Original Research

Microscopic and Electron Microscopic Studies of *Malassezia pachydermatis*

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Abstract

The Malassezia pachydermatis was isolated from clinical cases presented with signs of recurrent, moist, seborrhoeic dermatitis and transferred to sabourads dextrose agar. After several subcultures, pure colonies of the yeast were acquired which was confirmed under under high power field. The colonies were soft cream coloured and where as yeast appeared as dark blue coloured, foot print shaped when stained by diff quick method. Further, under electron microscope, the malassezia organism appeared as unique peanut or globose to ellipsoid in shape, round at one end and blunt at the other on SEM (Scanning electron microscope) and multilayered, thick cell wall with nucleus and vacuoles of various sizes and mitochondria of various shape and size and with bulgings between mother and daughter cell which was measuring about 3.19 to 4.56 µ in size, were the significant findings noticed on transmission electron microscope.

Keywords: *Malassezia pachydermatis*, Scanning Electron Microscope, Transmission Electron Microscope

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Introduction

Malassezia pachydermatis is a lipophilic, nonmycelial and saprophytic yeast. This thick-walled, ovoid to ellipsoid, unipolar budding yeast can be found in or on the ear canal, anal sac, lip, chin, vagina, rectum and skin of clinically normal dogs (Srikala *et al.*, 2010). It is a commensal yeast on canine skin (Brito *et al*., 2009), when factors favouring its overgrowth are present, *M. pachydermatis* is thought to act as a facultative pathogen on the skin of dogs. Earlier researchers have reported essential information regarding ultrastructure of *M. Pachydermatis.* In this present work the organism size was measured using SEM

(Scanning electron microscope) ranged between 3.19 - 4-56 µ and the ultrastructural characters of the yeast were clearly visualized using TEM (Transmission Electron Microscope).

Materials and Methods

The present investigation was carried out among the clinical cases of dogs that were presented with the history of chronic, recurrent and persistent skin and coat abnormalities to the Teaching Veterinary Clinical Complex, Bhoiguda and Campus Veterinary Hospital, College of Veterinary Science, PVNR Telangana Veterinary University, Rajendranagar, Hyderabad. Dogs presented at the above mentioned hospitals with the history of pruritus, erythema, alopecia, hyperpigmentation, offensive greasy rancid odour and any visible skin lesions, formed the basis for the present investigation. Detailed history regarding duration, severity and previous treatment was recorded and the samples were collected. Sample from dry seborrhoeic lesions were collected using cellophane tape impression and/or moistened sterile swab and whereas, those from greasy lesions were collected using cellophane tape impression and/or sterile swab and were transferred to sabourauds dextrose agar for further processing. However, cellophane tape impressions were stained with NMB by Diff-Quick method and examined under 100 x magnifications in oil immersion for identification. The colonies that were cultured on Sabourauds Dextrose Agar (SDA) with chloramphenicol (0.05%) were again subcultured till pure colonies were established. Later to study the ultra structural characters of the yeast they were subjected for scanning electron microscope (SEM) and transmission electron microscope (TEM) studies as follows;

Scanning Electron Microscopy (SEM)

The material was isolated under stereozome, mounted on double sided sticky carbon tape and exposed / fixed with 1% osmium tetroxide as a fume fixation. The processed samples were mounted over the stubs with double-sided carbon conductivity tape and a thin layer of gold coat over the samples were done by using an automated sputter coater (Model-JEOL JFC – JSM 5600) at required magnification as per the standard procedures given by John *et al.* (1998), at RUSKA laboratory, College of Veterinary Science, Rajendranagar, Hyderabad. Few samples that were found positive for *Malassezia* yeast were subjected for scanning electron microscopic procedure as described by John *et al*. (1998).

Transmission electron microscopy (TEM)

Samples were fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4^0C and washed with PBS for 4 times each 45 minutes, then post fixed in 1% aqueous Osmium Tetroxide for 24h later washed with deionised distilled water for 6 times each 45 minutes, dehydrated in series of graded alcohol, infiltrated and embedded in araldite 6005 resin or spur resin (Spur resin, 1969), incubated at 80^0 C for 72 h for complete polymerization. Ultra thin (60 nm) sections were made with a glass knife on ultra

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microtome (Leira Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous Urenyl acetate (UA) and counter stained with Reynolds lead citrate (LC). Viewed under TEM (Model Hitachi, H-7500 from JAPAN) at required magnification as per the standard procedures at RUSKA laboratory, College of Veterinary Sciences, PVNRTVU, Rajendranagar, Hyderabad.

Histopathology & Cytology

Skin biopsy samples were collected by using incised biopsy method from malasseziosis cases and were also subjected for histopathology as per the standard procedure (Chauhan and Dinesh, 2007). The tissue pieces from affected dogs were collected properly and fixed in a suitable fixative. Then these are processed and sections of 5-6 microns were cut and taken on to the slides. These sections were stained with H & E stain and mounted to make permanent slides.

Results

Microscopic and Cultural Characteristics of *M. pachydermatis*

In the present investigation, *Malassezia pachydermatis* was identified by all three diagnostic protocols adopted *i.e.* acetate tape impression, direct glass slide impression and sterile swab method. However, suspected samples were stained with new methylene blue (NMB) stain for 1 min, which were later examined under 100 x power of microscope showed blue coloured footprint shaped organisms confirmed as *M. Pachydermatis* (Fig. 1 & 2).

Fig. 1 Blue coloured footprint shaped Malassezia yeast on high power magnification

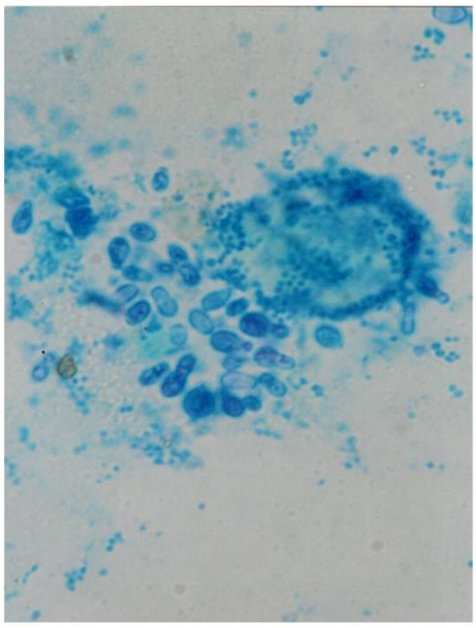

Fig. 1: Blue coloured footprint shaped Malassezia yeast on high power magnification)

Fig. 2 Blue coloured monopolar budding yeast on Methylene blue staining

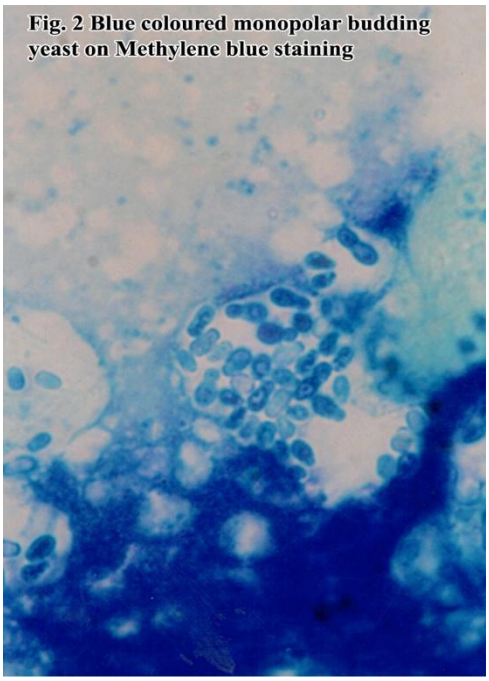

Fig. 2: Blue coloured monopolar budding yeast on methylene blue staining)

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Sterile cotton swab samples that were taken from malassezia dogs were also subjected to cultural examination by transferring or streaking on Sabourauds Dextrose Agar (SDA) with Chloramphenicol (0.05%) and Potato Dextrose Agar (PDA). Later these plates were incubated for 48 h at 37 °C in air supplemented with 5% CO_2, to observe the growth and to study the colony and microscopic morphology of the yeast. The colonies on Sabourauds dextrose agar were first white or creamy in colour then became buff to orange-beige and finally dark tan to brown by 5-7 days (Fig. 3). Whereas, the colonies on Potato dextrose agar were creamy in colour then became friable, shrink and finally (after 4-7days) dark tan to brown. The colonies were round, less convex with wrinkled margins, which were non pigmented on young culture, then became tan to brown (Fig. 4).

Fig. 3: Creamy, concave round Malassezia colonies on SDA

Fig. 4: Creamy, wrinked, shrinky Malassezia colonies on PDA

Scanning Electron Microscopic Aspects

The *Malassezia pachydermatis* colonies that were grown on Sabourauds dextrose agar were examined under scanning electron microscope showed a raised, doomed or high convex and smooth in appearance (Fig. 5). Similarly, the yeast appeared as unique peanut or footprint shaped, that was globose to ellipsoid in shape, round at one end and blunt at the other. The budding organisms were of different shapes starting from round to typical bipolar appearance with different stages of budding revealed by growing small knob of various sizes at the other end. Further, the yeast was measured between 3.19 to 4.56 µ in size (Fig. 6).

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Fig. 5 SEM image of Malassezia pachydermatis colony - different stages

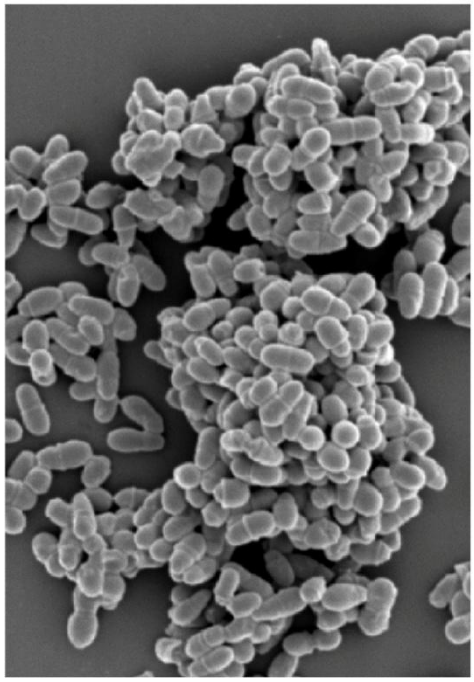

Fig. 5: SEM image of Malassezia pachydermatis colony – different stages

Fig. 6 Measurement of *Malassezia pachydermatis* - SEM image

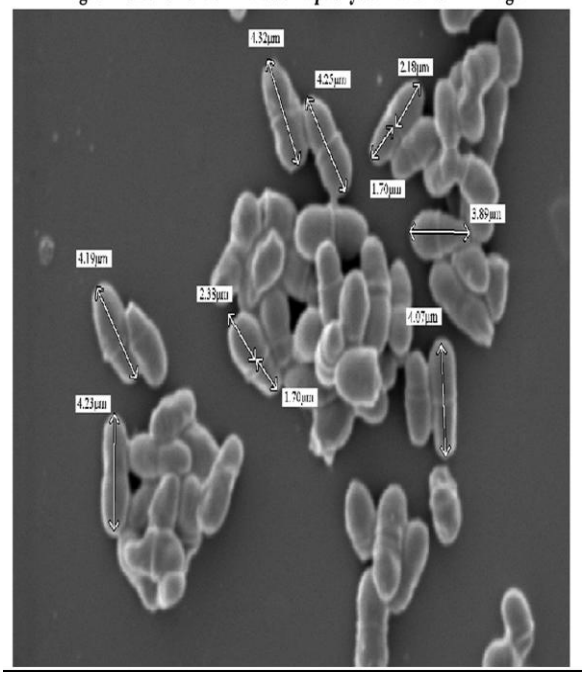

Fig. 6: Measurement of Malassezia pachydermatis – SEM image)

Transmission Electron Microscopic Aspects

The transmission electron microscopic aspects revealed thick cell wall that seemed to be composed of three layers. The nucleus of *Malassezia pachydermatis* showed a distinct nucleolus that was granular in appearance, deeply stained and centrally located. The vacuoles are of various sizes and appeared spherical in most sections. They showed light eccentric granulation and are separated from the other cell constituents by a membrane. The mitochondria vary in shape and size and are heavily granular. The intracellular structures were few but relatively large, considering the cell's size. Bulgings were present between each two grooves on the mother cell and gradually appeared on the bud during its growth (Fig. 7).

Histopathology

In our present study, biopsy specimens that were collected sterile surgical biopsy technique revealed hyperplasia, hyperkeratosis, infiltration of mononuclear cells and fibroblasts in the dermis as the significant histo-pathological findings (Fig. 8).

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Fig. 7 TEM image of *Malassezia pachydermatis*

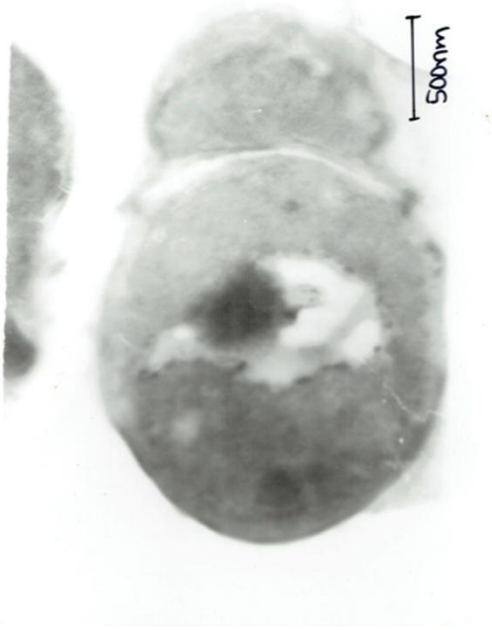

Fig. 7: TEM image of Malassezia pachydermatis

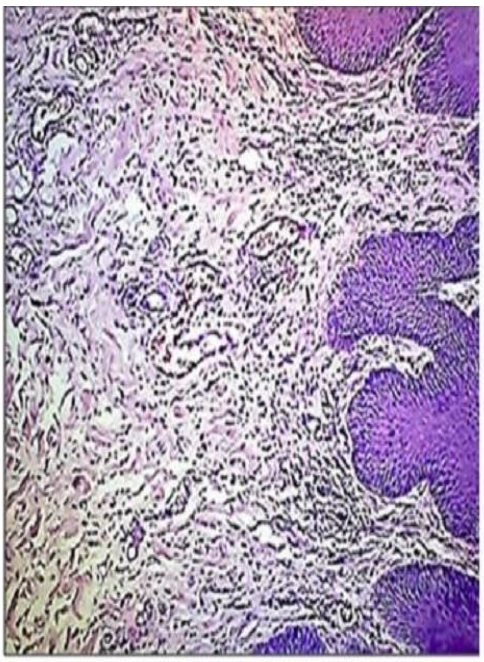

Fig. 8 Hyperplasia, infiltration of mono nuclear cells – Canine malasseziosis

Discussion

In our present investigation *Malassezia pachydermatis* was confirmed by different isolation and identification techniques viz. acetate tape impression, direct glass slide impression and sampling by sterile swab method showed blue coloured footprint shaped yeast organisms. These findings are in accordance with Nambi (2002) who reported that the organism *Malassezia pachydermatis* is a peanut shaped or foot print shaped yeast that measures about 2-5 microns in size that can be best diagnosed using cellophane tape impression or sterile swab method. The cultural and colony characters of the yeast on Sabouraud´s dextrose agar with 0.05% Chloramphenicol were white or creamy in colour initially, which then became buff to orange-beige and finally dark tan to brown (after 4-7days). These findings of our study is in accordance with Reddy and Kumari (2015) who isolated *Malassezia pachydermatis* from skin lesions on saborauds dextrose agar and reported that colonies were white in colour, convex, smooth and dry, which later turned to brown colour.

In our present study, ultrastructural characters of the *Malassezia pachydermatis* revealed typically foot print shape, round at one end and blunt at the other which were measuring about 3.19 to 4.56 µ in length on scanning electron microscope. These findings are in agreement with Jyothi and Kumar (2014) and Kumar *et al.* (2008) who reported that the yeast were ellipsoid to globose in shape measuring about 3.16 to 5.29 microns in length. Whereas, Akerstedb and Vollset (1996) reported that *Malassezia*

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pachydermatis were oval or ellipsoid in shape that were measuring about 2.5-6.5µm in diameter and 2-5µm in length. The transmission electron microscopic aspects of this yeast revealed a thick cell wall and seemed to be composed of three layers, the nucleus of *Malassezia pachydermatis* with a distinct nucleolus that was granular in appearance, deeply stained and centrally located vacuoles and mitochondria. The intracellular structures were few but relatively large, considering the cell's size. Findings of our present study is in accordance with David *et al*. (2003) who documented that they were 5 µm in length and 2.5µm in width and with a cell wall of thickness 0.2-0.3 µm, nucleus of 1µm, mitochondria of 0.7 µm and variable spherical vacuoles. However, Satish *et al.* (2008) documented that the yeast having multilamellar cell wall that was relatively thick, between 0.1-0.2µ and ultra thin sections of the organism revealed indistinct nucleus of 0.8µ, mitochondria of 0.5µ and with various size vacuoles. In our present study, biopsy specimens that were collected by punch biopsy technique revealed hyperplasia, hyperkeratosis, infiltration of mononuclear cells and fibroblasts in the dermis as the common histo-pathological findings, are in accordance with Maudlin *et al*. (1997) who reported an epidermal hyperplasia, superficial perivascular hyperkeratosis and lymphocytosis and opined that this might be due to delayed hypersensitivity reaction associated with IgE in malassezia affected cases.

Conclusion

The present investigation *Malassezia pachydermatis* was isolated from the clinical cases of dogs that were presented with the history of chronic, recurrent and persistent skin and coat abnormalities. The colonies of the isoates on Sabourauds dextrose agar were first white or creamy in colour then became buff to orange-beige and finally dark tan to brown by 5-7 days., whereas, the colonies on Potato dextrose agar were creamy in colour then became friable, shrink and finally (after 4-7days) dark tan to brown. Scanning electron microscope studies revealed unique peanut or footprint shaped yeast that was globose to ellipsoid in shape, round at one end and blunt at the other which measured between 3.19 to 4.56 µ in size. The transmission electron microscopic aspects revealed, thick cell wall that seemed to be composed of three layers and the nucleus of showed a distinct nucleolus that was granular in appearance, deeply stained and centrally located. The biopsy specimens subjected to histopathology revealed hyperplasia, hyperkeratosis, infiltration of mononuclear cells and fibroblasts in the dermis.

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