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Detection of American Foul Brood Disease (Afb) in Apis-Mellifera Colonies Using Ropiness Test in Division Kohat

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ABSTRACT

American foulbrood (AFB) is a highly contagious disease affecting European honey bees (Apis mellifera), caused by the spore-forming, Gram-positive bacterium Paenibacillus larvae. This pathogen primarily targets the larval stage, with infection possible from as little as one spore in 24-hour-old larvae, leading to high mortality and colony collapse. P. larvae thrive in both aerobic and anaerobic conditions and relies on host-derived nutrients. Due to its resilient spores, AFB contaminates hive products like honey, propolis, wax, and pollen. The disease severely impacts colony strength, as lost offspring cannot be replenished quickly enough by the queen, ultimately threatening apiculture worldwide. The study was conducted in Kohat Division, Khyber Pakhtunkhwa, Pakistan, covering five districts with favourable conditions for apiculture. A total of 300 Apis mellifera colonies from various apiaries were selected based on accessibility and beekeeper consent. Colonies were inspected for clinical signs of American Foulbrood (AFB), and 10–15 larvae per colony were sampled using sterile

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tools. Larvae were taken from different frames to ensure representative sampling. The ropiness test was conducted to identify AFB-positive cases, and descriptive statistics were used to analyse disease prevalence. A total of 650 samples were collected from three districts: Hangu, Kohat, and Karak, representing 12 aperies and 833 colonies. Out of these, 253 samples (38.92%) tested positive, while 397 samples (61.08%) were negative. Hangu contributed the highest number of samples (262), colonies (435), and positive cases (117; 44.27%). Kohat and Karak followed with moderate levels of both positive and negative samples. No samples were obtained from Kurram and Orakzai agencies due to unfavourable security condition in those regions.

Key words: American Foulbrood (Afb), Apis Mellifera, Ropiness Test, Honey Bee Colonies, Division Kohat, Paenibacillus Larvae.

INTRODUCTION

American foulbrood disease (AFB), caused by the bacterium Paenibacillus larvae, is a disease of the European honey bee (Apis mellifera) and other honey bee species (Apis sp.). It is an infection that can be seen at any time of the year when honeybee brood is present. It is distributed worldwide. (Ellis and Munn 2005) The bacterium responsible for American foulbrood (AFB) disease, Paenibacillus larvae, exhibits distinct morphological and physiological characteristics that contribute to its pathogenicity in honey bee larvae Morphologically, it is a Gram-positive, mostly motile, rod-shaped and spore forming bacterium that can form resilient endospores, enabling it to withstand extreme environmental conditions (Genersch, Yue et al., 2006).

Paenibacillus larvae, formerly Bacillus larvae White, was first identified by George F. White in 1906 as the causative agent of American foulbrood (AFB). This bacterium produces endospores that are commonly found in dried larvae and old cultures, with over one billion spores present in a single infected brood comb or dead larva. These elliptical spores are highly resistant to extreme temperatures and can remain infectious for 35 to 40 years in the environment, while spores in beeswax, propolis, and honey can remain viable for over 70 years. This makes the control of the disease difficult because human activity can spread the disease over long distances and previously dormant strains may cause an outbreak several years after the original outbreak (Ellis et al., 2005).

P. larvae are facultatively anaerobic, thriving in both aerobic and anaerobic conditions, and requires specific nutrients from its host for growth. Its rapid reproduction through binary fission and production of virulence factors, including toxins, significantly enhance its infectivity, leading to high mortality rates in susceptible larvae. Understanding these characteristics is essential for developing effective control measures against AFB in apiaries. This disease poses significant threats to bee populations and apiculture, and due to this disease, the main product, Honey and the biproduct like propolis, bee waxes and bee pollen are also contaminated due to the spore forming nature of the bacteria.

In recent years apiculture has become a profitable job in Pakistan. Approximately 7000 beekeepers are rearing the Apis mellifera species (non-native) in up-to-date beehives. A total of 300,000 colonies of Apis mellifera produce 7500 metric tons of honey each

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year. Honeybee flora is found throughout in Northern areas, Federally Administered Tribal Areas (FATA) and Azad Jammu and Kashmir (AJK) which can potentially support 1,000,000 honeybee colonies (Waghchoure-Camphora ES, Martin SJ). Sidr honey is made from the nectar of the honeybees collecting from the blossoms of berry trees (Ziziphus nummularia, Ziziphus jujuba and Ziziphus mauritiana var.), fortunately berry trees are produced in abundance in the study area (Anjum, 2015 #2).The Apis mellifera (Western honey bee) is a managed honey bee species of high economic importance to agricultural industries globally, generating over AUD $14.2 billion to the Australian economy due to pollination services alone (Karasiński et al., 2023).

The bacterium P. larvae effect the larval stage of the Apis species but in some cases, it also effects the pupal stage. The larvae can be infected by one spore when it is 24hours old, and the spore dose that kills 50% of the larvae is 8.5 spores per larvae for 24-48 hours old larvae (Bamrick and Rothenbuhler 1961) (Yue, Nordhoff et al., 2008). Once infected, larvae usually die within 3–12 days.(Genersch, Ashiralieva et al., 2005). Contaminated spores of the bacteria potently transmit a disease to the healthy brood. These spores germinate in the larval midgut, entering the body cavity, eventually killing the larvae. Then, they destroy the midgut peritrophic membrane and epithelium. This opens a path for the bacteria to enter the hemocoel, resulting in larval death (Piro et al., 2006). With increasing age of the larvae progressively more spores are needed to initiate an infection, and above 53 hours age from hatching a larva cannot be infected. (OIE Manual of diagnostic tests and vaccines for terrestrial animals). An infected larva is ultimately killed by the spores, generally in the pupation stage (Anjum et al., 2015). Infection not only dangerous and lethal for honey bee larvae Loss of offspring due to increased egg lying activity in the colony can no longer be replaced by the queen bee. As a result, the population decreases, which can lead to the collapse of the whole colony. The number of the adult bee in the colony constantly decreases the colony strength decreases. The decline in honey bee (Apis mellifera) populations has raised concerns about the future of pollination and food production (Alburaki et al., 2024).

Infected bee colonies can spread the disease between colonies and apiaries. Lack of nectar flow causing predation and improper beekeeping can accelerate the spread. The agent causing the infection is classified as a highly contagious pathogen. Death is inevitable for infected larvae. If the infection is not diagnosed at the early stage and suitable precautions are not taken, it will cause serious losses in infected honey bee colonies. Then the pupa changes into a glutinous mass containing up to a billion spores. Theses spores may be carried to young larvae by the nurse bees, and the infection can spread very quickly through the colony, finally leading to its collapse. This makes the control of the disease difficult because human activity can spread the disease over long distances and previously dormant strains may cause an outbreak several years after the original outbreak. Since an infected and diseased colony is weakened, bees as of neighbouring colonies enter and rob the colony. By doing this they take many spores with them, and can spread the disease rapidly. Spores of P. larvae are very persistent and long lived (Hansen and Brødsgaard 1999).

Transmission occurs through contaminated food sources, such as honey and pollen, or via direct spore transfer from infected larvae. Once ingested, the spores germinate in the gut of the larvae, leading to rapid bacterial proliferation. As the larvae develop, the

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bacteria invade their tissues, causing severe damage and ultimately resulting in death. The disease is highly contagious due to the resilience of the spores, which can survive in the environment for extended periods, facilitating the spread of infection between colonies. This mechanism underscores the importance of biosecurity measures in beekeeping to prevent the introduction and spread of AFB within apiaries (Piro et al., 2006).

The infection cycle of American foulbrood (AFB) begins when nurse bees feed young larvae, transferring spores of Paenibacillus larvae from their mouthparts. Larvae that are less than 24 hours old are particularly vulnerable, as only a small number of spores are needed to initiate an infection due to its week immune system and totally depended on nurse bee for feeding. Once ingested, the spores germinate in the larvae's gut, and the bacteria multiply rapidly, especially after the larvae are capped. Infected larvae typically die at the pre-pupal or pupal stage, and their remains become a source of billions of spores, which are then spread throughout the hive by adult bees attempting to clean out the dead brood. This process not only facilitates the spread of the disease within the colony but also increases the risk of transmission to neighbouring colonies through robbing behaviour or drifting bees. The resilience of AFB spores, which can survive for over 50 years in various environmental conditions, further complicates management efforts, making it crucial for beekeepers to adopt stringent biosecurity practices to mitigate the risk of infection.

Under normal beekeeping conditions, AFB is highly contagious since spread of the disease is facilitated by exchanging hive and bee material between colonies, managing numerous hives in a confined area and the trading of queens, colonies (‘‘package bees”) and honey. In many countries, AFB is a notifiable disease and measures are regulated by corresponding laws. Burning of colonies and contaminated hive material are widely considered the only workable control measure for diseased colonies whereas the shook swarm method (shaking the bees onto new comb foundation and destroying the infected comb) is sometimes applied to sanitize infected, although not yet clinically diseased. Thus, AFB is a serious problem in apiculture and causes considerable economic loss to beekeepers all over the world (Pernal et al., 2008).

The study on honey bee brood diseases in the USA The Bee Disease Diagnostic Laboratory (BDDL) in the USDA-ARS Beltsville Bee Research Laboratory received a total of 4790 brood samples between 2015 and 2022 from U.S. State Apiary Inspectors and beekeepers. Samples from 49 states were analysed by microscopy for the presence and prevalence of two bacteria, Melissococcus plutonius and Paenibacillus larvae, causing European foulbrood (EFB) and American foulbrood (AFB) diseases, respectively. Samples that tested positive for AFB were cultured and subjected to antibiotic susceptibility tests via the agar disc diffusion method to determine their resistance to tetracycline (Terramycin®) and tylosin (Tylan®) antibiotics. A comprehensive data analysis was conducted at multiple levels, including state, month, year, nationwide, and climate region. Among the 49 states, AFB was found in 31 states. Infection levels vary across states, ranging from 0% to 54.8% (Alburaki et al., 2024).

Furthermore, significant monthly variations were recorded for both brood diseases, with the highest occurrence of AFB observed from April to July. States with the lowest

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infection rates of both diseases were NV, ND, MS, AK, and AZ. On a nationwide scale, the AFB resistance to tetracycline (38%) was significantly ($p < 0.001$) higher than the resistance to tylosin (27%).In the United States alone, the value of pollination services rendered by honey bees is estimated at approximately US$17 billion annually, while the global figure reaches an impressive US$215 billion (Calderon, Schneider et al., 2017). However, the sustainability of these invaluable services is under threat, as evidenced by long-term declines in honey bee colony populations. Specifically, reports indicate a worrying decrease of 25% in European honey bee colonies and an even more concerning 60% reduction in U.S. colonies (Andreotti et al., 2013) & (Smith et al., 2015).

The cost of replacing lost colonies—including hives, frames, and bees—along with lost honey yield, can easily run into hundreds or thousands of dollars per apiary. In nations with regulations mandating hive burning, these losses extend to the cost of disposal and downtime. Additionally, colony mortality reduces pollination services provided to agriculture, diminishing crop yields indirectly. Given the high dependence of many crops on healthy hive services—and the expense of renting colonies for pollination—AFB represents both direct and ripple-effect economic challenges. Mass colony losses from AFB contribute to the broader agricultural economy's instability. Managed honey bees are responsible for pollinating many key crops worldwide—including almonds, berries, fruit, and vegetables—valued globally between $235–$577 billion annually In the U.S. alone, honey bee pollination generates roughly $20 billion in agricultural value per year (Anjum et al., 2015).

LITERATURE REVIEW

Beekeeping belongs among the oldest agricultural activities of man. Since at least 9,000 years before the present, humans have regularly collected honey and wax, an avocation that later developed into true beekeeping as we know it today. The long history of human interest in the life of honey bees is documented in ancient manuscripts such as Columella's (4 – c. 70 AD) monumental work De re rustica, which also includes vivid descriptions of honey bee diseases. In the aforementioned book, Columella discusses, among others, disorders and diseases such as starvation, dysentery, poisoning from plants, and possibly also conditions that we know today as European and American foulbrood, and tracheal mite infestation. However, it was not until much later that the earliest scientific enquiries into the causes of foulbrood were first made. The first use of the word 'foulbrood' has been traced back to the German apiculturist and clergyman Adam Gottlob Schirach (1724 – 1773) in 1769 (Phillips et al 1903). Looking back at the history of bee diseases before the age of modern veterinary medicine offers us a unique insight into the history of beekeeping but understanding how our ancestors dealt with this serious disease can also be inspiring for organic beekeepers today. American foulbrood (AFB) is a highly destructive and well-known disease affecting honey bee colonies. It is caused by Paenibacillus larvae, a Gram-positive, spore-forming, rod-shaped bacterium. This pathogen is notorious for its ability to destroy entire colonies by infecting the sealed brood of honey bees. Although AFB was not officially linked to its bacterial cause until 1907 in the United States, the disease was already known to be devastating. Prior to this discovery, beekeepers relied on observation, instinct, and trial-

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and-error methods to manage outbreaks. While similarly named diseases like European foulbrood (caused by Melissococcus pluton) exist, AFB remains a primary concern due to its persistence, severity, and global presence.

Early beekeeping authors used terms such as "foulbrood", "bee plague", "brood decay", or "sour disease" interchangeably, while others developed specific nomenclatural systems. Over the past 250 years, numerous and often conflicting theories have been proposed regarding the causes of foulbrood. Reverend Schirach, who first coined the term in the mid-18th century, believed that foulbrood resulted from larval malnutrition and the queen laying eggs with their heads facing inward in the cells. Following his observations, many other researchers suggested alternative explanations. Some hypothesized that foulbrood was caused by poor hive hygiene, environmental stress, or improper beekeeping practices. Others speculated that it was the result of parasitic infections, fermentation of stored pollen, or climatic factors such as cold and damp conditions within the hive.(Bailey and Collins 1982). These early attempts to understand the disease highlight the complexity of foulbrood and the evolving nature of scientific inquiry in apiculture. A popular explanation in the latter half of the 19th century claimed that a combination of malnutrition and neglect of the brood by nurse bees could result in foulbrood (Schirach et al 1787).

CLASSIFICATION

American foulbrood (AFB) is a disease of the brood of the honeybee, Apis mellifera, and is caused by the bacterium Paenibacillus larvae subsp. larvae (Pll). Within this species another subspecies P. larvae subsp. pulvifaciens (Plp) can be distinguished, which is associated with the so-called "powdery-scale disease" (Vauterin et al., 1996).

Taxonomic classification of Paenibacillus larvae is followed:

Historically, the species encompassed two main groups: P. larvae subsp. larvae and subsp. pulvifaciens, but those differences were later removed due to high genetic similarity and overlapping pathogenicity.

Taxonomic Classification of Paenibacillus larvae in Tabular Form (Ash et al., 1991).

Table No 1:

Kingdom	Monera
Phylum	Firmicutes
Class	Bacilli
Order	Bacilli
Family	Paenibacillaceae
Genus	Paenibacillus
Species	P. larvae

GEOGRPHIC DISTRABUTION OF AMERICAN FOULBROOD

American foulbrood (AFB) is the most destructive brood disease of honeybees (Apis

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mellifera) globally.(Morrissey et al., 2015) AFB is found on every continent where honeybees are kept (Matheson et al., 1993). The disease is spread by both humans and bees, and it is spread predominantly via horizontal routes although it has been shown to spread vertically (Genersch et al, 2008). Honey bee colonies both in the USA and globally, face a myriad of challenges, including diseases, pests, and environmental stressors, such as pesticide exposure, loss of habitat, insufficient forage, and the impacts of climate change, all of which adversely affect their performance and survival. In many countries, AFB is a notifiable disease since it is highly contagious, in most cases incurable, and able to kill affected colonies. Western honey bees, Apis mellifera (hereafter "honey bee") are among the most widespread organisms in the world, as their association with humans has led to their dispersal from their native range in Europe, the Middle East, and Africa to every continent except Antarctica (Crane et al., 2018).

Beekeepers around the world spend considerable time managing for these biotic colony stressors while scientists work to develop comprehensive research programs focused on the biology, pathology, and control of these organisms. Knowing the global distribution of the honey bee pests and pathogens is key to developing strategies for their control and limiting their further spread. Bradbear and Matheson were first to publish reviews of the global distributions of honey bee pests and pathogens (Crane et al., 2018). Their work was followed by Allen and Ball who developed a similar review, but for the major honey bee viruses. Subsequently, combined these reviews into a single treatise and included updated information on new pests/pathogens and the expanding distribution of established ones (Boncristiani et al., 2020).

In this way, according to the World Organization for Animal Health (OIE), there are cases reported in the first half of 2019 in Europe (with declared infection in Finland), South Africa, North America, South America and Australia. Despite those data, many countries have no information available for knowing the real state of this disease around the world as it shows figure 1(Mejias 2020). AFB is present in a considerable number of countries on all continents where honey bees and their subspecies are breed (Tests and Animals 2018). As it spreads rapidly on the continental level, the disease has a panzootic character (White 1906). There is not much data on the prevalence of AFB agents at both regional and national levels, except those published by (Morrissey et al., 2016).

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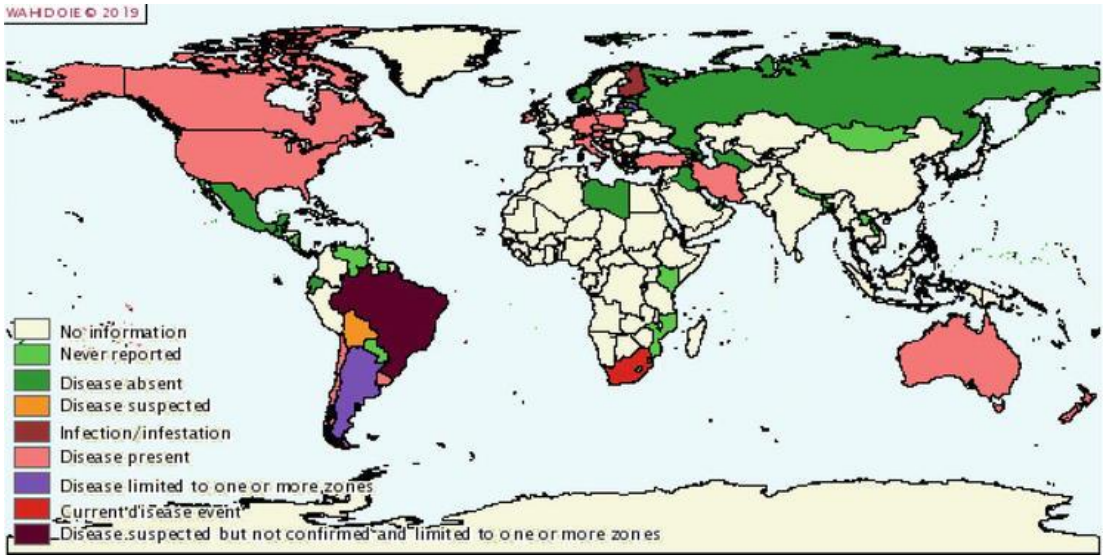


Figure 1: Dynamic maps showing the presence or absence of American foulbrood at the national and sub-national levels. Information based on 6-monthly reports (first half of 2019) according to the data base taken from World Organization for Animal Health (OIE).

HABITAT

Bacteria belonging to the genus Paenibacillus have been isolated from a variety of environments, with many of the species being relevant to humans, animals, plants, and the environment. The majority of them are found in soil, often associated with plants roots: these rhizobacteria promote plant growth and can be exploited for use in agriculture. Many species of Paenibacillus produce antimicrobial compounds that are useful in medicine or as pesticides, and many yield enzymes that could be utilized for bioremediation or to produce valuable chemicals. Some species are pathogens to honeybees or other invertebrates; while others are occasional opportunistic infectors of humans. In fact, many of these pertinent characteristics overlap within the same species. In accordance with their diverse characteristics, members of Paenibacillus have been discovered in disparate habitats, from polar regions to the tropics, and from aquatic environments to the desert.

The Paenibacillus larvae, attacks honey bee brood and renders entire hives dysfunctional during active disease states, but more commonly resides in hives asymptomatically as inactive spores that elude even vigilant beekeepers (Daisley et al., 2019). This pathogen often remains dormant in its spore-form and does not induce manifestations of AFB (Erban et al., 2017). It has been suggested that P. larvae may exist as a pathobiont in the native microbiota of adult worker bees, from where it is passively and constitutively transmitted throughout the hive to fresh brood cells.

The habitat of Paenibacillus larvae is highly specific and specialized. This Gram-positive, spore-forming bacterium is an obligate pathogen of the honey bee larva (Apis mellifera). It's only natural environment for germination and proliferation is inside the larval stage of honey bees. Spores are ingested by larvae through contaminated food

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fed by nurse bees, where P. larvae spores germinate in the larval gut, invade tissues, and multiply. Outside of the larvae, P. larvae spores persist in the hive environment, including honey, wax, pollen, hive surfaces, and contaminated beekeeping equipment. These spores are highly resistant, able to survive extreme conditions for decades (over 35 years), and serve as a durable environmental reservoir facilitating disease spread within and between colonies.

The natural habitat of Paenibacillus larvae larva is, internally within honey bee larvae, where it infects and multiplies. Internally within honey bee larvae, where it infects and multiplies

SIGNS AND SYMPTOMS

Much of our knowledge concerning the symptoms of American foulbrood has been gained through observations made by beekeepers while practicing their profession. The apiary in which the disease has been produced by experimental inoculations offers an opportunity to obtain a fairly complete picture of the disease. The present discussion of symptoms of the disorder is based upon observations made on the disease thus produced. During these studies it has been possible to duplicate observations already made by beekeepers on the disease as it occurs in nature and to make still others It is quite probable that a number of these additional observations could be duplicated on the disease in nature if a sufficiently close study were made. In American foulbrood the symptoms vary within wide limits.

The colony may or may not be noticeably weakened. If recently infected the strength will not be affected appreciably, but if the infection has been present for a considerable period the colony will be weakened as a rule Not infrequently during the course of the disease the strength of the colony is diminished and death is the result. Between these limits any degree of strength may be encountered. American Foulbrood (AFB) is a highly contagious and lethal bacterial disease of honeybee brood caused by Paenibacillus larvae.

The clinical symptoms are highly characteristic for the disease, uncapped brood pattern, darkness and shirking of brood cell invade and emitting foul Odor like a wet soil or decomposed object. The creamy or dark brown, glue-like larval remains of infected larvae continue to provide the most obvious clinical symptom of AFB, although it is not conclusive (De Graaf, Alippi et al., 2006). The caps over dead brood (Pl. II, B) appear in many instances similar to those covering healthy brood. In many instances, however, they are altered, being sometimes punctured (Pl. III, A; Pl. VI, C), sometimes sunken, and sometimes darkened. Sometimes these abnormal appearances are combined. The holes in the caps vary in number and dimensions (Bulletin 809, u. s. department of agriculture).

The body wall of a larva or pupa dead of the disease soon softens and is easily ruptured, making it impossible to remove the remains intact from the cell. As the process of decay continues the mass becomes viscid and before the scale stage is reached the viscidity is such that it is capable of being roped out into fine threads to a distance of 2 or 3 inches and sometimes even more. The scale when dry adheres more or less firmly to the cell wall.

The Odor of the brood combs, when it is present, is characteristic of the disease and

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may be spoken of conveniently as the foul-brood Odor. It is never detected in the early stages of the disease and if only scattering cells are present seldom is noticed. When much brood is dying and the disease has been present for weeks, the Odor is noticeable and sooner or later becomes marked. When marked it is recognized by its strong and penetrating character and is usually thought of as being disagreeable. The brood combs after their removal from the hive tend to lose this foulbrood Odor. The presence and extent of it in samples of the disease vary considerably, the variation depending largely upon the facts just mentioned. It is possible to recognize most cases of American foulbrood from the general symptoms that are present. On the other hand, many cases can be diagnosed only by an exact knowledge of the postmortem conditions of brood dead of the disease. A close study of these appearances, therefore, is advisable. The following somewhat brief description of larva and pupa dead of the disease will suffice in most instances and will serve as a guide to further observations.

Signs that will determine the exact time of death from American foulbrood have not yet been ascertained. As the larva and pupa that die of the disease do so at a period in their growth when healthy brood is motionless, lack of motion is no guide. In the descriptions made in the present paper it is assumed that a larva or pupa is dead if it shows a change from a bluish-white, more or less transparent appearance to one that is more nearly white, more nearly opaque, and shows at the same time a change from the normal turgidity to a slightly flaccid condition. The appearance of the larval and pupal remains changes gradually from day to day from that of a healthy brood to that of the dried residue the scale. A description, therefore, that would be correct for one day would be incorrect probably for the following day. Furthermore, all of these remains do not pass through the same changes. For convenience in description, therefore, the various appearances assumed by them are considered in five more or less arbitrary stages. The interpretation of the descriptions will be aided if the description of healthy brood (p. 3) is borne in mind, since the terms used are similar in both instances. The descriptions made of dead larva for the most part will be of those that have died during the 2-day quiescent period just preceding the transformation to the pupa, as most larva dying of the disease succumb at this age.

FIRST STAGE

The first symptoms of American foulbrood appear about the end of the first week after infection. From the bluish white of the healthy larva the colour changes during this first stage of the disease to a very light brown. The conelike anterior third (Pl. II, E) having settled somewhat, the apex is now slightly farther from the roof. The surface markings in each of the three thirds (Pl. II, H) are very similar to those of a healthy larva. The body wall is easily ruptured, but by care the larva may still be removed intact from the cell. In consistency the decaying tissues are soft and non viscid. Sometimes a considerable portion of a larva is removed piecemeal by the bees during the first stage of decay. The remnant (Pl. VI, D), consisting of the posterior third and usually much of the middle third, is found occupying normal position in the cell. The surface markings are similar to those of the middle and posterior thirds in the first stage of decay. The surface left by the removal of the fragments is somewhat roughened and transverse to the body.

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These larval remnants are similar to those seen in sac brood, but in American foulbrood there are fewer of them. When present they form one of the earliest symptoms of the disease.

SECOND STAGE

By the second week after death the larva shows a slightly de shade of brown, although still quite light. The anterior (Pl. II, F) is somewhat darker than the other two thirds. The is farther from the roof than in the first stage. The surface mar (Pl. II, I) are now less pronounced throughout. An attempt at this time to remove the dead larva from the cell results us in rupturing the body wall. The decaying mass can be removed, however. The consistency of the tissue mass in this is somewhat similar to that of moist dough. It has not yet rem the true viscid condition.

THIRD STAGE

By the third week after death the remains have assumed a me shade of brown, approximating that usually seen in brood cap. The apex of the anterior third (Pl. III, D) is now widely spread from the roof of the cell, exposing to view the ventral surface o remains. The transverse ridges and furrows marking the segments (Pl. III, G) are practically obliterated. The edges no longer the deep notches. The side-to-side convexity is decreased. Evade of developing head and thoracic appendages are sometimes Transverse trachea can usually be observed as white lines and the ventral surface of the abdomen, one in each segment. The wall is ruptured easily. The decaying mass shows some visa and adheres to the walls of the cell.

FOURTH STAGE

By the fourth week after death the colour of the dead larva reaches a deep brown, being in shade similar to that of the average older brood combs. The apex of the anterior third (Pl. III, E) is raised only slightly above the upper surface of the decaying larval mass. After being uncapped this third is the first to dry, becoming dark and scale-like. Surface markings practically have disappeared; trachea frequently are still visible, especially in the middle third. The ventral surface (Pl. III, H) is now slightly concave from side to side. posterior third lies upon the bottom of the cell and extends t roof. The decaying larval mass is now decidedly viscid in consistency. When tested with a toothpick, match, forceps, or any suitable object it can be drawn out into threadlike strings. viscid condition of decaying brood is referred to by beekeeper "ropiness." It is a well-known symptom and is much used in diagnosis of the disease.

FIFTH STAGE

One month or longer after the death of the larva the remains are found to be a thin mass more or less dry and covering most of the floor, some of the side walls, and the bottom of the cell. The dried mass is known to beekeepers as the "scale." It’s colourised dark brown, similar to that of old brood comb. That portion which was once the anterior third of the larva bears, if at all, only a slight elevation (Pl. III, C, F, I; Pl. VI, E, H). The ventral surface (Pl. III, I) is concave from side to side and quite uniform. The posterior third covers the bottom of the cell and extends to the roof. This can be seen especially

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well by cutting lengthwise the cell with a scale (Pl. VI, H) in it. It can be seen also that the thickness of a scale throughout the median line is approximately uniform.

As some larva die of American foulbrood before the quiescent stage is reached but after being capped, their dead remains may be found occupying not the uniform position just described but various positions in the cell. While the form of the remains, therefore, may vary materially, the colour and consistency pass through stages that are similar in all instances.

Occasionally death from American foulbrood takes place while the larva is still younger, i.e., before the time of capping has arrived and while it (Pl. VI, A) is coiled in the cell. This condition is comparatively rare. As the form of the remains depends upon the age of the larva at the time of its death, naturally, the form of the remains of a larva dying before the time for capping will vary materially from the descriptions above. The colour and consistency of such a larva during its decay, however, pass through stages that are similar to those already described.

EPIDEMIOLOGY OF AMERICAN FOULBROOD (AFB) DISEASE

The most severe bacterial disease of honeybees is American foulbrood (AFB). The epidemiology of AFB is driven by the extreme spore resilience, the difficulty of bees to remove these spores, and the considerable incidence of undetected spore-producing colonies. The demand for managed pollinators in agriculture has steadily increased during the past decades due to changing diets and an alarming decrease in natural pollinators in cultivated landscapes. A recent report indicated that 71 out of 100 important crop species are bee-pollinated At the same time, beekeepers worldwide are experiencing increased winter and seasonal colony losses (Stephan et al., 2020). One of the major threats to colony health and beekeeping viability is American foulbrood (AFB); a contagious, lethal bacterial disease of honeybee brood that is widely distributed across the world.

The disease causes great economic losses during outbreaks due to reduced productivity and material turnover. American foulbrood is caused by Paenibacillus larvae, a spore-forming bacterium that produces extremely resilient spores which can remain viable for decades. Within a colony, P. larvae spores are spread by nurse bees performing in-hive tasks, such as cleaning, but especially through the feeding of young larvae with spore-contaminated food. Billions of spores are produced in the dying larvae. The dried larval remains (scales) are difficult to remove by workers and are a continuous source of infection for new brood. The lethality and epidemiology of AFB are driven by the resilience of the spores and the fact that the removal of diseased brood, a communal bee hygienic behaviour (De Graaf et al., 2016).

One major problem for the control of American foulbrood is that even though clinical symptoms are highly characteristic for the disease, they tend to appear late during the epidemic, when the colony's hygienic behaviour to remove infected larvae before they produce spores, can no longer keep up with the epidemic. Estimations have been made that as much as 25% of spore-producing colonies remain undetected (Datta et al., 2013).

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ECONOMICAL LOSS

One of the major threats to colony health and beekeeping viability is American foulbrood (AFB); a contagious, lethal bacterial disease of honeybee brood that is widely distributed across the world. The disease causes great economic losses during outbreaks due to reduced productivity and material turnover (Gallai et al., 2009). The impact of pollination by honeybees upon the global economy has therefore been estimated to be hundreds of billions of dollars(Corbet et al., 1991) & (Gallai et al., 2009). Billions of spores are produced in the dying larvae. Besides fulfilling the critical role of pollinizing agricultural crops, A. mellifera are responsible for a number of economically valuable products, including propolis, royal jelly, bee venom, beeswax, bee pollen, and honey. Pollination and these apiculture products are made possible by adaptations that facilitate the collection and transport of pollen grains to the beehive. Behaviourally, bees improve pollen-transport efficiency by wetting pollen grains with nectar, thus creating a cohesive surface that increases the amount of pollen that can be carried during flight. Du to its anatomical skills, the characteristic buzz of bees facilitates the collection of pollen grains located on floral structures (Jonson R et al,2010).

In addition to pollen collection, young melliferous bees secrete a liquid from wax glands that, when exposed to air, hardens and forms small flakes that collect on the underside of the bee. This economically valuable natural substance is known as beeswax and is used by bees to construct hexagonal alveoli into honeycombs. The rigid structure of honeycomb cells serves to conserve honey and pollen. Likewise, alveoli serve as a place for the queen bee to deposit eggs and for larvae or pupae to develop (Maori et al., 2009). Beeswax contains carbohydrates (present in pollen and nectar) that have transformed into fats due to the presence of enzymes and enzyme precursors secreted by bees. More specifically, beeswax is constituted by water and minerals (1-2%), mono-esters and hydroxyl mono-esters, complex wax esters, hydrocar-bons, and free wax acids(Carreck et al., 2010).

MATERIALS AND METHODLOGY

The study was conducted in the division Kohat, Pakistan. These areas have diverse agricultural practices and provide an ideal site for apiculture. The region's climate and flora create a suitable environment for Apis mellifera colonies, making it an ideal location for investigating American Foulbrood Disease (AFB).

ETHICAL CONSIDERATION

The study was conducted with the consent of the participating beekeepers, ensuring that all ethical guidelines were followed. The researchers emphasized the importance of disease management and prevention in beekeeping practices.

STUDY AREA

This research was initiated in January 2025 and focuses on the Kohat Division, located in the southern region of Khyber Pakhtunkhwa, Pakistan. The Kohat Division encompasses five districts: Kohat, Karak, Hangu, and the tribal districts of Kurram and Orakzai. Spanning a total area of approximately 12,280.55 square kilometres

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Figure 2: Map of Study Area

SAMPLE SELECTION

A total of 300 Apis mellifera colonies were selected for the study, representing various apiaries in division Kohat. The colonies were chosen based on their accessibility and the willingness of the beekeepers to participate in the research. Each colony was assessed for signs of AFB, ensuring a mix of healthy and potentially infected colonies.

SAMPLING PROCEDURE

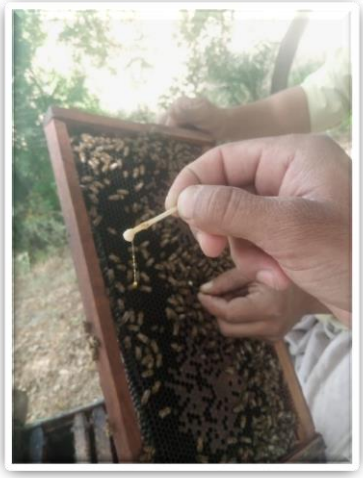
Each colony was inspected for clinical signs of American Foulbrood (AFB), such as sunken, perforated, or darkened caps on brood cells. From each colony, a sample of approximately 10–15 larvae ware examined using sterile wooden sticks or grafting needles. To ensure a representative sample, larvae will be selected from different frames within the hive.

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DATA ANALYSIS

The results of the ropiness test were recorded, noting the number of positive and negative cases for each colony. Statistical analysis was performed to determine the prevalence of AFB in the sampled colonies, with descriptive statistics used to summarize the findings.

Table No 1:

District	Aperies (N=12)	Percentage	Colonies (N=833)	Percentage	Samples (N=650)	Percentage
Kohat	4	33.33%	203	24.37%	213	32.77%
Hangu	5	41.67%	435	52.22%	262	40.31%
Karak	3	25.00%	195	23.41%	175	26.92%
Total	12	100.00%	833	100.00%	650	100.00%

A total of 12 apiaries were surveyed across the selected districts, comprising 833 colonies and 650 samples. The distribution varied significantly among districts.

The Hangu district exhibited the highest representation in all three categories. Out of the 12 total aperies, Hangu accounted for 5 (41.67%), followed by Kohat with 4 (33.33%) and Karak with 3 (25.00%). In terms of bee colonies, Hangu again led with 435 colonies, representing 52.22% of the total. Kohat followed with 203 colonies (24.37%), and Karak with 195 colonies (23.41%). Regarding sample collection, Hangu contributed the largest share with 262 samples (40.31%), followed by Kohat with 213 samples (32.77%) and Karak with 175 samples (26.92%). These findings indicate a concentration of apicultural activity in the districts of Hangu, Kohat, and Karak, with Hangu showing a notably higher engagement in beekeeping.

TABLE 3: PREVALENCE OF POSITIVE SAMPLES ACROSS SELECTED

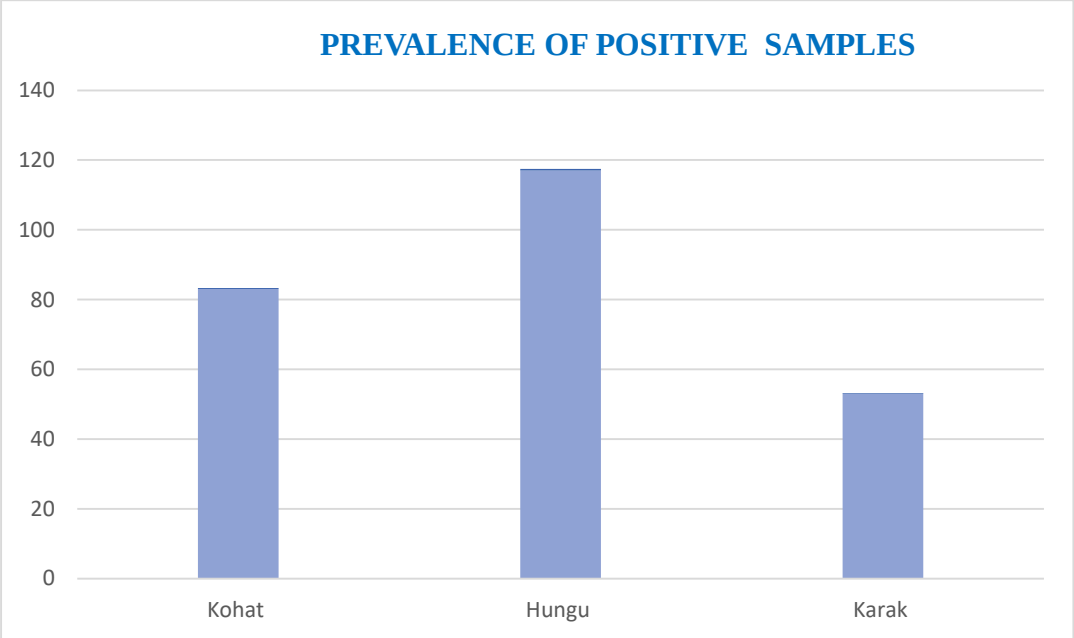
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DISTRICTS

Districts	**Sample**	**Percentage**
Kohat	83	32.81%
Hangu	117	46.25%
Karak	53	20.95%
Total	253	100.00%



Out of a total of 650 samples collected across the study area, 253 samples (38.92%) were found positive. These positive cases were distributed unevenly among the districts of Kohat, Hangu, and Karak, with no positive samples reported from Kurram and Orakzai agencies due to the absence of sampling in those regions.

Among the districts, Hangu recorded the highest number of positive samples, with 262 total samples collected, out of which 117 (44.27%) were positive. This also corresponds with Hangu having the highest share in colonies (435 out of 833; 52.22%) and apiaries (5 out of 12; 41.67%). Kohat contributed 213 total samples, with 83 (32.81%) testing positive. Kohat had 203 colonies (24.37%) and 4 apiaries (33.33%). Karak provided 175 samples, with 53 (20.95%) being positive. The district accounted for 195 colonies (23.41%) and 3 apiaries (25.00%). The Kurram and Orakzai agencies did not contribute any samples, colonies, or apiaries, and consequently had no positive cases.

These results indicate that the highest proportion of positive samples was observed in Hangu, reflecting its overall larger contribution in terms of beekeeping infrastructure

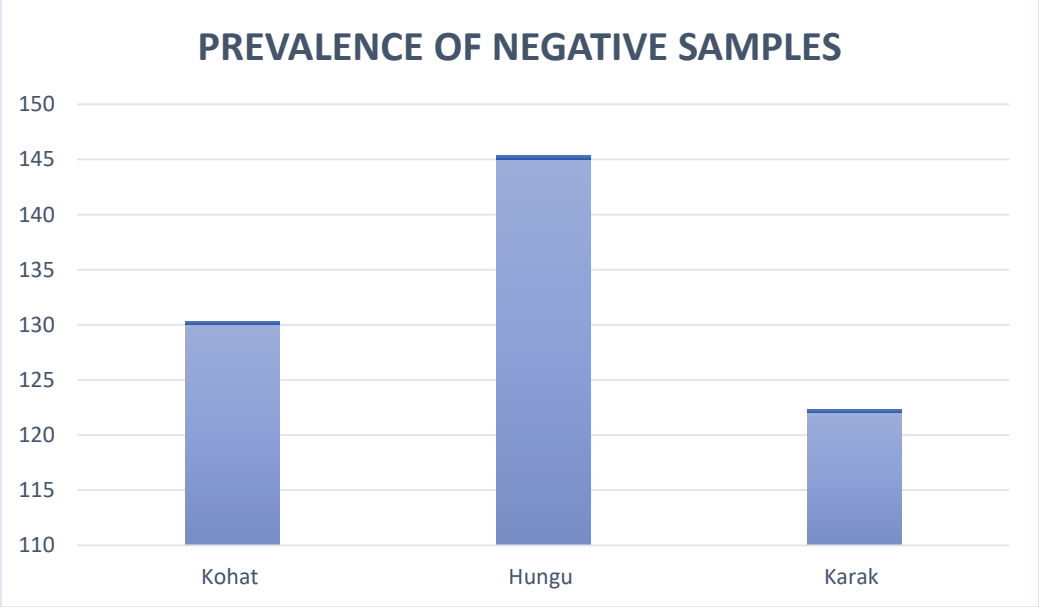
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and activity. Kohat and Karak followed, with lower but still significant positive detection rates. The complete absence of data from the Kurram and Orakzai agencies due to unfavorable conditions in those areas

TABLE 4: PREVALENCE OF NEGATIVE SAMPLES ACROSS SELECTED

DISTRICTS		
Districts	**Sample**	**Percentage**
Kohat	130	32.75%
Hangu	145	36.52%
Karak	122	30.73%
Total	397	100.00%



Out of a total of 650 samples collected across the study area, 397 samples (61.08%) were found to be negative. The distribution of these negative samples varied across the three districts from which data were successfully collected: Kohat, Hangu, and Karak. No samples were collected from Kurram and Orakzai agencies due to unfavorable conditions, including limited access. Hangu district recorded the highest number of

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negative samples, with 145 samples, accounting for 36.52% of the total negative cases. Kohat followed with 130 negative samples, comprising 32.75% of the total. Karak contributed 122 negative samples, representing 30.73%. The absence of data from the Kurram and Orakzai agencies is due to unfavorable conditions in that area during the study time frame. These findings suggest a concentration of sample collection efforts in more accessible and active beekeeping zones.

RESULTS

Overview Of Sampling and Regional Distribution

A comprehensive field survey was conducted across five districts in the study area: Hangu, Kohat, Karak, Kurram, and Orakzai. A total of 12 apiaries were visited, from which 833 colonies were inspected and 650 samples were collected for analysis. The distribution of samples, colonies, and apiaries varied across districts, with Hangu, Kohat, and Karak being the primary contributors. In contrast, the Kurram and Orakzai agencies did not contribute any data due to unfavorable field conditions, such as limited access and security issues.

Among the districts, Hangu showed the highest activity in terms of beekeeping operations. It accounted for 5 of the 12 apiaries (41.67%), 435 of the 833 colonies (52.22%), and 262 of the 650 samples (40.31%). Kohat followed with 4 apiaries (33.33%), 203 colonies (24.37%), and 213 samples (32.77%). Karak contributed 3 apiaries (25.00%), 195 colonies (23.41%), and 175 samples (26.92%). No apiaries, colonies, or samples were reported from Kurram and Orakzai, confirming the absence of active beekeeping or constraints that impeded data collection from these areas.

POSITIVE SAMPLE FINDINGS

Out of the total 650 samples, 253 samples (38.92%) were found to be positive for the condition under investigation. The distribution of positive cases was not uniform across districts and closely followed the trends in colony and sample density.

Hangu district recorded the highest number of positive cases, with 117 positive samples, accounting for 44.27% of all positive cases. The high positivity rate correlates with the district's dominant share in overall sampling and colony inspection, indicating a potentially elevated disease burden or greater sensitivity of detection in this region.

Kohat district followed with 83 positive cases (32.81%), out of the 213 samples collected. Although Kohat had fewer colonies and apiaries than Hangu, it still contributed significantly to the total number of positive detections, reflecting a moderate level of disease prevalence.

Karak district had 53 positive samples (20.95%) out of 175 total samples. This is the lowest among the three sampled districts but remains notable, given Karak's smaller scale of apiculture.

Kurram and Orakzai agencies had no reported positive cases, as no samples were collected from these districts due to unfavorable conditions and security issues.

These results indicate that Hangu district not only had the largest number of samples and colonies but also bore the greatest burden of positive detections. This strong correlation suggests a need for enhanced monitoring and potential intervention strategies in areas of higher beekeeping density.

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NEGATIVE SAMPLE FINDINGS

In contrast, a total of 397 samples (61.08%) tested negative, demonstrating that more than half of the collected specimens did not indicate the presence of the condition under surveillance. The distribution of negative cases followed a similar geographical pattern to the positive results, with Hangu again contributing the largest share. Hangu recorded the highest number of negative samples, with 145 cases (36.52%). While Hangu also had the highest number of positives, this also suggests variability in infection or contamination rates across different colonies or apiaries within the same district. Kohat had 130 negative samples, representing 32.75% of the total negative cases. The proportion of negatives is roughly aligned with its share in total sampling, reflecting a balanced distribution of both positive and negative outcomes. Karak contributed 122 negative samples (30.73%), making up a significant proportion of its total 175 samples and indicating a slightly lower positivity rate compared to Kohat and Hangu.

As with the positive cases, no negative samples were recorded from Kurram and Orakzai agencies due to the complete lack of sample collection in those areas. The negative results reinforce the observation that beekeeping-related disease or infection was not uniformly distributed. Although Hangu had the highest positive count, it also had the most negative cases, underlining the diversity in health status among colonies even within the same geographical region.

DISCUSSION

This study investigated the prevalence of American Foulbrood (AFB) disease in Apis mellifera colonies across selected districts of Kohat Division, Pakistan, using the ropiness test—a rapid and cost-effective diagnostic method. Out of 650 samples collected from 833 colonies in 12 apiaries across Hangu, Kohat, and Karak, 253 (38.92%) tested positive for AFB, while 397 (61.08%) were negative. Notably, Hangu exhibited the highest infection rate (44.27%), followed by Kohat (38.97%) and Karak (30.29%). The absence of data from Kurram and Orakzai due to security constraints highlights regional disparities in disease surveillance and apiary accessibility, which may influence the overall understanding of AFB distribution in the region.

The findings reveal significant variations in AFB prevalence compared to previous studies in Pakistan. While earlier nationwide research reported a 37.30% positivity rate across 15 apiaries (Anjum et al., 2015), the current study identifies Hangu as the most affected district, contrasting with prior reports where Kohat and Bannu had higher prevalence. This discrepancy may stem from differences in sampling scope, methodological approaches, and regional beekeeping intensity. The earlier study employed symptomatological, biochemical, and molecular techniques (16S rDNA PCR) for confirmation, whereas our research relied on field-based ropiness testing with a smaller sample size (650 vs. 1276). Additionally, Hangu higher AFB prevalence could be attributed to its dense beekeeping activity, accounting for 52.22% of surveyed colonies and 40.31% of samples, factors that likely amplify disease transmission due to colony density and management practices.

The uneven AFB distribution in this study also reflects broader challenges in disease control, including migratory beekeeping, inadequate sterilization, antibiotic misuse,

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and socio-political instability restricting access to high-risk areas like Kurram and Orakzai. These constraints limit the representativeness of the data compared to more comprehensive national or international studies.

When compared to the Asian context, Pakistan's AFB prevalence (38.92%) exceeds reports from China (22.5%) (Tian et al., 2012) and India (26.9%) (Kumar et al., 2015), where lower rates are linked to climatic diversity, structured apiculture programs, and better diagnostic coverage Similar to our findings, studies in Thailand, Nepal, and Iran associate AFB outbreaks with poor hive management and migratory practices (Sartori et al., 2016; Adjlane et al., 2016). However, unlike these countries, Pakistan's surveillance gaps—particularly in conflict-affected regions—likely result in underreported cases and skewed prevalence estimates.

Globally, the AFB prevalence in Kohat Division is markedly higher than in countries with stringent regulatory frameworks, such as Germany (5–10%) (Genersch et al., 2005) and New Zealand (<1%) (Goodwin et al., 2006), where early detection and hive destruction policies curb outbreaks Conversely, our results align with high-prevalence regions like Ethiopia (33.3%) (Ejigu et al., 2021) and Argentina (40%) (Reynaldi et al., 2008), where limited beekeeper training and weak surveillance contribute to persistent AFB transmission The absence of a national monitoring program in Pakistan further exacerbates the challenge, emphasizing the need for policy interventions to improve diagnostics, beekeeper education, and regional cooperation.

In conclusion, this study underscores the high AFB burden in northwest Pakistan, particularly in high-activity zones like Hangu. The disparities in prevalence compared to global and regional data highlight the impact of sampling biases, diagnostic limitations, and socio-political barriers to surveillance. Addressing these gaps requires expanded monitoring, standardized molecular diagnostics (e.g., PCR), and targeted awareness programs to mitigate AFB's economic and ecological threats to Pakistani apiculture.

CONCLUSION

This study highlights the significant prevalence of American Foulbrood (AFB) in Apis mellifera colonies across Kohat Division, Pakistan, with an overall infection rate of 38.92%—substantially higher than global averages. The highest prevalence was observed in Hangu (44.27%), followed by Kohat (32.81%) and Karak (20.95%), emphasizing regional disparities in disease distribution. The absence of data from Kurram and Orakzai due to security constraints underscores the challenges in comprehensive disease surveillance, potentially masking higher infection rates in inaccessible regions.

The findings align with previous studies on AFB's devastating impact on apiculture but reveal critical gaps in Pakistan's current monitoring and control strategies. The reliance on the ropiness test, while cost-effective, may underestimate subclinical infections compared to molecular methods (e.g., PCR). Furthermore, high colony density, migratory beekeeping, and poor hive hygiene likely contribute to the elevated AFB prevalence observed. Without immediate intervention, the unchecked spread of AFB could lead to long-term economic losses for beekeepers and threaten pollination-dependent agriculture in the region.

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RECOMMENDATIONS

For Local Researchers (Pakistan-Specific Focus)

Expanded Surveillance: Future studies should prioritize sampling in Kurram and Orakzai once security conditions permit, as exclusion may bias prevalence estimates. Molecular Confirmation: Supplement ropiness tests with PCR or ELISA-based detection of Paenibacillus larvae to identify asymptomatic carriers and improve diagnostic accuracy. Beekeeper Surveys: Investigate local beekeeping practices (e.g., antibiotic misuse, hive sterilization) through interviews to identify high-risk behaviors driving AFB transmission. Spatial Mapping: Use GIS technology to track AFB outbreaks in real time and correlate prevalence with environmental factors (e.g., humidity, floral resources).

For global researchers

Comparative Genomics: Sequence P. larvae strains from Pakistan to assess spore virulence and compare with global isolates (e.g., European or American strains). Alternative Treatments: Evaluate phage therapy or probiotic supplements as sustainable alternatives to antibiotics, which face resistance issues. Climate Change Effects: Study how rising temperatures and erratic rainfall in South Asia may influence AFB spore survival and transmission dynamics. Machine Learning Models: Develop predictive algorithms using hive health data (e.g., brood patterns, bee behavior) to flag early AFB outbreaks before clinical signs appear.

For Policymakers and Beekeepers

National AFB Monitoring Program: Pakistan's government should establish a centralized database for AFB cases, mandating reporting from all districts. Beekeeper Training: Launch workshops on hygienic hive management, including: Regular equipment sterilization (e.g., flame torches for hive tools). Avoiding honey or pollen exchanges from infected colonies Recognizing early AFB symptoms (e.g. sunken, perforated brood capping) Regulate Antibiotic Use: Ban over-the-counter sales of oxytetracycline to curb resistance and encourage biocontrol methods. Incentivize Hive Destruction: Offer compensation for burning AFB-infected colonies (modeled after New Zealand's eradication program).

Final Remarks

AFB remains a critical threat to Pakistan's apiculture, exacerbated by limited surveillance, fragmented beekeeper knowledge, and logistical barriers. While this study provides a snapshot of Kohat Division's AFB burden, long-term solutions require collaborative efforts between researchers, policymakers, and beekeepers. Global insights—such as molecular diagnostics from Europe or phage therapy trials from the U.S.—can be adapted to local contexts, but community engagement is paramount to ensure adoption. Future work must bridge the gap between academic research and on-ground implementation to safeguard honey bee populations and food security.

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