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An Ultrastructure of Microsporidia in Honey Bees (Apis mellifera) by microscopic techniques

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***Abstract:** This paper describes the microsporidian parasite morphology and developmental stages that were isolated from honey bees (Apis mellifera) and identified by microscopic techniques. It was observed under light bright field microscope that the mature spores were ovo-cylindrical in shape and shining florescent green, with an inimitable Brownian motion and measured 5.65±0.60 (4.98- 6.63; n=5) µm in length and 2.04±0.11 (1.87-2.21; n=5) µm in width. Observation of mid-gut smears revealed different developmental stages of the parasite. Primary small spores were also observed that had a diameter of 1.90±0.25 (1.55-2.21; n=5) µm. Giemsa staining exhibited various life stages of the intracellular parasite undergoing merogony and sporogony. Germinated empty spores with its polar tubule were observed under phase contrast microscope and imaging microscope. Scanning electron microscope represented the spore morphology and interaction of the parasite with its host. The overall morphology and development stages of the parasite microsporidia isolated from Honey bees, as studied by microscopic techniques, relate it to the genus Nosema.*

***Keywords:** Microsporidia; Honey bee; Apis mellifera; Meront; Sporont; Nosema*

I. INTRODUCTION

Honey bees (*Apis mellifera*) are essential pollinators for agriculture and an important source of honey as well as a number of valuable non-food products such as bee wax. It plays an important role in the conservation of biodiversity and has become an obligatory part of the ecosystem. However, an inexplicable illness called Colony Collapse Disorder (CCD) has been threatening the health of honey bees and the beekeeping industry since late 2006 (Chen et al., 2007) and contributing substantial bee die off phenomena. One of the major cause of Colony Collapse Disorder are the Microsporidia, an obligate intracellular parasite, that are usually considered as primitive eukaryote without mitochondria. These are very common insect parasites and play an important role in regulating insect population (Tsai et al., 2003). In adult honey bees, *Nosema* disease is most prevalent and caused by *Nosema apis* and *ceranae* (Bailey, 1981; Matheson, 1993; Chen et al., 2008). Microsporidia, *Nosema ceranea*, was a wide spread infection of European honey bees as reported by Klee et al (2007). The disease nosemosis in honey bee colonies is spread mainly via the fecal-oral route, resulting in the Colony Collapse Disorder (CCD) **(**Ptaszynska., 2014). There are two primary stages in the microsporidian life cycle, a vegetative stage that yields merozoites, and a sporogonic phase that produces mature spores (Higes et al., 2010). The infective life stage of all species is a spore which contains a coiled polar filament, a unique morphological character of the Microsporidia. Inside the host cell, sporoplasm matures and replicates as meronts that further develop into sporonts. The sporonts divide to become sporoblast which further matures into spores (Futerman et al., 2005). Infected bees do not show any visible symptoms of the disease but their life span reduces and they become lethargic. *Nosema* disease is distributed universally and probably the most severe disease problem for the beekeeper. Being an unusual eukaryotic parasite, microsporidian infection resides in the midgut of the bees and utilizes the contents of these cells as a food supply. As a result, the infection plummets the efficiency of the gut in the process of digestion and absorption of food. Because it is an insidious, persistent disease with no outward symptoms, the beekeeper is ignorant that the bees are infected (Higes et al., 2007). However, *Nosema* disease affects nearly every aspect of the bee's life. In the present study, microsporidian infection was investigated in honey bees collected from different areas of Lucknow by using microscopic techniques. The various life stages of the parasites have been observed and their morphological description has been shown in order to understand the pathogenicity of the parasite to its host. Severity of infection by the parasite has been studied and presented by Scanning electron microscopy and gives a precise idea of interaction of the parasite with its host.

II. MATERIALS AND METHODS

A. Samples

Honey bee specimens were collected from established hives on trees and buildings during autumn season (Dec-Jan) from different areas of Lucknow, and frozen at -20˚C. Individual Honey bee sample was warmed to room temperature and dissected to separate the abdomen from rest of the body. The abdomen sample were macerated separately in 3ml ddH_2O with a mortar and pestle, filtered and

centrifuged at 8000rpm for 10mins. The samples (pellets) were washed thrice by discarding the supernatant and homogenate(spore pellet diluted with distilled water) was examined for spore detection using bright field (Olympus) microscope and photographed in EVOS XL Imaging system with a magnification of 400X. The extent of infection of typical spores in the abdomen was measured by counting the spores by Neubauer hemocytometer.

B. Staining

Giemsa staining was done by fixing the air dried slides of the spore in 10% methanol followed by washing and staining it in freshly prepared 5% (V/V) Giemsa stain for approximately 10hrs. The slides were rinsed in running water for 5 mins, air dried and then first observed under bright field microscope then in imaging microscope to study the different developmental stages of the parasite.

C. Scanning Electron Microscopy

Additionally, SEM analysis of honey bee abdomen homogenate samples was done by fixing the the sample in 2.5% (V/V) glutraldehyde mixed in 0.1M PBS at pH 7.3 for 24hrs, followed by washing with phosphate buffer thrice of the above fixed samples. After washing, post fixation was done in 1% OsO_4 in 0.1M PBS for 24hrs. Dehydration was done with different grades of acetone (30%, 50%, 75%, 90% and 100%) for 15mins each. Dried samples were mounted on specimen stubs and coated samples were viewed under a scanning electron microscope (Jeol, Japan; JSM 6490 LV) at 20K, and specimens were measured and photographed.

III. RESULTS

We observed the same kind of stages and the same pattern of development of the pathogen in the host species studied. The accumulation of the parasite caused fragility of the host cells and insects adipose tissue was the predominant site of microsporidia infection. As a result, amorphic spore clumping (AMC) was observed in the homogenate sample (Fig.1A), that may be due to the development of host derived membrane around them. The spores observed were shinning fluorescent green with an inmitable Brownian motion and were ovo-cylindrical in shape (Fig. 1A) measuring 5.65±0.60 µm (4.98- 6.63µm; n=5) in length and 2.04±0.11 µm (1.87-2.21µm; n=5) in width. The fresh smears of abdomen samples observed, also had very small spores that appears almost spherical with a diameter of 1.90±0.25 (1.55-2.21; n=5) µm. The calculated hemocytometer spore count average values for mature large spores were found to be approximately 3.38×10^6 spores/ml.

Dividing sporonts were more commonly observed than dividing meronts. The sporonts divided at least once yielding two sporoblast (SB) (Fig. 1B). Mature spore formed free in the host cell cytoplasm without any sporophorous vesicle. Spore germination and its extruding polar tubule (PT) was observed (Fig. 1A) under imaging microscope (40X). Giemsa staining could distinguish the microsporidia internal contents by thick unstained wall and a blue stained featureless interior (Fig. 2A). The merogonial stage of microsporidia was the very first stage that was recognized as round and slightly oval structures. Primary meronts with two nucleus dividing by simple binary fission (binucleate meronts; BM) were observed (Fig. 3) and later merogonial stages were found with larger cells with two or four nuclei (tetranucleate meronts ;TM)) in the Giemsa stained slides. Diplokaryon appeared separated in immature spores while in mature spores nuclei it was not distinguishable (Fig 3). Many empty spores were also observed (Fig. 2B) that indicated germinated spores.

Scanning Electron Microscope revealed that the honey bee abdomen had higher incidence of infection with microsporidian spores. Some areas of epithelial cells of the host also showed developmental stages. The three dimensional spore morphology revealed the ovo-cylindrical shape of the microsporidian spores (Fig. 4A). It was observed that all stages of the parasite were in direct contact with host cell cytoplasm and the end of the polar tubule remained hidden inside the host cell (black arrow). It was observed that both polar tubule and spore wall are involved in host cell invasion (Fig. 4B) as spores were seen adhering to the walls of the intestine. After ejection of sporoplasm in to the host cell (Fig. 5) the surface of the spores became completely distorted leaving behind the empty spores.

IV. DISCUSSION

The microsporidia detected in honey bee (*Apis mellifera*) from different areas of Lucknow is an obligatory intracellular parasite that multiplies in the host cell. The morphological and developmental character as revealed by light and electron microscopy supports the fact that microsporidia belongs to the genus *Nosema*. Similar findings were reported by Higes et. al (2007) who reported that during the early development of an infection of *N. ceranae* in *A. mellifera*, the presence of merogonial plasmodia with four diplokarya and endogenous cytoplasmic development were typical of the genus *Nosema*. The genus *Nosema* is often a constant pathogen of both valuable and detrimental insects groups, being prevalent especially in Lepidoptera (Cheung and Wang, 1995).

In the present study, observation with light bright field microscopy showed that spores are ovo-cylindrical in shape. Moreover, the variations in sizes are observed that might be different developmental stages of the spores in the mid-gut epithelial cells of the host, which remains to be verified.

Life cycle reflected the merogony and sporogonic stages that are typical of microsporidia of *Nosema* genus as reported by Hylis et al (2006). Diplokaryotic stages are observed in all stages of development. Binucleate spores germinating in the gut lumen set down the sporoplasm directly into the mid-gut epithelial cells by means of an extruding polar filament. Each binucleate sporoplasm grew in size and developed into a meront. Primary very small spores that were observed could be considered as primary meronts. Meronts divided and differentiated into sporonts, and each sporont promotes sporoblasts formation. A related finding has been described in Apis mellifera infected with Nosema apis, where long chains of schizonts or merogonial plasmodia, were reported (Gray et al., 1969). Enthrallingly, it has been observed that the infected abdomen of Apis mellifera had some spores aggregation in the prepared macerated homogenate of gut that resembles pansporoblast of the genus Pliestophora but proper microscopic observation hints that these aggregated spores is not covered by an ensheathing membrane as found in pansporoblast and possibly could be well thought-out as an old wall of epithelial cells of insect intestine. The spore morphology studied by Scanning electron micrograph had particularly shown the spore ovoid surface and invagination explaining the information reported by Solter and Maddox (1998) on primary spore germination in the midgut that allow the polar tubes to infringe the basal membrane resulting in the placement of sporoplasm in tissue that are injected to the midgut. The spore layer covering the bee intestine is the cause of diarrhoea like symptoms and mortality faced by bees. Undergoing different stages of life cycle the accumulation of parasites in the cytoplasm caused enlargement and fragility of host cell. It was also observed that spores are lying on the monolayer of host cells and after ejection of its sporoplasm the spore wall was completely collapsed. A related finding was presented by Schottelius et al (2000) on life cycle of microsporidia of the genus Encephalitozoon by scanning electron microscope describing developmental stages of microsporidia describing the collapsing spore after extrusion of polar tube in the host cell. Presences of empty spores disclosed the completely germinated spores and spread of infection in the epithelium of midgut in honey bees. The microsporidia detected in honey bee abdomen isolate appears to be apansporoblastic and spores were in direct contact with host cell cytoplasm sharing silent features of genus Nosema. Sprague and Vernick (1971) distinguished Nosema from the genera Glugea and Encephalitozoon based on their nuclear arrangement.

V. CONCLUSION

By observation from the present study the resemblance in the development of parasite and three dimension spore morphology by electron microscopy correlates it with *Nosema* genus. Moreover, electron microscopy has become a prominent technique to study the pathogenesis and spore developmental cycle of microsporidia. Detection of pathogen in bees stored over long time suggests that microsporidia has adapted to and established an infection in honey bees.

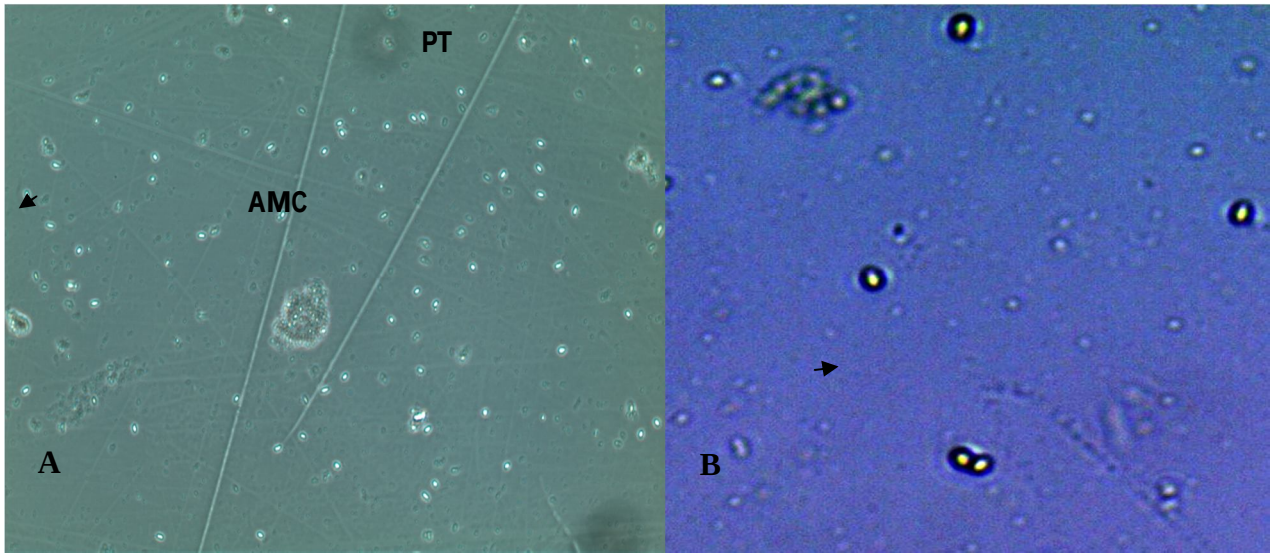

Fig. 1. Light microscopy images of microsporidian spores in honey bee mid gut. (A) Fresh smears of microsporidian spores under phase contrast microscope (black arrow); Spore with its ejecting polar tubule (PT); few spores showing amorphic spore clumping (AMC) (B) Sporonts forming sporoblasts (black arrow).

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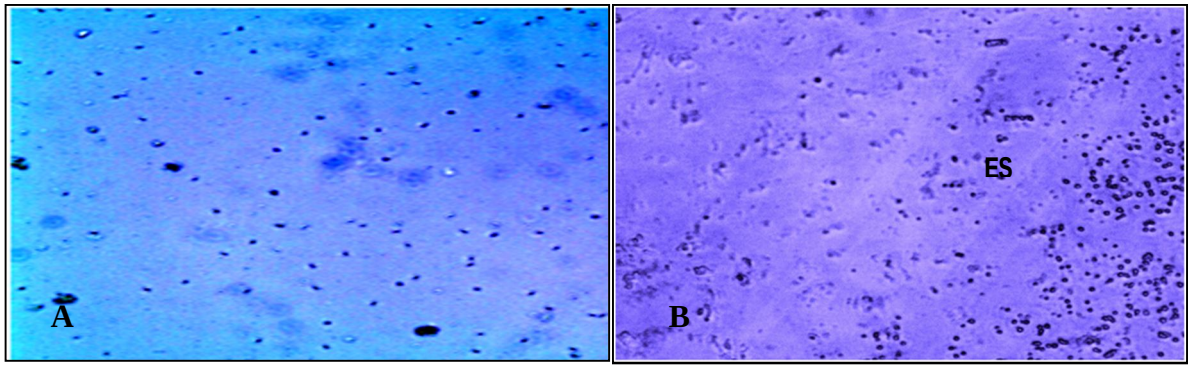

Fig.2. (A) Methanol fixed smear of microsporidia stained with Giemsa stain (B) Germinated spores that are unstained empty spores (ES)

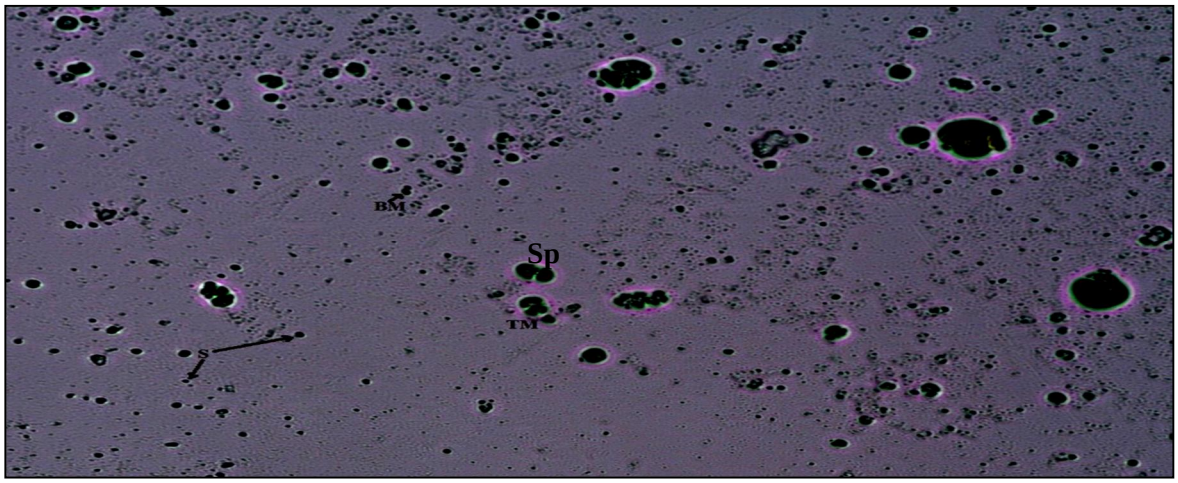

Fig. 3. Different developmental stages of microsporidian spores ; Mature spores (S); Binucleate meronts (BM); Tetranucleate meronts (TM); Sporonts (Sp)

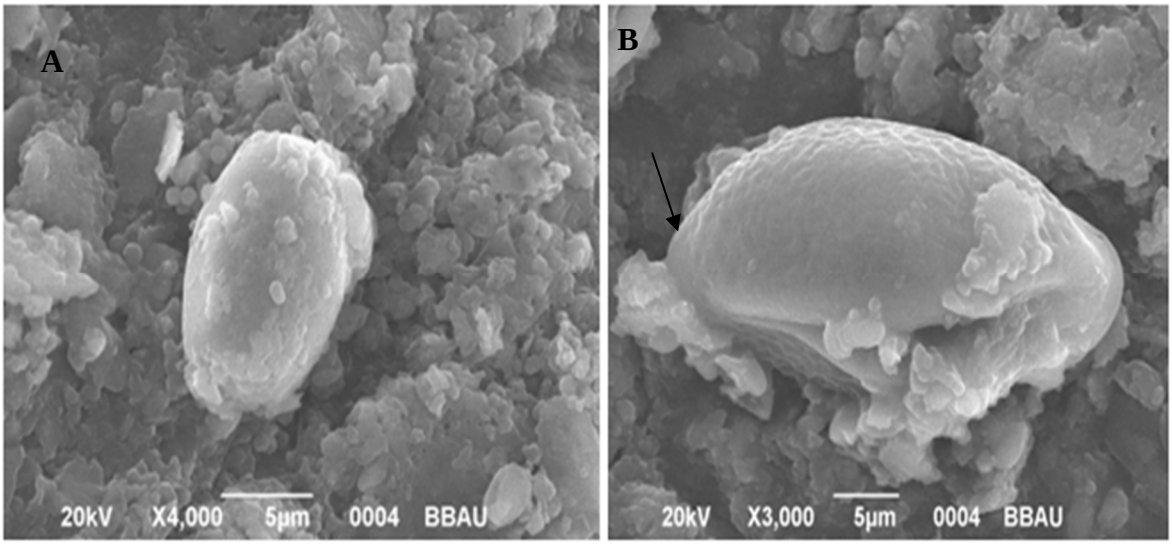

Fig. 4. Scanning electron microscopy of microsporidian spores (A) mature spores infecting the epithelium of *Apis mellifera* intestine (B) The end of the polar tubule is hidden inside the host cell (black arrow) and both polar tubule and spore wall are involved in host cell invasion

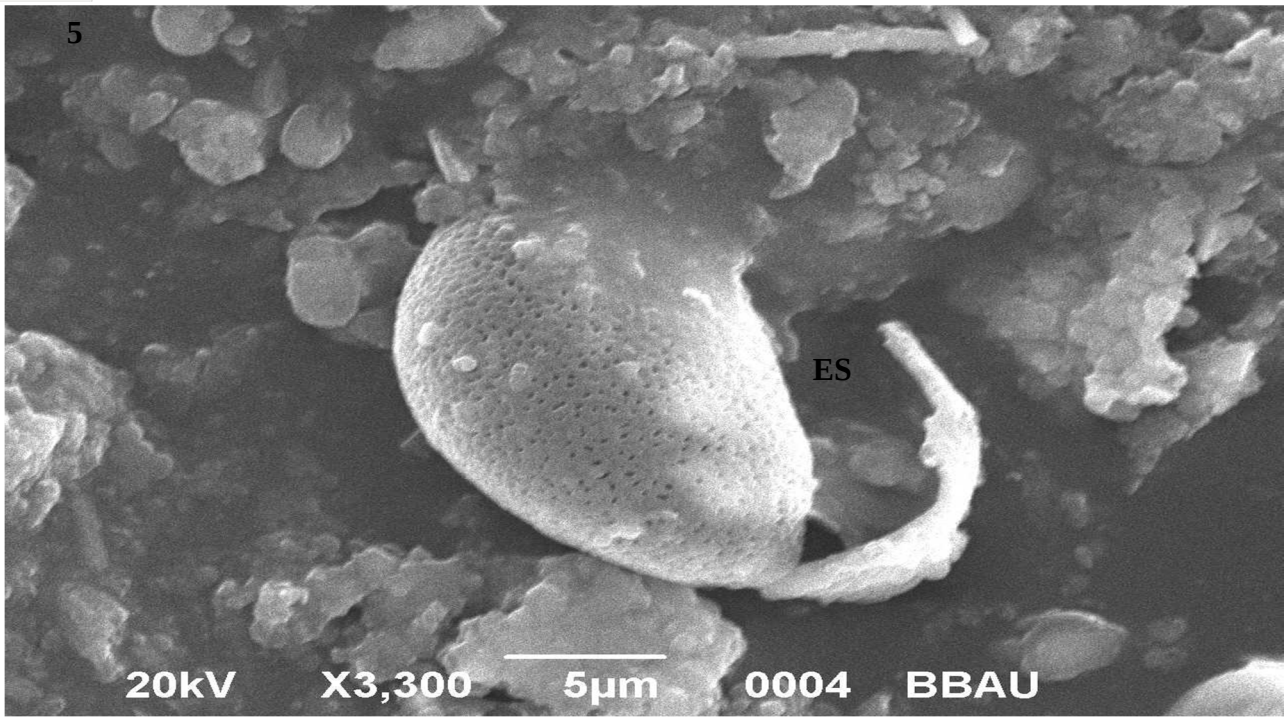

Fig. 5. Scanning electron micrograph of microsporidian spore liberating its spore content (sporoplasm) inside host cell; empty spore (ES)

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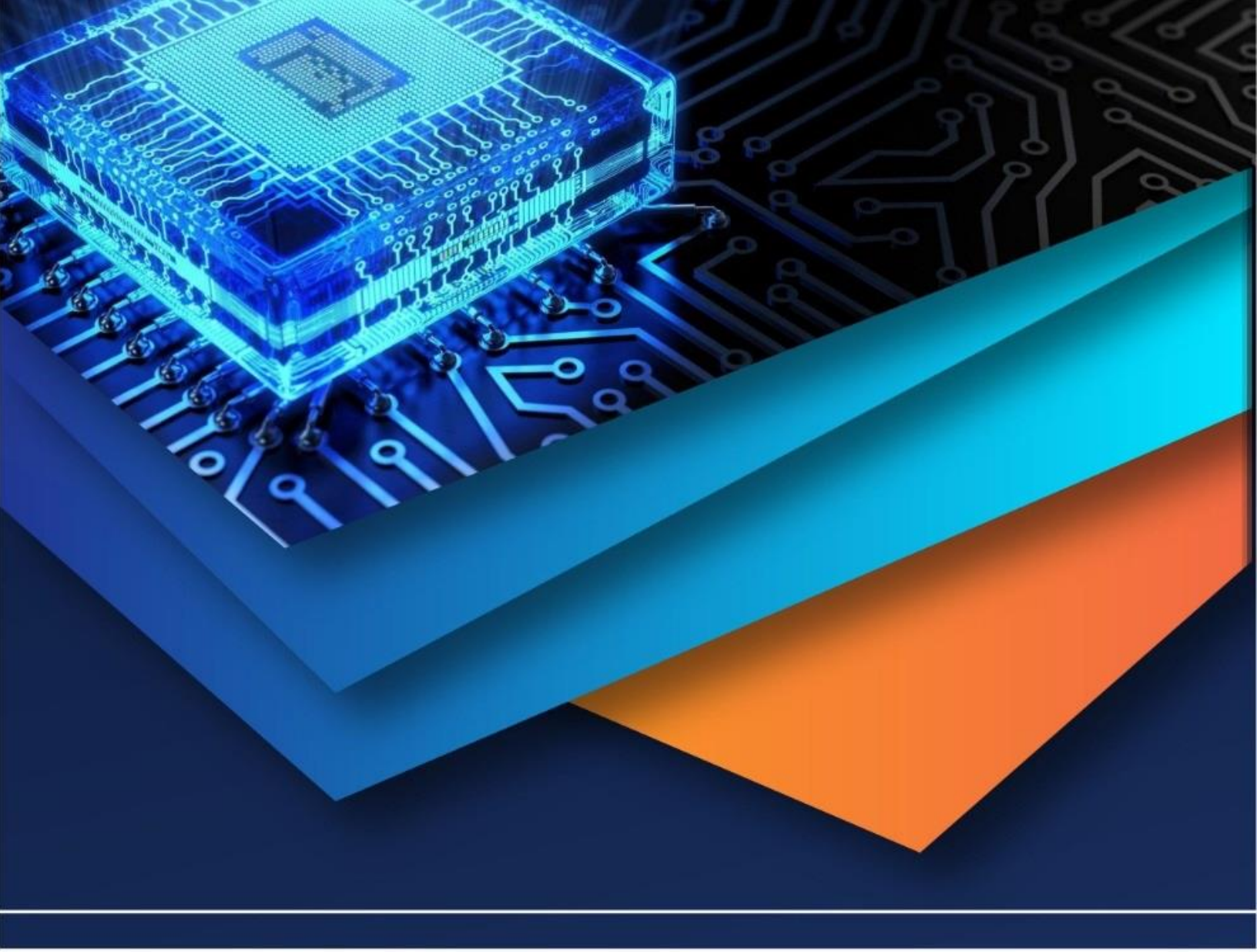

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