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In vivo and Laboratory Studies of Tetracycline Prevention of Foulbrood disease of *Apis cerana indica* Fab. and its Effects on Honey

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Abstract

Laboratory and field studies were carried out to assess the efficacy of the antibiotic tetracycline in preventing and managing American foulbrood (AFB) and European foulbrood (EFB) diseases in honey bees. Experiments with immature worker bees in the laboratory demonstrated that honey bee larvae could endure a broad range of antibiotic doses in their diet. The recommended dose of tetracycline (100 µg/ml) effectively protected young larvae from infection. However, once the dose exceeded this recommended level, it reached a point where it became lethal.

Keywords: *Apis cerana indica*; Honey; Tetracycline; ELISA; Unintentional adulterant

1. Introduction

The Asian honey bee (*Apis cerana indica*) plays a vital role as a pollinator in India, contributing significantly to the country's ecological health, biodiversity preservation, and agricultural productivity. Thriving across India's varied climates, these bees are central to apiculture practices nationwide. India ranks eighth globally in honey production, consumption, and export, with major honey-producing states including Punjab, Uttar Pradesh, West Bengal, Bihar, Rajasthan, and Karnataka. According to the National Bee Board, there are currently around 12,699 beekeepers and 1.934 million honey colonies in India, producing approximately 133,200 metric tons of honey as of 2021-2022.

However, the global decline in bee colonies over the past decade is concerning, with a significant reduction in native and wild bee populations each year [1]. Honey possesses natural antibacterial and antimicrobial properties due to its hydrogen peroxide content, glucose oxidase, and low pH, which inhibit the growth of harmful bacteria and fungi [2]. Despite these benefits, apiculture faces increasing challenges from pests and diseases, exacerbated by climate change, which threatens global honey production. The study area, south Karnataka is typically a plain area, which includes a considerable area of natural forest and tree plantations, besides large extent of land is under the cultivation of agrihorticultural crops. The presence of large number of bee-flower species in the area suggests that the plains of south Karnataka is undoubtedly suitable for commercial beekeeping. The diversified bee flora of the area supports beekeeping throughout the year. The result supports the views expressed many years ago by Zamarlicki (1984)[3].

Honey bees are susceptible to a range of diseases caused by bacteria, fungi, viruses, and protozoa. Notably, the Gram-positive bacteria *Paenibacillus larvae* and *Melissococcus plutonius* are responsible for American foulbrood (AFB) and European foulbrood (EFB), respectively[4], leading to substantial economic losses. Infected colonies often necessitate the destruction of hives and disinfection of equipment to control outbreaks. Colony collapse disorder (CCD) also remains a significant concern, with no single cause identified but with several pathogens and parasites contributing to the risk of collapse [5].

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The Karnataka State has all the four species of honeybees, viz. *Apis dorsata, A. cerana, A. florea and A. mellifera*. However, *A. mellifera* is newly introduced into the state after the bee keepers lost *A. cerana* colonies due to sudden outbreak of Thai sac brood disease (TSBV) during late 1980's [6]

The health of honey bees is closely linked to their digestive system, which interacts with pathogens and xenobiotics. Studies have shown that exposure to therapeutic doses of tetracycline can alter the gut micro biome of worker bees, affecting their behavioral and mortality rates [7,8,9]

Bangalore is the capital of the Indian state Karnataka and has an estimated (2014) population of 11,440,000 in the metro area. It is located in the south-eastern region on the state on the Deccan Plateau, and it is the third most populous city and the 5th most populous urban area.

This study aims to investigate the effects of tetracycline at various concentrations dose of tetracycline on honey bee larvae (*Apis cerana indica*). The research will assess how tetracycline influences the bees ability to nectar foraging and lethality. Tetracycline, used since the 1950s for their broad-spectrum antimicrobial activity, low toxicity, and affordability, remain a common choice for treating microbial diseases in food-producing animals [10]. However, the effects of Tetracycline on *Apis cerana indica*, particularly the areas of in and around Bangalore, Karnataka, have not been extensively studied to date.

2. Material and methods

The study was conducted during April, 2020 to March, 2023. Survey studies were conducted in five places of Bangalore district of Karnataka State (Table-2). Information collected on various parameters and practices followed for *Apis cerana indica* rearing viz., brood diseases, awareness, and management practices adopted by beekeepers for maintenance of bee colonies and honey storage practices.

One hundred beekeepers of the selected area were used as the main tool for data collection. The majority of the respondents was males and well distributed in all considered group ages. The questionnaire is composed of the demographic characteristics and the knowledge of the risk posed by antibiotics of the beekeepers were collected

Antibiotics: Tetracycline, used to control the bacterial disease of honey bees in Karnataka collected from local medical store.

ELISA Test Kit for Honey: from secure Bio diagnostic Company, India. The honey sample (1 g) was added with 9 mL of diluted wash buffer warmed to 37°C. The preparation was diluted with an equal amount of diluted wash buffer (1 mL + 1 mL).

well mixed 200 µg/ml diluted honey sample was incubate for 3 minutes at 40 degree C and visual interpretation was done by color contrast test line, after about 5 minutes at 40 C

2.1. Honeybees colonies

Healthy honeybees, *Apis cerana indica* colonies were carefully collected and selected with no apparent infections and with apparent infection from local bee keepers apiary separately. Selected colonies were grouped and kept in a controlled laboratory condition at 34 ± 1^0C and 75% ± 5% relative humidity (RH) in Agri consultant, Hootagalli, Mysore.

Four deferent colonies were used as four experimental replicates. Worker bees were raised in separate boxes and incubated at 31^0C ± 1^0C. Dead bees were counted and removed daily. All pollen and syrup supplied to workers were UV irradiated and sterilized.

In the treatment group, larvae were fed a meal containing various antibiotic concentrations (Tetracycline) were orally exposed at concentrations (above and below recommended dose 100 µg/ml). Groups were made in to four, the first was control group (G0) in complete sterilized hives box and were fed a normal diet without Tetracycline. The Second one (G1) was complete sterilized hives box and another without sterilized hives box (G2) with healthy bees. Last one was complete sterilized hives box with infected bees(G3) and were fed a normal diet with Tetracycline [11]. These larvae were fed with UV irradiated and sterilized syrup once a day and the larvae in the control group were fed a normal diet without Tetracycline [12].

2.2. Honey samples

Twenty different honey samples obtained from five different regions of Bangalore district (Fig-1) of Karnataka state, India. by local bee keepers. Presence of antibacterial agent tetracycline was evaluated for two important honey bee bacterial diseases. *Apis cerana larvae* as the etiological agents of American foulbrood (AFB) and European foulbrood (EFB) diseases were subjected to biochemical assay.

2.3. The mortality

Groups of 50 worker Honey bee larvae were exposed to four different concentrations of the antibiotic (Tetracycline) orally 48 hours and the mortality was assessed and determined,

2.4. Analysis of residues

There are forty samples (20 X 02) of natural and apiculture honey was aimed to investigate the antibiotic (Tetracycline) effects at different concentrations of recommended dosage, 100 µg/ml (25%, 50%, 100%, 125%, 150%, and 200 µg/ml) on the honey bees (*Apis cerana indica*) larva, effect on the Bio-efficiency, in terms of knowing how long they survived after being exposed to it 48 hours, also the amount of Tetracycline was calculated by Liquid chromatography

2.5. Statistical Analysis

All data were subjected to statistical analysis wherever necessary using SPSS Package 11.5. 12 samples were used in each of two replicates for each treatment and the experiment was repeated twice. A two-way analysis of variance was used to evaluate and the significance level was set at $P<0.05$.

3. Results and discussion

The results of the analysis conducted using the Evidence Investigator Anti-Microbial Arrays indicated that Tetracycline (TC) residues were found only in honey samples collected from the most densely populated and polluted apiary location. In contrast, 12 *in vivo* samples showed no detectable levels of TC residues and were reported as negative (-),The honey samples collected in the year 2021 from Anekal (A4) and Nelamangala (N1) showed the absence of any of the detectable antibiotic molecules but samples collected from the same study places in the year 2023 were showed the presence of tetracycline molecules (Table- 1).

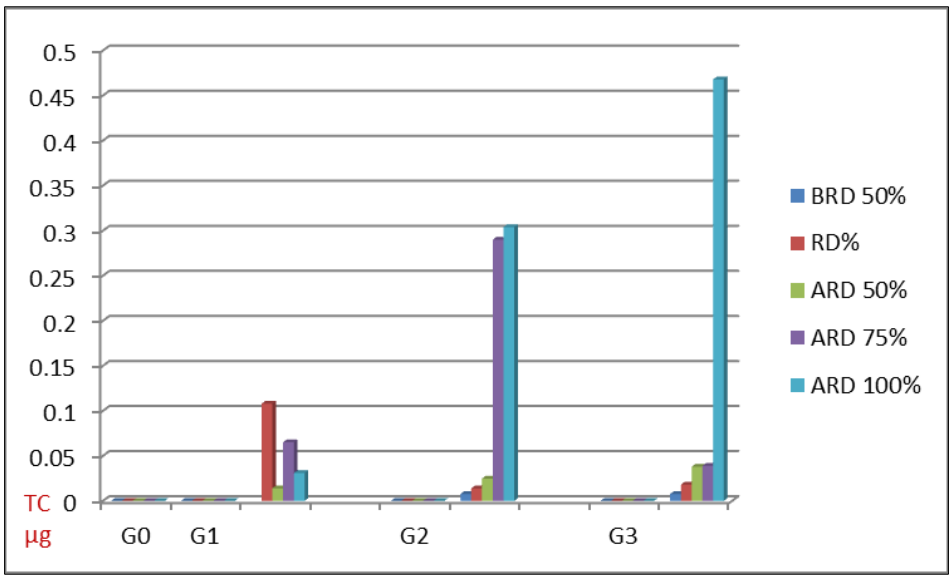
Table 1 Comparison of the antibiotic concentrations detected in *in vivo* honey samples

Bengaluru And Its Environs	Study sites Code No. and Name	Year 2021-2022	Year 2022 -2023
Doddaballapur	D1-Aralumallige	-	-
	D2-Doddaballapura Town	0.0038±0.0093	0.0219±0.0132
	D3-Rajanukunte	-	-
	D4-Industrial area	0.0026±0.0058	0026±0.0002
Anekal	A1-Banneraghatta	-	-
	A2-Chandapura	-	-
	A3-Athibele	-	-
	A4-Anekal Town	-	0.0027±0.0023
Nelamangala	N1-Nelamangala Town	-	0.0036-0.0011
	N2-Arashinakunte	-	-
Bengaluru North	BN1-Yelahanka	0.0092±0.0025	0.0022±0.0008
	BN2-Byatarayanapura	-	-
	BN3-Heggadenagar	-	-
	BN4-GKVK Campus	-	-
Bengaluru South	BS1-Kengeri	0.0249±0.00265	0.0066±0.0055

	BS2-Jayanagar	-	-
	BS3-Padmanabhanagar	-	-
	BS4-Vijayanagar	-	-
	BS5-Chamarajapete	-	0.0012±0.0004
	BS6-Nagarabhavi	-	-

Mean (±SD) antibiotic concentration in samples of honey; Mean of 4 replicates, significant at P < 0.05; Total No. of samples 4 samples detected positive (+) samples detected negative (-)

The analysis of experimental laboratory-treated honey samples revealed that Tetracycline (TC) were detected in all groups treated with the recommended and above-recommended dosages (G2 and G3). However, in the control group (G0) and the groups treated with lower dosages (G1), the levels of antibiotics (TC) were not detected (Fig-1).



(G0) control group sterilized hives box with healthy bees; (G1) sterilized hives box with healthy bees; (G2) without sterilized hives box with healthy bees; (G3) sterilized hives box with infected bees

Figure 1 Tetracycline (TC) concentrations (µg) detected in experimental bee honey samples

At higher concentrations of TC, the larvae died before or during the pupal stage and fails to undergo metamorphosis into adult worker bees (Table -2) and or inactive.

Table 2 Mortality percentage (%) of Tetracycline at different concentration on *Apis cerana indica Larvae*

Apis c. indica	**Tetra Cycline Percentage (%) Mortality**				
	BRD 50%	**RD%**	**ARD 50%**	**ARD 75%**	**ARD 100%**
Control G0	-	-	--	-	-
Groups G1	-	-	-	16	18
Groups G2	-	-	03	12	25
Groups G3	-	05	05	32	45

N= 50 average of 3 replicates

4. Discussion

Disease prevention and manage the transmission of pathogens within and across apiaries, beekeepers often resort to treating their hives with antibiotics. This practice aims to minimize the spread of infections and protect the health of bee colonies, though it may also contribute to antibiotic residue issues in honey[13].

Survey conducted was revealed that Antibiotic residues in honey are frequently due to inadequate awareness of proper antibiotic use among beekeepers. A online questionnaire was designed and distributed to beekeepers of 20 selected area of in and around Bangalore. The results showed that 56.6% of beekeepers use antibiotics to treat their bees. However, 69.7% are unaware of the risks associated with consuming honey containing antibiotics. Furthermore, 37.4% of beekeepers do not follow the labeled instructions for antibiotic use, and around 15% obtain information about antibiotics from the unreliable sources. These findings reveal a significant knowledge gap among beekeepers and raise concerns about potential antimicrobial resistance in consumers. This issue is essential for enhancing food safety and quality policies in that area.

TC on immature worker bees maintained in the laboratory revealed that honey bee larvae could tolerate quite a range of doses of antibiotic in their diet. Recommended doses of TC protected very young larvae from becoming infected by *Bacillus.*

Antibiotic treatment had no measurable effects on larval or pupal developmental rates until the dose reached above 25% recommended (ARD) dosage and Bees Larvae treated with TC in the laboratory were kept to the field after attain maturity, noted no negative effects at any dosage rates.

Overtly diseased bee colonies with larvae treated with TC in the laboratory were reintroduced to the field after reaching maturity. The treatment was effective in eliminating signs of foulbrood (FB) infection in the overtly diseased colonies.

The primary aim of larval treatment is to protect both larvae and adult bees from bacterial diseases. However, a secondary concern is the impact on honey, as residues of antibiotics from larval treatments can accumulate in the honey. Larvae are considered suitable hosts for maintaining bacteria, even in the absence of external bacterial sources. By treating these larvae with antibiotics, they are safeguarded from bacterial attacks.

A direct correlation was observed between different concentrations and their occurrence in the sample. The results of the study are conclusive of the significant Tetracycline occurrence in *Apis cerana indica* honey samples against the treated bees. Etiological agents of the mentioned honeybee diseases, suggesting their probable use as a safe biological agent to control AFB and EFB diseases [14,15,16]. Antibiotics effects not only bees but also most important symbiotic microbes of the honey bees [17,18]. Understanding the source of pollution is crucial for preserving the quality of honey and ensuring its safety [19]. Furthermore, given the high presence of antibiotic residues in honey is a unintentional adulterant [20].

5. Conclusion

The data obtained indicates that inappropriate percentage of antibiotic useage were affected with the differences in the concentration under investigation. Unintentional adulterants of antibiotic residues in honey may be one of the reasons for its therapeutic properties. The use of rapid and simple screening methods for detecting multiple analytes is essential for maintaining product integrity and consumer safety.

Compliance with ethical standards

Authors declare that there is No conflict of interest

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