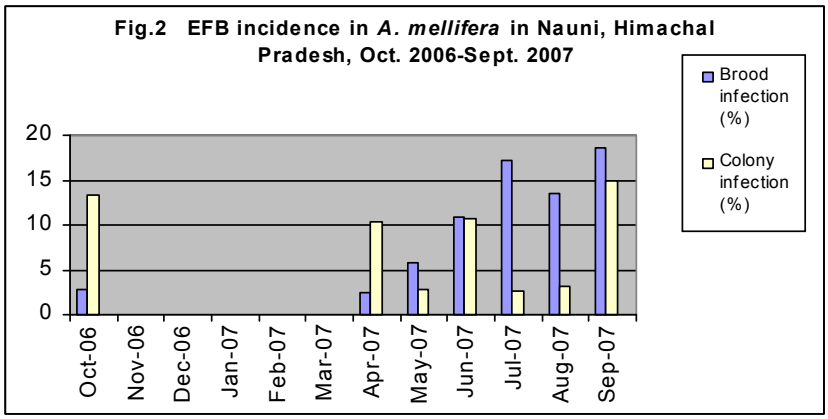
RESULTS AND DISCUSSION

Symptoms similar to typical European foul brood disease recorded presently in *A. mellifera* larvae were: pale yellow in colour (Fig. 1), died at 3-5 days age, few slightly displaced in their cells, rotten mass emitted vinegar like smell and finally dried down to soft scales. Sometimes death of the brood (1-2%) was also noticed at pre pupal and pupal stages with no colour change.

FIGURE 1. EFB infected larvae

Disease incidence- The disease incidence was recorded during October 2006 and April to September 2007 with 2.87 and 2.52 to 18.52 per cent brood infections, respectively in the colonies (Fig. 2). On an average, 13.25 per cent (range 3.14 to 15.00%) colonies were infected in the apiary during respective period. No disease was recorded during November 2006 to March 2007. Earlier, variations in seasonal incidence and severity of this disease have been recorded in different parts of the world which may be attributed to variations in agro- climatic conditions from place to place, year to year and casual bacterium strain (Buza & Kovacs, 1969; Knox *et al*., 1971; Bailey,1977). Generally during winter months, less number of available nurse bees results in production of less amount of glandular food which causes the diseased larvae to consume more food. Such larvae are immediately detected and removed (Bailey, 1960; Simpson, 1960). Therefore, presently no disease out break was recor

ded during winter months.



Isolation of bacteria: Two types of bacterial colonies designated as M_1 and M_2 were isolated on specific basal medium after 1-6 days of incubation period under anaerobic conditions at 34°C from the inoculum of *A. mellifera* larvae. However, from the disease suspected

prepupae and pupae, only M_1 type of colonies were isolated that too very easily.

Characteristics- Present bacterial isolates were different (Table.1) with regard to some cultural characteristics as M_2 grew well on bacteriological medium even under anaerobic as well as aerobic conditions and on 6.0 per cent sodium chloride + specific basal medium having pH 6.0-11.0 with an incubation period of 24 h. The isolate M_1 gave negative response regarding its growth on 6.0 per cent sodium chloride + specific basal medium and bacteriological medium. This isolate required an incubation period of 3-4 days under anaerobic condition (about 10% CO_2 added) at pH 6.6-6.7. Morphologically, M_1 bacterial isolate colonies were dense white in colour, round in shape with entire margins and having convex elevation. The size varied between 0.57 - 1.5 mm. The texture of the colonies was opaque and soluble in water. While the colonies of M_2 isolate were round in shape with 1.0 - 1.5 mm size and had smooth sides/margins with flattened elevation. Microscopically, M_1 isolate was Gram positive, lanceolate in shape, 0.5-0.9 µm in size and arranged in long chains (Fig. 3) while M_2 isolate was ovoid in shape, size 0.5-1.0 µm, occurred singly, in pairs, or in small short chains. The pure cultures were got identified from Indian Institute of Microbial Technology, Chandigarh. These isolates confirmed to be M_1 as *Melisococcus pluton* and M_2 as *Enterococcus faecalis* with accession number MTCC 6433 and MTCC 3159, respectively. Similar cultural characteristics have been reported for *M. pluton* isolated from *A. mellifera* of the same region by Bahaman and Rana (2002).

TABLE 1. Characteristics of M_1 and M_2 bacterial isolates from diseased *A. mellifera* brood with regard to various parameters

Bacterial isolate	BLM	SBM	SBM + 6.0 % NaCl	Shape of cells	Size of cells (µm)	pH requirement	Arrangement of cells	Growth under anaerobic conditions	Growth under aerobic conditions	Size of bacterial colony (mm)
M_1	_	+	_	Lanceolate	0.5-0.9	6.6-6.7	Long chains	+	_	0.57-1.50
M_2	+	+	+	Ovoid	0.5-1.00	6.0-8.0	Single, Pairs or short chains	+	+	1.00-1.50

M_1 and M_2 from *A. mellifera*
BLM= Bacteriological medium,
SBM=Specific basal medium,
SBM+6% NaCl=Specific basal medium + 6.0 % sodium chloride
– No growth
+ Growth present

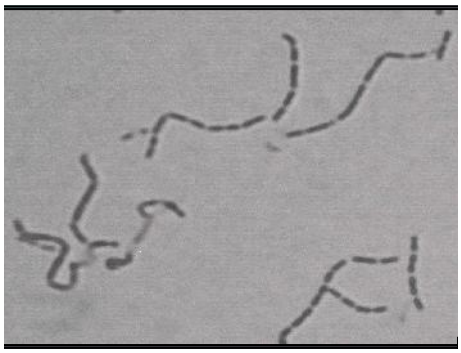


FIGURE 3. Lanceolate cells in long chains (M_1)

Since M_1 isolate was possibly *M. pluton*, the main causative bacterium of the present disease, indicating the incidence of European foul brood in *A. mellifera* while M_2 isolate *E. faecalis*. These results get further support from the earlier studies conducted by various workers (Bailey, 1957, 1963; Bailey and Collins 1982a; Allen and Ball, 1993).

Pathogenicity – Pathogenic studies showed that after feeding M_1 isolate, either larval brood was ejected out by the bees or disease symptoms were noticed on 6^{th} day i.e 2^{nd} day of larval stage. Typical symptoms of the disease were observed on 8^{th} day after the feeding. M_1 type bacterium was reisolated from the diseased brood of treated *A. mellifera*. Thus M_1 isolate was pathogenic in nature. Earlier, Bailey (1957), Krasikova and Naumova (1957) and Glinski (1972) have recorded the appearance of diseased symptoms on 6-8 days after feeding pure culture of *M. pluton* in *A. mellifera*. The results further confirmed that the isolate M_1 was the causative bacteria of European foul brood disease.

Antiserum Production- Homologous titre of the present antiserum tested through agglutination slide test was found to be 1:200 against *M.pluton* of *A.mellifera***.** Earlier, Bailey and Collins (1982b), Allen and Ball (1993) had also produced the antiserum against different isolates of *M. pluton* infecting hive bees with variable homologous and heterologous titres ranging from 4 to 2048 depending upon their antigenicity.

ELISA Production- The present ELISA product was tested against the pure culture of *M. pluton* from *A. mellifera* through DAC-ELISA test. The positive reaction at 1:1000 dilution of EC (Enzyme Conjugate) and IgG (Immunoglobulin) in ELISA plate on the basis of visual observations, confirmed the successful production of ELISA product.

Serological characters- *M. pluton* was also confirmed through Immunodiffusion gel and ELISA tests.

In immunodiffusion gel test**,** development of sharp precipitin bands between present M_1 isolate and the antiserum was confirmed the presence of *M. pluton*.

Similarly, through DAC-ELISA test, development of yellow colour (1.799 nm) between the ELISA product and the isolate indicates the presence of *M. pluton*.

Characterization through polymerase chain reaction- Finally, polymerase chain reactions were conducted to characterize and confirm *M. pluton* at molecular level. Studies through the use of 92 DF/93DR primer pair were resulted in the amplification of genome of the isolate M_1 in the form of clear and distinct bands (amplicon) of 812 bp size (Fig. 4 **).** The size of 812 bp as per the earlier findings of Govan *et al*. (1998) confirmed the identity of the present isolate as *M. pluton*.

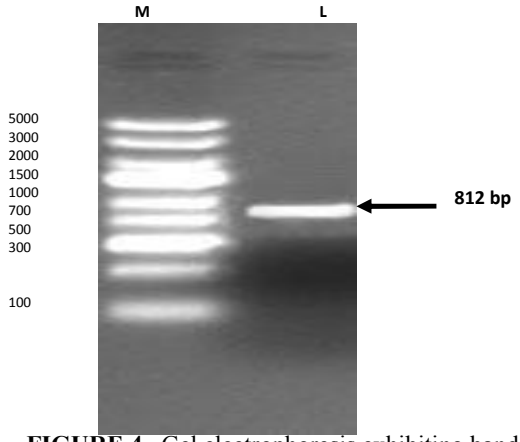


FIGURE 4. Gel electrophoresis exhibiting band of PCR Product using *M pluton* specific primers
Lane M :Marker 1 kb ladder
Lane L :Amplified product of 812 bp of *M. pluton* from *A. mellifera* using 92 DF/93 DR primers

Further, the use of Intergenic Transcribed Spacer (ITS) primers (BCF_1/BCR_2) also resulted in the amplification of the genome in the form of 550 bp size (Fig 5). Both the amplicons (812 and 550 bp) were sequenced which yielded 395 (Fig. 6) and 436 (Fig. 7) bp nucleotide lengths, respectively. The sequenced data of 436bp nucleotide length was submitted to EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) Cambridge, United Kingdom and got accession number FN 297846. According to Daffonchio *et al.* (2003), ITS between 16S and 23S rRNA genetic loci are frequently used in PCR finger printing to discriminate bacterial strains at the species and intra- species levels.

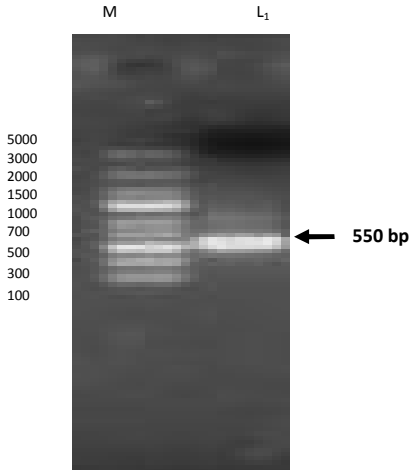


FIGURE 5. Gel electrophoresis exhibiting bands of PCR product Using BCF_1/BCR_2 primers
Lane M : Marker 1kb ladder
Lane L : Amplified product of 550 bp of *M. pluton* from *A. mellifera*

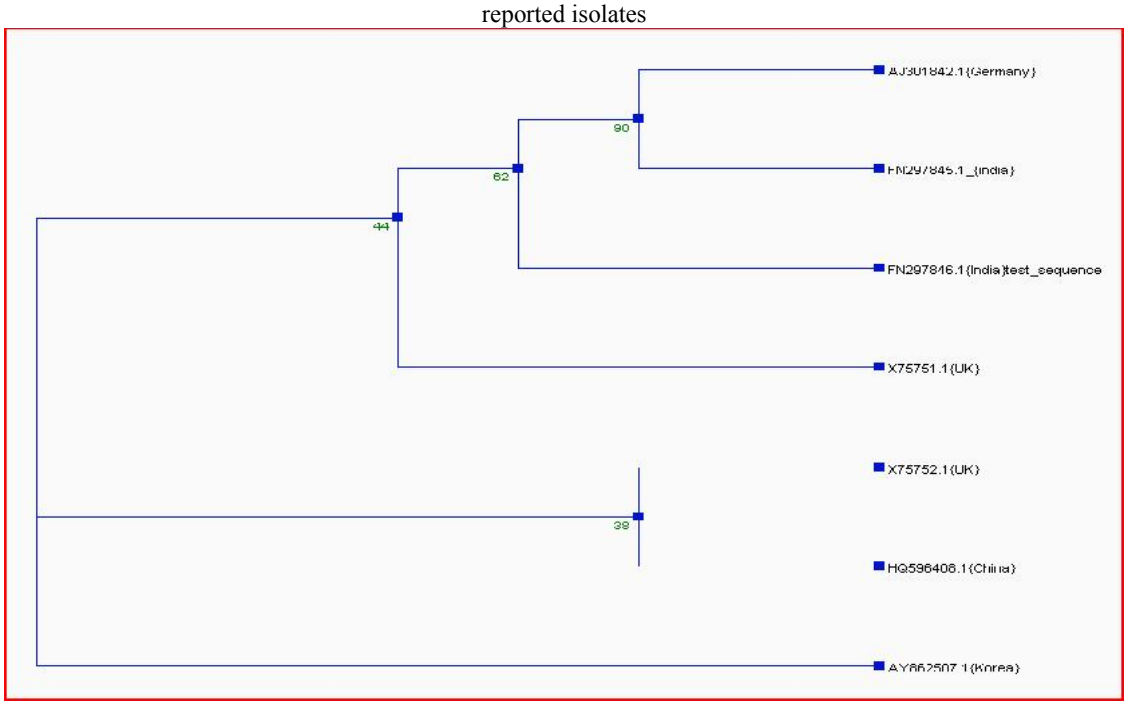
FIGURE 6. Nucleotide sequence (partial) of *M. pluton* isolated from *A. mellifera* (395 bp)

CCGAC CTGAG AGGGT GATCG GCCTC ACTGG GACTG AGACA CGGCC
CAGAC TCCTA CGGGA GGCAG CAGTA GGGAA TCTTC GGCAA TGGAC
GAAAG TCTGA CCGAG CAACG CCGCG TGAGT GAAGA AGGTT TTCGG
ATCGT AAAAC TCTGT TGTTA GAGAA GAATA GGGGA AAGAG TAACT
GTTTT CCTCG TGACG GTATC TAACC AGAAA GCCAC GGCTA ACTAC
GTGCC AGCAG CCGCG GTAAT ACGTA GGTGG CAAGC GTTGT CCGGA
TTTAT TGGGC GTAAA GCGAG CGCAG GCGGT TTTTT AAGTC TGATG
TGAAA GCCCC CGGCT CAACC GGGGA GGGTC ATTGG AAACT GGAAG
ACTTG AGTGC AGAAG AGGAG AGTGG AATTC CATGT

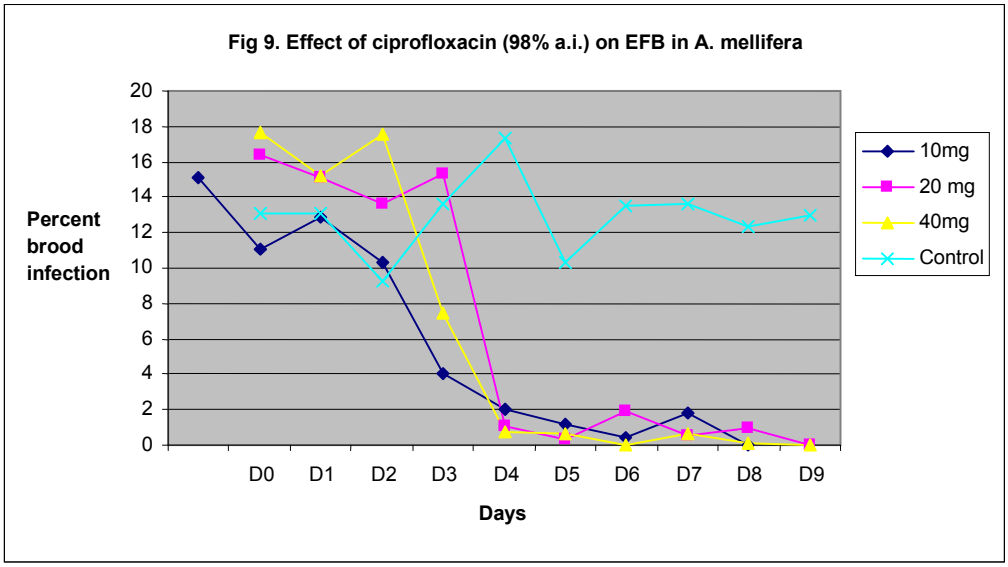
FIGURE 7. Nucleotide sequence of *M. pluton* isolated from *A. mellifera* (436 bp)

AACGC	CGCGT	GAGTT	GAAGA	AGGTT	TTCGG	CATCC	TAAAA	CTCTG
TTGTT	AGAGA	AAGAA	TAGGG	GAAAG	AGTAA	CTGTT	TTCCT	CGTGA
CGGTA	TCTAA	CCAGA	AAGCC	ACGGC	TAACT	ACGTG	CCAGC	AGCCG
CGGTA	ATACG	TAGGT	GGCAA	GCGTT	GTCCG	GATTT	ATTGG	GCGTA
AAGCG	AGCGC	AGGCG	GTTTT	TTAAG	TCTGA	TGTGA	AAGCC	CCCGG
CTCAA	CCGGG	GAGGG	TCATT	GGAAA	CTGGA	AGACT	TGAGT	GCAGA
AGAGG	AGAGT	GGAAT	TCCAT	GTGTA	GCGGT	GAAAT	GCGTA	GATAT
ATGGA	GGAAC	ACCAG	TGGCG	AAGGC	GGCTC	TCTGG	TCTGT	AACTG
ACGCT	GAGGC	TCGAA	AGCGT	GGGGA	GCAAA	CAGGA	TTAGA	TACCC
TGGTA	GTCCA	CGCCG	TAAAC	GATGA	GTGCT	A		

FIGURE 8. Phylogenetically, present isolate showed 99% identity to German isolate (AJ301842) and 98% to other reported isolates



Phylogenetic tree (NJ) illustrating the genetic relationship of present (Indian) isolate (FN297846) with other *M pluton* isolates.



Field evaluation of ciprofloxacin

In vitro evaluation of 22 antibiotics was carried out by paper disc agar diffusion method against the isolate.

Keeping in view the high sensitivity of the isolate to ciprofloxacin, it was selected to test its efficacy against EFB in the colonies. Treatment of the diseased colonies with ciprofloxacin during August 2008 indicated that there was a continuous reduction in brood infection in all the treated colonies (Fig.9) and complete control was recorded on 9th day after each treatment.

Fast recovery was recorded due to 40 mg dose of ciprofloxacin as compared to 20 and 10 mg doses. The respective reduction in percent brood infection being from 17.63 to 0.00 (99.65% recovery), 16.43 to 0.00 (97.99%) and 15.14 to 0.00 (96.96%). However, on an average uniform brood infection (12.92 %) persisted in control colonies. After treatment, either no or negligible infection of disease was recorded till December 2008. In India, Bahaman and Rana (2002) have been able to control European foul brood disease in *A. mellifera* in 8 days by feeding a dose of 200 mg OTC (5 % a.i.) in sugar syrup. Such antibiotic treatments are suggested to adopt only during prolonged dearth period so as to avoid residues in honey.

ACKNOWLEDGEMENT- Authors are thankful to the authorities of Dr Y S Parmar UHF, Nauni, Solan, CRI. Kasauli and CPRI, Shimla,Himachal Pradesh for providing necessary facilities. Financial assistance from ICAR, New Delhi through Project Coordinator, AICRP (Honey Bees & Pollinators) is also duly acknowledged.

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