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Prevalence of American Foulbrood Disease in Local Honeybee Colonies; Targeting Incidences of *Paenibacillus Larvae*

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Article Details

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ABSTRACT

American Foulbrood (AFB) represents a formidable threat to honeybee populations, profoundly impacting larvae and contributing to a significant decline in overall bee numbers. This study focuses on the causative agent of AFB, the spore-forming bacterium Paenibacillus larvae, and evaluates the prevalence of this devastating disease in Apis mellifera colonies situated in the Malakand district of Pakistan. We employ biochemical analyses, Gram's staining, and microscopic and molecular techniques to assess the presence of AFB. A total of 800 honeybee colony samples were collected from 35 apiaries across the region. The findings reveal an alarming 35.47% incidence of AFB symptoms within the sampled colonies. Local variations in disease prevalence are underscored, with Thana exhibiting significantly higher rates ($P < 0.01$) compared to Batkhela, which records the lowest incidence. Furthermore, the temporal dynamics of AFB are explored, highlighting a substantial increase in prevalence during the warmer months of June and July ($P < 0.01$). Maximum prevalence is observed at temperatures ranging between 41°C and 45°C, emphasizing the influence of climatic conditions on disease dynamics. This study provides crucial insights into the widespread presence of Paenibacillus larvae in *Apis mellifera* colonies within the Malakand district, posing a severe and immediate threat to the already declining honeybee populations in the area.

INTRODUCTION

Honeybees (Apididae: Hymenoptera) are significant insects for the economy because they produce valuable goods including honey, propolis, bee venom, wax, royal jelly, pollen, and increase agricultural production by providing pollination services [1]. A hive can include numerous bees. They use pheromones and dancing language to interact with one another in a special way. Pakistan has four honeybee varieties: *Apis cerana* (Rock bee), *Apis florea* (small bee), *Apis dorsata* (Oriental bee), and *Apis mellifera* (Occidental bee). Through the support of a UNHCR initiative, *in* 1981–1982, *Apis mellifera* was brought to Pakistan in an effort to boost bee populations. The value *of Apis cerana* is unknown because there hasn't been enough research on it [2]. However, due to this bee's extremely low honey production, Pakistani farmers and beekeepers hardly ever handle it. While *Apis florea* is unharmed by the introduction of *Apis mellifera*, an economically controlled bee, and its associated parasites and diseases endanger local Asian bee species, notably in Pakistan [3], [4].

Apiculture, often known as beekeeping, is raising honeybees for both domestic and commercial use, producing honey, beeswax, and other products, and offering honeybee hives for sale. Honeybees are well-liked and beneficial to the country's economy. The world's bee population totals more than 20,000 different species. The fourth species, which is native to America and was imported into Pakistan, is not an indigenous species; the other three are. In Pakistan, keeping honeybees is a lucrative business. Approximately seventy thousand beekeepers presently raise *Apis mellifera* species in modern hives. In 2023, Pakistan produced 15,750 metric tons' honey according to Pakistan's Honey Market report 2023. District Malakand, Pakistan offers a prime location for beekeeping due to its abundant natural resources, abundant crops, water supplies, and vast bee colonies [5]. Beekeepers' competence is necessary for apiculture thus they have to carefully examine new developments in this field [6]. If the bees disappear from the earth, life will be impossible. Additional research is needed to differentiate among colonies that are wild and those that are not due to the enormous global decline of the species [7], [1]. Climate change significantly impacts honeybee colonies due to its impact on pollinator activities and beekeeping, leading to high destruction rates [8]. Disease in honeybees is caused by a variety of pathogens and pests [9]. One of the most important issues for *Apis mellifera* is infection, which includes bacterial diseases like AFB, Varroa destructor (mite), fungal infections like *Nosema*, and viral infections like DWV [10].

American foulbrood (AFB), which frequently results in the total eradication of entire honeybee colonies, is a serious threat to honeybee larvae and a huge threat to beekeeping globally [11], [12]. Bee larvae are exposed to the bacteria *Paenibacillus larvae*, which is the pathogen that causes sickness, through contamination of food. American foulbrood (AFB) is a highly contagious disease, and routine beekeeping practices make it simple for spores to spread between apiaries [13]. There haven't been any confirmed sightings of *Paenibacillus larvae* in Malakand, despite their widespread presence and frequent honeybee fatality.

The study area is an ideal environment for the development of apiculture. Many beekeepers work in apiculture in this area. Because of a dearth of scientific knowledge, sustainable apiculture has not yet been established in this area. The study objective is to ascertain the prevalence of AFB in the honeybee colonies at Malakand, Pakistan. This is the first study on this aspect in the area.

MATERIALS AND METHODS

STUDY AREA & STUDY PERIOD

The study was carried out from March to September 2022 at Malakand, Pakistan. Five localities were selected in district Malakand (Batkhela, Thana, Dargai, Palai and Malakand Top) (Fig. 1).

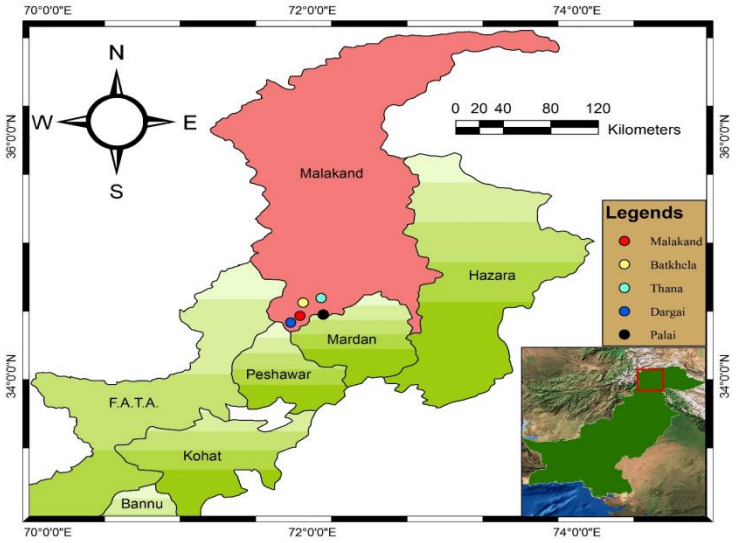
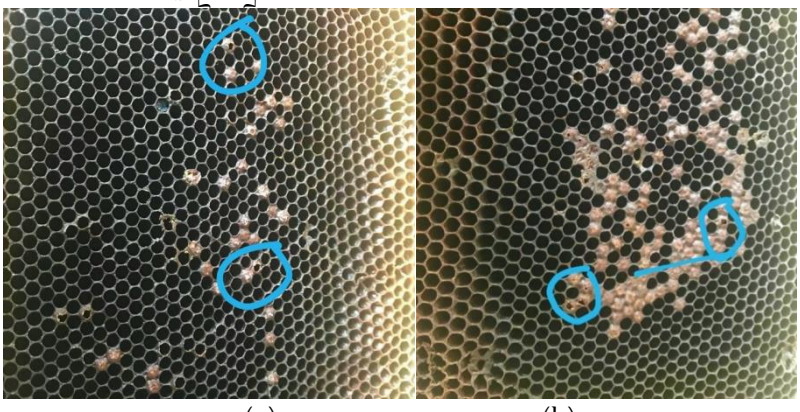


FIGURE 1. ***MAP OF THE STUDY AREA, MALAKAND, PAKISTAN***

COLLECTION OF DISEASED SAMPLES

Swab sticks and combs (20 cm) were both employed for the collection of infected samples. A swab stick was dipped in the colony's contaminated comb. When the matchstick test was used on those colonies where infections were identified, it revealed ropy-looking results. We used the matchstick test to look into the brood comb, and we put the ropey larval remains in sterile bags so we could identify them in the lab. The brood comb was subsequently enclosed in a paper bag and placed within a wooden box for the purpose of further examination. All samples were recognized before being sent to the lab by tags that included the type, bee farm, hive number, location, and dates of collection [14].



(a) (b)

FIGURE 2. ***(A) AND (B) SHOW INFECTED SAMPLES COLLECTED FROM THE BEEHIVES***

MEDIA FOR BACTERIAL GROWTH

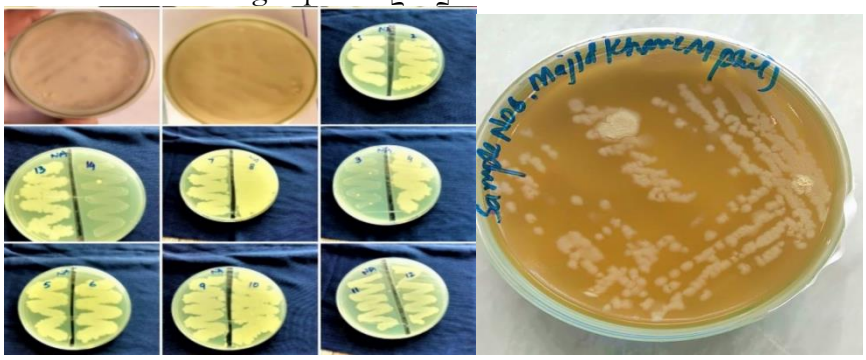
TABLE 1. ***THE INGREDIENTS OF BACTERIAL CULTURE MEDIUM***

Different Media used for the culturing of the Bacteria			
S. No	**Media**	**Contents**	**Concentration (gm/L)**
1	**Muller Hinton Broth**	Dehydrated infusion from beef	300
		Casein	17.5
		Starch	1.5

Muller Hinton (MH) Broth is summarized in Table 1 as regards to its chemical composition. This microbiological preparation explained herein is the production of bacterial cultures through MH Broth. This defined medium was prepared by combining 800 ml of deionized water, 300 mg of beef dehydration infusion, 17.5 grams of casein hydrolysate, and 1.5 grams of starch. Following the adjustment of the medium's volume to one liter through the incorporation of deionized water, the medium underwent autoclaving at 121 °C for a duration of 20 minutes. A number of bacterial isolates were grown in Mueller-Hinton agar (MHB), which was sterilized by the method provided by the manufacturer of this substance. Application of selective media has simplified the purification of target organisms under diverse sources.

CULTURE OF *PAENIBACILLUS LARVAE*

Subsequent to additions of 9 ml sterile distilled water into test tubes stocked with contaminant materials of preserved specimens, suspensions were created. Different dilutions were made, including 1/10, 1/100, and 1/1000. MH A 100 l aliquot of the diluted material was used to inoculate already-prepared, nutrient-filled broth plates. The plates were incubated in an incubator at 37°C for 18 to 24 hours shown in figure 3. Visual distinctions between the colonies on the master plates allowed for the selection of different colonies for pure culture. When these colonies were cultured at 37°C, it was revealed that the pure colony had grown significantly after being streaked on fresh nutrient agar plates [16].



(a) (b)

FIGURE 3. (A) & (B) CULTURE PLATES OF *PAENIBACILLUS LARVAE*

POLYMERASE CHAIN REACTION (PCR) AND GEL ELECTROPHORESIS

PCR was employed with the gradient PCR method to optimize the primer annealing temperature. The PCR reaction mixture composition consisted of Mater Mix (6.5 µ L for the 13 µ L reaction and 12.5 µ L for the 25 µ L reaction), Forward Primer (1 µ L for the 13 µ L reaction and 2 µ L for the 25 µ L reaction), Reverse Primer (1 µ L for the 13 µ L reaction and 2 µ L for the 25 µ L reaction), PCR Water (2 µ L for the 13 µ L reaction and 3.5 µ L for the 25 µ L reaction), and Template (2.5 µ L for the 13 µ L reaction and 5 µ L for the 25 µ L reaction), resulting in total

volumes of 13 µ L and 25 µ L, respectively. The cycling conditions for the 16S gene included a holding stage at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds (with different temperatures for different primers), extension at 72°C for 90 seconds, final extension at 72°C for 10 minutes, and a hold at 4°C.

Purified PCR products were run on a 1.5 % agarose gel. 30mL of 1x TBE buffer and 0.45g of agarose were combined to yield 30 mL of 1.5 % gel. The solution was microwaved for one minute at a time and then allowed to partially cool before adding 5 L of ethidium bromide. 2µ L of loading buffers were added, and then 5µ L of PCR-purified samples were placed onto the gel.

BIOCHEMICAL TESTS

Biochemical tests played a crucial role in affirming the presence of American Foulbrood (AFB) in the collected samples. Various diagnostic assays were employed, including the Catalase test, which assessed the presence of the catalase enzyme; the Oxidase test, which determined the activity of cytochrome c oxidase; the String test, which examined the characteristic ropey consistency indicative of AFB; and sugar fermentation tests involving Sucrose, Lactose, Glucose, and Mannitol. These tests provided comprehensive insights into the biochemical profile of the samples, aiding in the accurate identification and confirmation of AFB presence through a systematic evaluation of enzymatic activities and fermentation reactions.

STATISTICAL ANALYSIS

The data collected from both laboratory and field investigations were meticulously organized using Microsoft Excel (version 2019). Subsequently, a Chi-square Test was employed through Graph Pad Prism to scrutinize the data and ascertain the presence of any statistically significant differences. This comprehensive statistical analysis facilitated a rigorous examination of the datasets, enabling the identification of meaningful patterns or variations that contribute to a deeper understanding of the research findings.

RESULTS

In this study 800 or more colonies that had been tested, the absence of acid-fast bacilli (AFB) of the 1088 samples screened was clear in 386 of the cases with visual symptoms. Results were confirmed by the methods of biochemical examination and Gram stain with the general detection rate of 35.47/. Batkhela recorded the lowest prevalence rate of 20.8 % and Thana recorded the highest prevalence rate of 49.2 %. Other areas per prevalence ranks were Palai in 40 percent, Malakand in 30.49 percent, and Dargai in 30.12 percent. An overall statistical analysis indicated that the level of positive cases was significantly higher in Thana as opposed to Batkhela, Malakand and Dargai. The results corresponding tables and figure 6 are shown in Table 2 and figure 6 (Chi-square = 53.93, P value = 0.0001). The current study provides an in-depth image of AFB prevalence in the different geographical locations thus providing important evidence that can be used in control and check of AFB among the honeybees.

TABLE 2. OVERALL AND LOCALITY-WISE PREVALENCE OF AMERICAN FOUL BROOD DISEASE

S. No	Location of Apiary	Colonies in Apiary (n)	Examined Colonies (n)	Positive for AFB (n)	Prevalence (%)	*P* value
1	Thana	730	250	123	**49.2***	
2	Palai	451	180	72	40	<0.01
3	Malakand	570	223	68	30.5	
4	Dargai	370	239	72	30.12	

5	Batkhela	490	196	51	**29.14**
Grand total		**2611**	**1088**	**386**	**35.79**

The outcomes of the biochemical and Gram staining tests conducted on samples collected from diverse colonies across multiple apiaries are presented in Table 3.

TABLE 3. RESULTS OF GRAM'S STAINING AND BIOCHEMICAL TESTS OF BACTERIAL ISOLATES

S.No	I.D	Catalase test	Oxidase Test	Gram Staining	Spores	Glucose fermentation test	Sucrose fermentation test	Lactose fermentation test
1	3.2	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
2	4.2	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
3	5.1	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
4	5.2	Positive	Positive	-ve, rod shape	No	Positive	Positive	Positive
5	6.2	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
6	7.2	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
7	8.6	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
8	9.1	Positive	Positive	+ve, rod shape	Yes	Positive	Positive	Positive
9	9.2	Positive	Positive	-ve, rod shape	No	Positive	Positive	Positive

* = ($P<0.05$)

MONTH-WISE PREVALENCE OF AMERICAN FOUL BROOD (AFB) DISEASE

The highest prevalence of American Foulbrood (AFB) was observed in July, with subsequent elevated occurrences noted in June and September, as outlined in Table 4. A statistically significant difference in prevalence was confirmed through Chi-square statistics (Chi-square 14.67, df=6) ($p < 0.05$). This temporal distribution of AFB prevalence sheds light on the seasonality of the disease, providing valuable insights for potential mitigation and control strategies during these specific months.

ASSOCIATION OF PREVALENCE RATE WITH TEMPERATURE

As shown in Table 5, the recorded temperature range exhibits a noticeable skew towards higher temperatures, surpassing the lower temperature ranges.

TABLE 4. ***MONTH-WISE PREVALENCE OF AFB DISEASE IN THE STUDIED LOCALITIES***

	Studied Localities										
	Thana		Palai		Malakand		Dargai		Batkhela		
Months	Total frames in	Infected frames (n) Prevalenc	Total frames in	Infected frames (n) Prevalenc	Total frames in	Infected frames (n) Prevalenc	Total frames in	Infected frames (n) P	Total frames in	Infected frames (n) Prevalenc	Overall Prevalence (%)
March	4	1 (38)	7	2 (25)	5	2 (18)	7	1 (13)	5	2 (18)	15
April	8	1 (24)	8	3 (33)	9	1 (23)	7	2 (25)	5	2 (18)	22
May	5	4 (46)	8	5 (43)	9	4 (40)	8	3 (29)	6	2 (25)	35
June	9	6 (65)	9	4 (50)	8	4 (30)	9	4 (56)	7	2 (29)	47
July	79	7 (95)	8	6 (56)	9	4 (41)	9	2 (33)	8	5 (56)	55*
Aug	5	5 (47)	9	4 (44)	9	2 (38)	9	3 (25)	9	3 (34)	38
Sep	7	1 (30)	9	3 (30)	9	1 (24)	10	2 (30)	9	2 (25)	28
Overall	49.2		40		30.5		30.12		29.14		35.79

TABLE 5. ***ASSOCIATION OF PREVALENCE RATE WITH TEMPERATURE***

Temperature Range (n)	Range of Temperature (°C)	Prevalence (%)	P value
1	15-20	22.5	
2	25-30	25.5	<0.01
3	30-35	30.6	
4	36-40	35.8	
5	41-45	59.2*	

The steric (*) shows significantly higher prevalence (P<0.01)

DISCUSSION

The Gram-positive, spore-forming bacterium Paenibacillus larval sub specie Larvae is the causative agent behind American foulbrood, a severe bacterial disease that proves fatal to honeybee larvae [17]. Recognized under list B of the Office International des Epizooties (OIE), a global organization for animal health, American Foulbrood holds significance due to its substantial impact on a country's socioeconomic status, public health, and international trade in animals and animal products [18]. The disease spreads readily among bee colonies through various means, including infected bees, beekeeping equipment, pollen, and honey, with contaminated combs harboring the remains of diseased brood emerging as the most common transmission route [19]. Notably, the use of wax comb foundations contaminated with *Paenibacillus larvae* spores possess an additional risk for disease dissemination.

While indicative signs such as the matchstick test for ropiness and the mottled appearance

of brood combs assist in diagnosing AFB, laboratory studies on pathological samples are imperative for a definitive diagnosis due to overlapping symptoms with other bee infections [20]. Intriguingly, despite suggestive clinical signs, the American Foulbrood disease has not been documented in the bee colonies of Khyber Pakhtunkhwa's Malakand region. This investigation, according to existing research records, represents the first exploration into AFB sickness in the Malakand district, offering valuable insights into the prevalence and potential impact of this honeybee disease in the region.

This study established a prevalence rate of 35.47 percent in Malakand district which was similar to the results of 37.30 percent, 30 percent, and 32.4 percent represented in a study conducted by and [4], [21] and [22], in three districts of Khyber Pakhtunkhwa, mid- northerner Algeria, and South Africa respectively.

Conversely, lower prevalence rates were documented by [23], [24], [25], [26], and [27] in the Kurdistan area of Iran, the Duhok province of the Kurdistan region of Iraq, Belgium, New Zealand, and the USA. Discrepancies in these rates may be attributed to variations in the methods used, the number of samples investigated, the sampling period, and geographical differences.

The notably high disease rate observed in our area, located southeast and southwest of Mardan and Charsadda in Pakistan, can be linked to the illegal entry of bee colonies from infected nearby regions, coupled with beekeepers' lack of awareness about disease transmission methods. [28] reported a prevalence incidence of 51% in Uruguay, and [29] reported a prevalence rate of 55.6% in the Buenos Aires province of Argentina. The higher humidity and warmer climate in these locations, akin to the Malakand district, could contribute to the elevated prevalence observed in studies by Antnez et al. and Allipi et al. Additionally, the inexperience and lack of competence among beekeepers, along with the use of contaminated equipment in these areas, may further contribute to the disease's heightened incidence.

The examination of locality-based prevalence in the current study unveils an elevated frequency of American Foulbrood (AFB) in the Thana and Palai regions. Palai, characterized by an extended summer duration and a higher number of colonies, emerges as a hotspot for AFB incidence. Similarly, the Thana region, in close physical proximity to Palai and separated only by mountains, experiences an increased colony incidence. Notably, the heightened occurrence of colonies in Thana is further compounded by the frequent relocation of these colonies to Palai and other regions, particularly Punjab (Talagan).

Expanding on the temperature-wise prevalence analysis, the study indicates a notable surge in AFB prevalence during the months of June and July in the Malakand region. These months coincide with the peak of summer in the area, characterized by high temperatures and humidity. The positive correlation between higher temperatures and increased prevalence implies a potential influence of climatic conditions on the proliferation of AFB. This association underscores the importance of considering seasonal variations in devising strategies for AFB management and prevention in the Malakand region.

CONCLUSIONS

In conclusion, this study on the prevalence of American Foulbrood in the Malakand district revealed an overall rate of 35.47%. Thana and Palai regions emerged as hotspots for the disease's incidence, linked to factors such as the extended summer duration in Palai and the geographical proximity of Thana to Palai. Furthermore, the relationship between temperature and disease prevalence was highlighted, with a higher incidence observed during the warmer and more

humid months of June and July, the peak of summer in the region. The results also suggest that the frequent mobility of colonies between Thana and Palai, as well as other regions in the country, contributes to the spread of the disease. Ignorance among beekeepers regarding disease transmission methods and the illegal entry of infected bee colonies from neighboring regions are significant contributing factors. For future perspectives, increased efforts are needed to raise awareness among beekeepers regarding disease prevention and the adoption of safer beekeeping practices. Targeted training programs on early detection of American Foulbrood, along with guidelines on best practices for hive management and colony movement, could help mitigate the spread of the disease. Additionally, in-depth studies on the genetics of Paenibacillus larval sub specie Larvae strains in the region could provide crucial insights into disease variability and inform the development of more specific prevention strategies. Finally, initiatives aimed at strengthening border control measures could help limit the illegal entry of infected bee colonies into the Malakand district.

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DISCLOSURE AND CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest in this study. No conflicting financial, professional, or personal relationships influenced the design, execution, or interpretation of this study. Furthermore, the authors affirm that this paper has not been previously published and is not being considered for publication elsewhere. The manuscript includes all of the data and materials that support the study's findings.

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