Incidence of *Malassezia pachydermatis* in The Ears of Apparently Healthy Cattle

By
Fedyas Yousif Abd El Salam
(B.V.Sc.2004 University of Bahr El Ghazal)

Thesis submitted for partial fulfillment of the requirement for the degree of Master of Science by Courses and Complementary Research in Microbiology
(M.Sc. Microbiology).

**Supervisor:**
Dr. Abdel Hafeez Hassan Nimir
Faculty of Veterinary Medicine
University of Khartoum

Department of Microbiology
Faculty of Veterinary Medicine
University of Khartoum
April 2008
Dedication

To my parents who brought me to life, and taught me to presume
my ancestors devotion to knowledge and science

To the soul of my beloved uncle Elshibli, who encouraged me
endlessly to carry out my study.
Acknowledgement

I would like to express my gratitude to many persons who gave me an indispensable help in writing this research. Ahead of them is my Supervisor Dr. Abdel Hafeez Hassan Nimir who, despite his crowded timetable, was too patient and kind to go over and comment on what I did and wrote fact that made possible the accomplishment and presentation of the work in its present shape and contents.

My great indebtedness is extended to my parents, my brothers, my sisters and my uncle Dr. Ibrahim Abd El Salam.

I am also deeply indebted to all of the teaching and technical staff of the Department of Veterinary Microbiology for their help and encouragement.

Last, but not least, my gratitude is extended to my friends who kept encouraging me during my study and provided me with moral impulse until the last stage of submitting this thesis.
# List of contents

Dedication                                      I  
Acknowledgement                                  II  
List of contents                                  III  
List of Figures                                    V  
Thesis Abstract                                   VI  
Thesis Abstract in Arabic                          VIII

**Chapter One: Introduction**                   1  
  Objectives of the study                           3

**Chapter Two: literature Review**              4  
  2.1 Background                                    4  
  2.2 *Malassezia pachydermatis* in cattle          7  
  2.3 *Malassezia pachydermatis* in goats           7  
  2.4 *Malassezia pachydermatis* in pigs            8  
  2.5 *Malassezia pachydermatis* in dogs            9  
  2.6 *Malassezia pachydermatis* in human           12

**Chapter Three: Material and Methods**        15  
  3.1 Sampling procedures                           15  
  3.2 Media and reagents                             16  
    3.2.1 Corn Meal Agar                              16  
    3.2.2 Sabouraud Dextrose Agar + Chloramphenicol + Cycloheximide 16  
    3.2.3 Yeast Nitrogen Base                         17  
    3.2.4 Carbohydrate Assimilation Tests             17  
    3.2.5 Sabouraud Dextrose Agar (SDA) + Chloramphenicol 18  
    3.2.6 Sabouraud Dextrose Agar                     18  
  3.3 Identification of the yeast isolates          19
3.3.1 Colony characteristics 19
3.3.2 Morphology of vegetative cells 19
3.3.3 Mycelium or pseudomycelium 19
3.3.4 Growths at 37 °C and elevated temperature 20
3.4.5 Carbohydrate Assimilation (Auxanographic) Tests 20
3.4.6 Germ Tube Test 21

Chapter Four: Results 22

Chapter Five: Discussion 28

Conclusions and Recommendations 31

References 33
List of Figures

Figure No 1: Micromorphology of Malassezia pachydematis. (Dr.Fungus website), for comparison.....................................................24

Figure No 2: Malassezia pachydermatis (Specimen No.103), grown at 35°C on Sabouraud Dextrose Agar. ………………………..25

Figure No 3: Malassezia pachydermatis from specimen No.108 showing globose to ellipsoidal cells budding from one pole at the same location. Gram stain × 1000…………………………… 26

Figure No 4: Malassezia pachydermatis in exudates from the external canal of cattle. (Specimen No. 103) with “peanut shape” Gram stain, x 1000…………………………………………27
Abstract

One hundred and fifty bovine ear swabs from crossed breeds were investigated for the presence of *Malassezia pachydermatis* in the external ear canals of apparently healthy cattle. The study included 25 calves, 100 dairy cows from different farms in Khartoum North and 25 oxen from the El kadaro slaughter house.

Collection of the specimens was made by sterile swabs; after cleaning of the auricle with alcohol solution, recording amount and the nature of the wax within the ear canal. Specimens were transported immediately after collection to the laboratory for investigation.

Specimens were inoculated onto Sabouraud Dextrose Agar (SDA) \(\text{pH} \ 5.6\); supplemented with Chloramphenicol (150 mg\(\text{l}\)) and Cyclohexamide (0.05 mg\(\text{l}\)) to suppress the growth of bacteria and saprobic fungi. Olive oil was added as a growth factor and incubation was made at 35\(\text{°C}\) for at least a week.

Two samples yielded yeast growth representing 1.3 % of the total samples. These isolates were identified as *Malassezia pachydermatis*. The isolates were identified to the species level according to the methods described by Lodder (1974) and Geuho and Guillot, (1996).
The relatively lower existence of *Malassezia pachydermatis* in this study could be due to anatomical structure of the external ear canal in these breeds also could be due to environmental temperature and humidity.

Macroscopic morphology revealed that rapid growth occurred at 35°C, reaching a maximum after 72hrs on Sabouraud dextrose agar, supplemented with Chlroamphenicol, colonies were round, convex and smooth, white or creamy in colour then became buff to orange-beige. Growth was good but slightly less than without antibiotics and was enhanced when olive oil was added.

The microscopic morphology indicated that budding occurs from one pole which gives a typical shape resembling footprint or a peanut. Moreover *Malassezia pachydermatis* does not form pseudomycelia.

Thus, judging by results in the present study it appears that the *Malassezia pachydermatis* may be considered as a possible source of infection in the auditory canal of cattle.
ملخص الدراسة

اجريت هذه الدراسة على 150 عينة تم جمعها من القناة السمعية الخارجية للاذن. Malassezia لايفار هجين، سلامة ظاهرية لاستبان وجود نوع من انواع الخمائر يعرف بـ Malassezia pachydermatis. 

الدراسة اجريت على 25 عجل و 100 بقرة حلوب بالإضافة إلى 25 ثور. تم اخذ العينات من أماكن مختلفة من محلية بحرية تضمنت:

1- مزرعة جامعة الخرطوم (شمبات)
2- المحلب (حلة كوكو)
3- بعض المزارع الخاصة (شمبات)
4- سلخانة الكنرو

تم اخذ العينات بواسطة مسحات معقمة بعد نظامية وتطهير صوان الأذن بحلول الكحول وتم اخذ العينات مباشرة الى المعمل لإجراء الاختبارات المطلوبة للكشف عن Malassezia pachydermatis.

تمت زراعة العينات في مستنبس خاص بالفطريات (سابرود) ذو الاس الهيدروجيني (Chloramphenicol) بتركيز 150 ملغ للتر و مضاف اليه مضاد حيوي لمنع نمو البكتيريا. ايضا تم اضافة زيت Cyclohexamide بتركيز 500 ملغ للتر و تم إجراء تحليز نمو الخميرة (Malassezia pachydermatis) في درجة حرارة 35 درجة مئوية لمدة أسبوع على الاقل قبل اعتبار ان العينة سالبة.

تم عزل Malassezia pachydermatis من عينتين من اصل 150 ما يوازي 1.3%.

النسبة المنوية في هذه الدراسة أقل من النسبة المنوية التي تم رصدها من قبل بعض الباحثين الآخرين وقد يعزى ذلك إلى التركيب التشريحي للذبابة الخارجية للسلالات التي تم تناولها في هذه الدراسة أو قد يكون بسبب التغيرات المناخية الموسمية التي تشمل تغير في درجات الحرارة والرطوبة. اظهرت هذه الدراسة أيضاً امكانية هذا الفطر في التسبب بالتهاب الأذن الخارجية إذا تواجد بعداد كبيرة داخل الأذن وذلك بسبب افراده لبعض الانزيمات التي تودى إلى تهيج والتهاب الأذن.
CHAPTER ONE

Introduction

The word "yeast" comes from the Old English language "gist", "gyst", ultimately from the Indo-European root "yes-", meaning boil, foam, or bubble (Wikipedia.com). Yeasts are probably one of the earliest known organisms. People have used yeasts for fermentation and baking throughout history. Archaeologists digging in Egyptian ruins found early grinding stones and baking chambers for yeasted bread, as well as drawings of 4,000-year-old bakeries and breweries (Cited by Such, McHugh and Pollock .2005). In 1680, the Dutch naturalist Antonie van Leeuwenhoek was the first to observe yeasts microscopically, but at that time he did not consider them to be living organisms but rather globular structures (Leeuwenhoek, 1721). In 1815, Guy-Lussac understood how yeasts convert the simple sugar, glucose to ethanol (Such, McHugh and Pollock .2005).

In (1837) Cagnianrd demonstrated that the beer yeasts were spherical bodies capable of multiplying and they belonged to the plant Kingdom (Fritz Schlek .1997). In 1857 the French scientist Louis Pasteur proved in his paper "Mémoire sur la fermentation alcoolique" that alcoholic fermentation was conducted by living yeasts and not by a chemical catalyst. Pasteur showed that by bubbling oxygen into the yeast broth, cell growth could be increased, but the fermentation inhibited - an observation later called the Pasteur Effect (Barnett. 2003).

After Pasteur, there was a period of intensive activity during which yeast taxonomy and morphology flourished. Jorgensen (1886) wrote on yeast fermentation. In 1912, the first treatise devoted entirely to yeasts
was published by Guilliermond. He was the first to include keys for identification of yeasts. (Nimir, 1980).

The yeast research in Delft (The Netherlands) has been developed quite separately from the classical morphological methods used for filamentous fungi. The yeast taxonomy was based mainly on the fermentation and assimilation of certain carbon compounds. This way of classifying yeasts is now generally known as the "Dutch School". J. Lodder, a member of the yeast division published a revision of the imperfect (non ascosporogenous) yeasts in cooperation with H.A. Diddens in 1942 (Cited in WWW.cbs.knaw.nl). In 1946, N.J. W. Kreger-van Rij who joined the yeast department in 1946, compiled the monograph "The yeasts, a taxonomic study" in cooperation with J. Lodder in 1952. This book has been reprinted and revised several times and is still considered as a major textbook in yeast taxonomy. Other valuable texts dealing with the biology and the taxonomy of yeasts include those by Ingram (1955), Roman (1957), Cook (1959), Luer and Lindemann (1960, 1962), Rose and Harrison (1969, 1970) and Barnett and Pankhurst (1974).

Over the years, more and more yeast genera and species have been described; Lodder (1970) listed 341 species in 39 genera. Later, Barnett and Pankhurst (1970) added 10 new genera and 93 new species to those already reported. Kreger-van Rij (1986) recognized over 500 yeast species grouped into 60 genera. Presently approximately 1,500 species of yeasts and 6900 strains have been described (Kurtzman and Fell 1998).

Yeasts have asexual and sexual reproductive cycles; however the most common mode of vegetative growth in yeast is asexual reproduction by budding (Balasubramanian, Bi and Glotzer. 2004) where a small bud, or daughter cell, is formed from the parent cell. The nucleus of the parent cell splits into a daughter nucleus and migrates into the daughter cell. The bud continues to grow until it separates from the parent cell, forming a new cell.
The bud can develop on different parts of the parent cell depending on the genus of the yeast.

Under high stress conditions haploid cells generally die; however under the same conditions, diploid cells can undergo sporulation, entering sexual reproduction (meiosis) and producing a variety of haploid spores, which can go on to mate (conjugate), forming the diploid spore, Neumann (2005).

Yeast are predominantly saprobic and widely distributed in nature; they are found in both terrestrial and aquatic environments (Phaff and Starmer, 1987). They inhabit soil, plants, atmosphere, fresh and salty water, insects and vertebrates; they are known to exist as commensals on or in the animal's body and have been isolated from domesticated, zoo and wild animals (Plant, Rosencrantz, and Griffin. 1992; Balasubramanian et al., 2004).

Objectives of the study:-

The purpose of this study was to investigate the prevalence of Malassezia pachydermatis in the ear canal of apparently healthy cattle, and correlate the findings to the possibility of this species causing otitis externa in cattle.
CHAPTER TWO

Literature Review

2.1 Background

Yeasts of the genus *Malassezia* are known as members of the skin microflora of human and other warm-blooded vertebrates (Leeming and Notman. 1989; Gueho and Guillot. 1995; Faergmann. 1985; Midgley. 1989).

The earliest report of *Malassezia*-like yeasts was made by Eichstedt in 1846. In 1853, Robin described round cells on the skin of patients with superficial fungal infection (dandruff). In 1873, Rivolta described the presence of round double-contoured budding cells in a patient with psoriasis.

Baillon (1889) accommodated Robin's *Microsporn furfur* (1853), the etiologic agent of Pityriasis Versicolor, which has been described from its appearance in epidermal scales of patients with this superficial fungal infection. This disease is common in late teens and young adults of both sexes and characterized by well-demarcated scaling patches with variable pigmentation and its frequency depends on climatic, occupational and socio economic condition (Fitzpatrick, Johnson, Wolff and Suurmond, 2001; Borelli, Jacobs and Nall .1991).

In 1889 Bizzozero found both spherical and oval budding yeast-like cells in epidermal scales from normal human scalp and named them *Saccharomyces sphaericus* and *Saccharomyces ovalis*, respectively (Gordon.1979). Similar form had been described earlier by Malassez (1874), who later called his organism (spore). However, Malassez was never able to culture the microorganism and Von Sehlen and Unna (1881) were probably the first to culture the organism. (Faergemann. 2002; Lodder. 1974).
In (1904) Sabouraud proposed the binomenclature *Pityrosporum Malassezi* to encompass globose, ovoid and elongated forms of these “spore of Malassez”. Castellani and Chalmers (1913) accepted the new generic term but combined it with Bizzozero’s species designation to form *Pityrosporum ovale*, which Acton and Panja in (1927) converted it into *Malassezia ovale*.

Weidman, in 1925, found numerous bottle-shaped budding yeast-like cells in scales from exfoliative dermatitis of Indian rhinoceros and was able to culture this microorganism on media without fatty acids or other special nutritional additives. Because of its resemblance to *Pityrosporum ovale* Weidman proposed the name of *Pityrosporum Pachydermatis* for the new species. (Cited by Salkin, Gordon and Stone. 1978)

Salkin and Gordon (1977) have shown that *P.orbiculare* and *P.ovale* are morphologic and physiologic forms in the life cycle of a single microorganism. Since the term *Malassezia furfur* has priority over *P.orbiculare* and *P.ovale*, and the acceptance of *P. pachydermatis* as a congeneric with the two later species, there remain two species in the genus *Malassezia*, namely *furfur* and *Pachydermatis*, a combination devised by Dodge in (1935).

The genus *Malassezia* contained only two species for many years (Yarrow and Ahearn, 1984), *Malassezia furfur*, a lipophilic yeast that requires long-chain fatty acids for growth (lipid-dependent species) and *Malassezia pachydermatis*, which can take advantage of the short-chain fatty acids present in basic mycological media such as Sabouraud glucose agar (non-lipid-dependent species) (Ahearn and Simmons, 1998; Slooff, 1970; Yarrow and Ahearn, 1984). However, there was no consensus about this limited number of species. Some authors demonstrated clearly that *M. furfur* was a polymorphic species. Midgley (1989) identified two groups of lipid-dependent yeasts on morphological, physiological and immunological
grounds (Cunningham, Leeming, Ingham, and Gowland. 1990), also showed that *M. furfur* could be subdivided into three serotypes on the basis of group-specific surface antigens.

The historical development of the taxonomic classification of the genus *Malassezia* yielded a description of many different species based upon molecular, biological, morphological and biochemical parameters (Ingham and Cunningham. 1993; Weiss, Raabe and Mayer. 2000; Boekhout, Kam and Gueho. 1998).

In the last reclassification by Gueho, Midgley and Guillot .(1996) and Gueho (1995), seven distinct species were recognized within this genus, namely *M. furfur* (Robin) Baillon 1889, *M. pachydermatis* (Weidman) Dodge (1935), *M. sympodialis* (Simmons and Guého. 1990; Boekhout and Bosboom. 1994), *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* Simmons and Ahearn (1998). Furthermore, four new species were included in this genus recently, *M. dermatis*, *M. equi* (Nell, James, Bond, Hunt and Herrtage. 2002), this species has not formally been described, and *M. nana* (Hirai, Kano, Makimura, Duarte, Hamdan, Lachance, Yamaguchi and Hasegawa. 2004). It is possible that this species is the same as that presented by Duarte, Lachance and Hamdan. (1999) as atypical strains of *M. sympodialis*. Recently Sugita, Takashima, Kodama, Tsuboi, and Nishikawa. (2003) have described another new species, *M. japonica*. However, the acceptance of these new species is still under investigation. There is only scanty information about the epidemiology and ecology of *Malassezia* species available and the clinical significance of these species is not completely recognized.
2.2 *Malassezia pachydermatis* in cattle

Little is known about the significance of *M. pachydermatis* in cattle. Ear swabs were collected from 192 cattle by Duarte *et al.*, (2003) from both european and zebu breeds and used to evaluate the epidemiology of *Malassezia* spp. High numbers of *Malassezia* isolates were significantly associated with maturity of the animals and with diagnosed otitis. Identification and distribution of *Malassezia* spp. from the animals revealed 3 isolates of *Malassezia pachydermatis*. In healthy animals, low prevalence of *M. pachydermatis* particulary was found in Holstein cows in summer months, a finding perhaps correlating with the open air-exposed ear of this breed.

Dufait. (1985), investigated 50 cows for the presence of *M.pachydermatis* in the external auditory canal and *M. pachydermatis* was obtained from 2 samples (4%) besides two positive culture of *M.pachydermatis* from the skin of six sampled cows.

Duarte *et al.*, (2002), reported about the occurrence of *Malassezia* species in the external ear of 200 cattle (143 healthy and 57 with otitis) in Minas Gerais, Brazil, and found that 4 isolates of *M.pachydermatis* came from healthy cattle's, one from a cow with otitis, although the strain from the cow with otitis was identified as a lipid-dependent variant of *M. pachydermatis*.

2.3 *Malassezia pachydermatis* in goats

Didier Pin. (2004) described a case of a 6-year-old female goat with seborrhoeic dermatitis. The condition developed following severe enteritis associated with weight loss. Dermatological examination showed a generalized greasy seborrhoeic dermatitis, which spared the head and limbs. Microscopic examination of impression smears of skin revealed numerous yeasts typical of *Malassezia*. spp.
Culture on Sabouraud's dextrose agar yielded *Malassezia pachydermatis* growth. Histopathological examination of haematoxylin/eosin and safranin (HES) stained sections of biopsies showed mild lymphocytic superficial perivascular hyperplastic dermatitis. Numerous budding yeasts were visible both on the surface, and follicular keratin, in sections stained with HES and periodic acid Schiff (PAS).

A dramatic response was observed after 1 week of a topical anti-*Malassezia* treatment, and the resolution of the condition was complete after 4 weeks (Didier Pin. 2004). Scott. 1988, reported a case of yeast dermatitis in a goat very similar to the case reported by Didier Pin (2004), but the fungal cultures were negative. The diagnosis was candidiasis, based on the presence of numerous budding yeasts and pseudohyphae in direct smears and skin biopsy specimens.

2.4 *Malassezia pachydermatis* in pigs

There are scanty data on the ecology of *M. pachydermatis* in pigs. (Gustafson 1960; Guillot, Chermette and Guého. 1994). In 1959, Gustafson reported for the first time the presence of yeasts of the genus *Pityrosporum* spp. in pigs. One year later, Gustafson 1960; studied the prevalence of *Pityrosporum* yeasts from the external ear canal of 30 pigs, and isolated *Pityrosporum* spp. in 33% of the studied animals. In 1985, Kuttin and Glas, isolated *P. pachydermatis* from the external ear canal from pigs with otitis externa. The most recent study dates from 1994 and was developed by Guillot, Chermette, Guého. 1994, who investigated the occurrence of *Malassezia* spp. from 356 domestic and wild mammals. They studied a total of 40 healthy pigs and observed that 57% of the ear specimens were colonized by *M. furfur*. 
2.5 *Malassezia pachydermatis* in dogs

*Malassezia pachydermatis* is the only member of the genus that does not require lipid supplementation for development in culture medium, traditionally and it has been considered to be zoophilic, and frequently found on wild and domestic carnivores including dogs, cats, bears, pinnipeds, ferrets and foxes (Bond and Anthony.1995; Guillot, Gue’ho and Chermette. 1999; Guillot and Bond .1999; Crespo, Abarca and Cabaned es. 2002). It has been incriminated as an aetiological agent in otitis externa and different kinds of dermatitis in domestic animals especially in dogs, Guillot *et al.*, (1999). This species is more frequently isolated from dogs than from cats and appears to be a relatively infrequent pathogen in other animals, (Guillot and Gue’ho. 1999; Crespo *et al.*, 2002).

According to Nimir .1980, the increased incidence of isolation of *M.pachydermatis* from diseased dogs' skins is nearly five times its incidence of isolation from normal skin; probably indicates an abnormal proliferation of this yeast.

The pathogenic role of *M. pachydermatis* has been exhaustively investigated by Castellà *et al.*, (2005) in a Rapid amplified polymorphic DNA (RAPD) analysis on yeast isolates from different domestic animals and body sites found that the same animal could harbour more than one type of *M. pachydermatis*, and different genetic types were present in the same body site.

Several authors have mentioned the high incidence of this yeast in canine otitis externa (Abou-Gabal.1979; Batex.1976; Akerstedt and Vollset.1996) especially in chronic otitis. Although the pathogenic role of *M. pachydermatis* in otitis externa has been a matter of controversy, it was demonstrated that the yeast could induce inflammatory changes in the normal canine external ear canal in the presence of moisture (Mansfield, Boosinger, and Attleberger. 1990), and the disease was experimentally induced with *M.*
Several studies have shown that *Malassezia pachydermatis* is a member of the normal cutaneous flora of the dog; around 50% of healthy dogs are carriers for this yeast, which can be found in the external ear canal (Didier-Noël, 2002; Carter, Chengappa, Williams and Yasuko, 1995). The alterations of the cutaneous microclimate or host defense mechanisms allow *M. pachydermatis* to multiply and become pathogenic in certain canine breeds such as poodles, boxer, and silky Terries (Craig, 2006; Bond, Curtis, Lloyd, Craig and Faerquson, 1996).

Cutaneous factors enhancing the multiplication of *Malassezia pachydermatis* reported by Didier-Noël Carlotti (2002) include:

- An excessive production or a modification of sebum and/or cerumen.
- An excess of moisture.
- A rupture of the epidermal barrier (skin injury)
- Cutaneous folds.

These changes may be due to underlying causes, of which the following are most common:

- Cutaneous hypersensitivity including atopic dermatitis.
- Pyoderma.
- Ectoparasitic skin diseases.
- Endocrine disorders, particularly hypothyroidism.
- Keratinization disorders: epidermal dysplasia of the West Highland and white terrier, idiopathic seborrhoea,
- Treatment with glucocorticoids or antibiotics.
Immunological dysfunction (cell-mediated immunity, IgA secretion) could also promote growth of the *M. pachydermatis* population on the skin (Morris. 2003).

*Malassezia pachydermatis* produces many enzymes (including lipases and proteases) that can contribute to cutaneous inflammation through proteolysis, lipolysis (which alters the lipidic cutaneous film), changes of cutaneous pH, and complement activation (Durate, Lachance and Hamdan. 2002). In addition, it has been shown that *M. pachydermatis* can play an allergenic role, in about 30% of dogs with “seborrhoic dermatitis”. Skin testing with *M. pachydermatis* extract shows immediate hypersensitivity reactions (Didier-Noël Carlotti, 2002). There are higher levels of specific IgG in atopic dogs (with or without concurrent *M. pachydermatis*) than in non-atopic dogs with *M. pachydermatis* or normal dogs (Zargri, Midgly, Back, Johason and Scheynius. 2003). Dogs with atopic dermatitis and *M. pachydermatis* have a high level of specific IgE whereas atopic dogs have a low level and normal dogs have no specific IgE. (Pier, Caban~es, Chermette, Ferreiro, Guillot, Jensen, and Santurio. 2000; Morris. 1998).

Primary diseases which cause inflammation and increased sebum production provide a cutaneous microenvironment that encourages overgrowth of this yeast. *Malassezia pachydermatis* is the most common yeast that contributes to otitis externa as a perpetuating factor in dogs (Scott, Miller, and Griffin. 1995).

According to Simona, Monica, Fabrizio and Francesca Mancianti. (2007), the Interdigital spaces and external ear canal of dogs were significantly more colonized rather than the other body sites. The simultaneous colonization of right and left ear, right and left axilla, right and left groin, front and hind interdigital spaces, perineum and right groin, perineum and hind nails, , was also statistically significant. Although the
recovery of *M. pachydermatis* was higher from lesion-free ears rather than from animals with otitis, the difference was not statistically significant (Giusiane. 2006).

### 2.6 *Malassezia pachydermatis* in Human

Reports of human infections with *M. pachydermatis* have been fewer than those with *M. furfur* according to Gueho, Simmons, Pruitt, Meyer, and Ahearn. (1987); Larocco, Dorenbaum, Robinson and Pickering (1988); Mickelsen, Viano-Paulson, Stevens, and Diaz. (1988), except for a single report of *M. pachydermatis* canaliculitis in a 61-year-old man reported by Romano, Segal, and Blumenthal. (1978), the two reports of confirmed clinical cases involved infants in Neonatal Emergency Care Unit (NICU), Marcon and Powell (1992).

Larocco, Dorenbaum, Robinson, and Pickering. (1988), retrospectively reviewed their clinical microbiology laboratory records of cultures obtained from 507 infants hospitalized in their NICU from October (1985) to January (1987). They identified eight infants (1.6%) from whom *M. pachydermatis* had been recovered. Three infants had single isolates from cerebrospinal fluid, urine, or eye discharge; one infant had positive urine and ear discharge cultures; and four infants had multiple isolates, including isolates from catheter blood (four cases), peripheral blood (two cases), central line catheter tip (three cases), urine (two cases), and tracheal aspirate (one case). All infants were premature and had multiple complications of prematurity; all but the infant with the cerebrospinal fluid isolate had been receiving broad-spectrum antibiotics and parenteral lipid emulsions for 18 to 35 days through a central vascular catheter (CVC). Clinical symptoms occurred in all but the infant with the cerebrospinal fluid isolate and included apnea and/or bradycardia or temperature instability.
The four patients with fungemia recovered without antifungal therapy when their CVCs were removed. The two patients with a single positive eye culture and a single positive urine culture died from cardiac and pulmonary decompensation, respectively. Because autopsies were not performed, the role of *M. pachydermatis* in causing these deaths is speculative. Mickelson *et al.*, (1988), reported details of *M. pachydermatis* sepsis in three infants in their NICU. All infants had birth weights of <1,000 g and had numerous complications of prematurity.

Lipid emulsions had been given for 8 weeks to 5 months prior to development of symptoms, which included fever (two cases), lethargy (one case), respiratory distress (one case), and repeat bouts of bradycardia (one case). One infant developed erythema at the catheter insertion site 2 days after onset of symptoms. Chest roentgenograms were normal in all three patients. Laboratory findings included thrombocytopenia (all three infants), leukocytosis (two infants), and leukopenia (one infant). All had multiple positive blood cultures obtained from a CVC, and two infants had positive cultures of peripheral venous blood. Two infants recovered promptly after removal of the CVC and short courses of amphotericin B, while one infant recovered after a 10-day course of amphotericin B infused through the infected catheter.

Mickelson *et al.*, (1988) also reported isolating *M. pachydermatis* from an additional 30 patients over a 3-years period; 28 of the 30 patients were infants in the NICU. Clean-catch or bagged urine samples accounted for 80% of the isolates; other samples included endotracheal aspirates and swab specimens from the nares, rectum, and vagina. None of the 30 patients had symptoms or signs of illness. These data suggest that *M. pachydermatis* may colonize various anatomic sites in hospitalized patients, particularly those patients in an NICU.
A survey of yeasts received at a reference center confirmed the observation that multiple anatomic sites can be sources of *M. pachydermatis*, as it was reported by Gueho et al., (1987). Because clinical descriptions were not included in the survey, it is impossible to determine whether most isolates were causing an infection or merely colonizing the sites; however, 4 of 15 isolates were obtained from blood culture (Chang, Miller, Watkins, Arduino, Ashford, Midgley, Aguero, Pinto-Powell, Von Reyn, Edwards, McNeil and Jarvis.1998).

*Malassezia pachydermatis* has also been implicated in the pathogenesis of other superficial dermatitis, the most important ones being seborrheic dermatitis (severe and difficult to treat when associated with AIDS) Bergbrant and Faergemann. 1990, folliculitis (Back, Faergemann and Hornqvist. 1985), and atopic dermatitis. The mechanisms by which the commensal microorganisms cause these dermatoses, however, are not yet clear and several investigations failed to demonstrate any significant difference in humoral and cell-mediated immune responses specific to *Malassezia* in patients suffering from *Malassezia*-associated dermatoses ( Faergemann. 1999).

Zoonotic transfer has been documented by Daniel, Frances, and Shelley. (2005), from dogs to immunocompromised patients by healthcare workers who own dogs, although there is only one report of *M. pachydermatis* culture from normal adult skin (Bandhaya.1993). They investigated the role of pet dogs as risk factors for mechanical carriage of *M. pachydermatis* on human hands. Dogs and their owners were sampled as pairs, by fungal culture and nested polymerase chain reaction (PCR). Although fungal culture was not a reliable means by which to detect carriage of the yeast on human hands, PCR identified *M. pachydermatis* on most human participants (93%), (Daniel et al.,2005)
CHAPTER THREE
Material and Methods

3.1 Sampling procedures

One hundred and fifty bovine ear swabs were collected from apparently healthy cattle and investigated for the presence of *Malassezia pachydermatis*. Animals were three months to six years old and of both sexes (125 female, 25 male) from crossed breeds. The samples were obtained using sterile swabs to collect cerumen from the external ear canal; the cotton tip of the swab was rolled and rubbed firmly against right or left external ear canals of each animal for 10 seconds, then the collected samples were immediately transported to the laboratory for culture. Amounts of wax within the ear canal were reported as none, slight, moderate or heavy.

Microscopic examination

Swabs were rolled on a clean grease-free glass slide, fixed by gentle heating stained by Gram method and examined microscopically.

Cultivation

The 150 samples were inoculated onto Sabouraud Dextrose Agar supplemented with Chloramphenicol (final concentration 150 mg\L), Cycloheximide (final concentration 0.0 5 mg\L) as previously described (Durate *et al.*, 2004; Laura, Charles and Arnold . 1998; Kiss, Radvanyi and Szigetti. 1996) and sterile olive oil as growth factor and incubated at 35 °C, with daily inspection for growth for at least 7 days .
Samples yielding yeast growth were further investigated and identification methods were applied, including macroscopic appearance of colonies and microscopic cell morphology. *Malassezia pachydermatis* was identified by serial transfers on a lipid-free culture medium. Amounts of yeast growth were recorded as moderate (1-20), heavy (21-50) or profuse (over 50 colonies). Stock cultures were maintained on SDA slopes in universal bottles at 4°C with monthly sub culturing. (Crespo, Abarca and Cabanes, 2000).

### 3.2 Media and reagents

The following media were used in the study for isolation and maintenance of the yeast species. Commercial media were prepared according to manufacturer's recommendations.

#### 3.2.1 Corn Meal Agar

- Corn meal extract (oxoid) 0 2.00 g
- Agar 15.00 g
- Distilled water 1000 ml

**Preparation**

Seventeen gram of dehydrated medium were suspended in a liter of distilled water, brought to boiling temperature to dissolve and sterilized by autoclaving at 15 lb/sq.in. (121°C ) for 15 minutes. pH 6.0.

#### 3.2.2 Sabouraud Dextrose Agar (SDA) supplemented with Chloramphenicol and Cycloheximide

- Mycological peptone 10.00 g
- Dextrose 40.00 g
**Preparation**

Sixty five g. Of dehydrated medium were suspended in a liter of distilled water, dissolved by heating and sterilized by autoclaving at 15 lb/sq.in (121°C) for 15 minutes. Then Chloramphenicol and Cycloheximide were added in water bath (56 °C). pH. 5.6.

### 3.2.3 Yeast Nitrogen Base

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto –yeast nitrogen base (difco)</td>
<td>67.00 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.00 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation**

Yeast nitrogen base powder (67g.) were suspended in a liter of distilled water, 15 g agar were added, dissolved by heating and sterilized by autoclaving at 15 lb/sq.in (121°C) for 15 minutes. pH. 5.4.

### 3.2.4 Carbohydrate Assimilation Tests

Saturated aqueous solutions of glucose, trehalose, sorbitol, sucrose, D-galactose, inositol, lactose and maltose, were prepared and pipetted on 6×6 mm filter paper discs in Petri dishes, the excess solution was removed and the discs were dried at 56 °C. The impregnated discs were used as a carbohydrate sources in the assimilation tests.
3.2.5 Sabouraud Dextrose Agar supplemented with Chloramphenicol

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological peptone</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>40.00 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.00 g</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.50 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation**
Sixty five g. Of dehydrated medium were suspended in a litter of distilled water, dissolved by heating and sterilized by autoclaving at 15 lb/sq. in. (121°C) for 15 minutes. Then Chloramphenicol and was added in water bath (56 °C). pH 5.6.

3.2.6 Sabouraud Dextrose Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological peptone</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>40.00 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.00 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation**
Sixty five g. of dehydrated medium were suspended in a litter of distilled water, dissolved by heating and sterilized by autoclaving at 15lb/sq. in. (121°C) for 15 minutes. pH 5.6.
3.3 Identification of the yeast isolates

After primary isolation, yeast cultures were purified by streaking onto SDA containing chloramphenicol, and then single colonies were selected to obtain pure cultures. These pure cultures were used for preparation of inocula and identification which was carried out according to the criteria defined and methods described by Lodder (1979); Rose and Harrison (1987); Gueho et al., (1996); Gueho et al., (1998) and Simona et al., 2007.

3.3.1 Colony characteristics:

Yeast isolates were grown on SDA plates at 35°C for 3-4 days, colonial characteristics were noticed, the surface of the growth, the margin; appearance, consistency, smell and pigmentation were all recorded.

3.3.2 Morphology of vegetative cells:

Wet mounts of 3-4 days old pure cultures were examined microscopically to determine the shape and the type of budding of the vegetative cells.

3.3.3 Mycelium or pseudomycelium

The ability to form pseudo or true mycelium was determined by culturing on corn meal agar. Plates were inoculated from the young yeast cultures by streaking over the agar surface. A sterile cover slip was placed over a portion of the streak. Inoculated plates were incubated at 28°C for 7 days with daily examination for the presence of filamentation.
3.3.4 Growths at 37 °C and elevated temperature

Yeast isolates were tested for growth at 37°C on SDA plates held for 3-4 days. A visible growth was regarded as positive. In case of week growth the test was repeated.

When it was necessary to test for growth at temperatures above 37°C, subculture were made on SDA slants in universal bottles which were incubated in water bath adjusted to the required temperature. After 3-4 days incubation the slants were examined for the presence of growth, and doubtful results were repeated.

3.4.5 Carbohydrate Assimilation (Auxanographic) Tests

The ability of a yeast species to utilize a specific carbohydrate compound as a sole carbon source was tested by the auxanographic method using Difco yeast nitrogen base as the basal medium.

Seven carbohydrates compounds, namely glucose, trehalose, sorbitol, sucrose, D-galactose, inositol, lactose and mannitol were used in the primary tests for yeast isolate.

Inocula for the assimilation tests were prepared by making aqueous suspensions of the yeasts, from 3-4 days old culture on SDA slants in universal bottles grown at 28°C, by adding 3 ml of sterile distilled water and carefully washing the yeast growth from the agar surface. Twelve drops of the yeast suspension were then added to 20 ml of melted nitrogen yeast base agar previously cooled to 45°C in water bath, using sterile Pasteur pipette. The yeast suspension and agar were well mixed, poured into 2 sterile Petri dishes (10 inoculated medium per plate) and allowed to solidify. The carbohydrate-impregnated discs were then placed on the agar surface with sterile forceps, using 7 discs per plate, one central and 6 peripheral, with the sugar numbers marked on the back of the plate.
The inoculated plates were incubated at 28°C, with daily examination, for 7 days. A heavy yeast growth around the disc was regarded as positive. In doubtful cases, the test was repeated.

3.4.6 Germ Tube Test

0.5 ml of sterile horse serum was inoculated with small inoculum from a young culture, incubated at 37°C for 3 hours and examined for germ tube production.
CHAPTER FOUR

Results

Direct examination of 150 swabs from external ear canal of apparently healthy cattle investigated for the presence of *Malassezia pachydermatis* (figure 1) revealed the presence of the yeast in three specimens. Two specimen yielded yeast growth (1.3\%) and the isolates were identified as *Malassezia pachydermatis*. Positive Gram stains of cerumen with dominant population of yeasts were considered as a possible source of infection. There was always large amount of thick wax or macerated tissue in the external canal. Both microscopic and cultural indications of the yeast noted in these specimens were in the sparse or moderate categories.

Macroscopic morphology

Colonies on Sabouraud dextrose agar (supplemented with Chloramphenicol and Cycloheximide) were first white or creamy in colour then became buff to orange-beige, and finally (after 4-7 days) dark tan to brown; The reverse was non pigmented in young culture, then became tan to brown, colonies were round, convex and smooth to slightly wrinkled with lobate margins (figure 2). Rapid growth occurred at 35°C, reaching a maximum after 72hrs. The growth was slightly less rapid at 42°C, no growth was obtained at 45°C and growth was sparse at 25°C however at 34°C growth was good but slightly less than without antibiotics. There was good growth on corn meal agar after one week. This species is the only non-lipid dependant isolate in the *Malassezia* genus. Adequate growth occurred without addition of olive oil but growth was enhanced when olive oil was added.
Microscopic morphology

Cornmeal preparations revealed ellipsoidal cells. The daughter cells being produced from a very broad base and leaving behind distinct collarettes.

The budding occurred from one pole and from the same location (unipolar budding) (figure 3). This gives a typical shape (resembling a footprint or a peanut) (figure 4). *Malassezia pachydermatis* size is small (2 to 7 µm); does not form true or pseudohyphae (pseudomycelia); and does not form germ tube.

Assimilation of glucose, mannitol, sorbitol was observed, and all strains were characterized by no fermentation.
Figure 1: Micromorphology of *Malassezia pachydermatis* (Dr.Fungus website). For comparison.
Figure 2: *Malassezia pachydermatis* (specimen No. 103), grown at 35°C on Sabouraud Dextrose Agar
Figure 3: *Malassezia pachydermatis* from specimen No.108 showing globose to ellipsoidal cell budding from one pole at the same location. Gram stain, × 1000.
Figure 4: *Malassezia pachydermatis* in exudates from the external ear canal of cattle (specimen No. 103) with “peanut shape”. Gram stain, × 1000.
CHAPTER FIVE

Discussion

The habitat of Malassezia pachydermatis is not completely known and the yeast survives for only short periods in the environment (Gabal, 1988). However, it is known that the yeast is part of the mucosa and skin flora of mammals; colonized stratum corneum in low numbers, and has been frequently isolated from the auditory canal and skin of healthy dogs and cats (Bond et al., 1996; Coutinho, 2006) and from other domestic and wild animals (Guillot et al., 1994; Kuttin and Müller, 1994; Gue´ho et al., 1998, Guillot and Bond, 1999). Malassezia pachydermatis is non-lipid-dependent species, which can take advantage of the short-chain fatty acids present in basic mycological media such as Sabouraud glucose agar (Ahearn and Simmons, 1998; Sloof, 1970; Yarrow and Ahearn, 1984). However, the original isolation of the yeast was made by Weidman (1925) from the inflamed skin of an Indian rhinoceros (Rhinoceros unicornis) and he was able to culture his organism on media (Malt agar) apparently without addition of fatty acids.

In the present study, the incidence of Malassezia pachydermatis in the external auditory canals of apparently healthy cattle was found to be 1.3 %, and appears to be lower than that reported by Guillot et al., (1994) and findings of Duarte et al., (2003) who reported the occurrence of the yeast in (1.56) % of the external canal of healthy cattle. Also the presence of yeast growth in this study appears to be lower than the findings of Dufait. (1985), who reported the occurrence of yeast in 4% of the external canal of cattle, and the findings of Duarte et al., (2002), in Minas Gerais, Brazil; reported the occurrence of the yeast in (2.09) % of animals.
Although *M. pachydermatis* is a commensal microorganism, some conditions may lead to its exacerbated development, causing disease. Predisposing factors and an imbalance between microorganism populations are fundamental for the multiplication of *Malassezia* spp. (Bond *et al.*, 1996). The high isolation rates of these organisms could be explained by factors such as humidity, breed, age, pendulous ears, season, intercurrent diseases (Duarte *et al.*, 2002; Machado, Appelt, Ferreiro and Guillot. 2003).

*Malassezia pachydermatis* was observed microscopically in three specimens and confirmed by cultivation of two specimens. The absence of this yeast in the culture from the 3rd sample is considered false negative; this may be explained by the presence of non vital yeast cells or they were inhibited or incapable of growth in cultivation medium by the presence of contamination.

The relatively low prevalence of *Malassezia pachydermatis* in this study may be explained by anatomical characteristics of the external ear. Ears in these breeds are short and horizontal, this open horizontal anatomy could promote the circulation of the air and increase variation of humidity and temperature (*Malassezia pachydermatis* does not tolerate temperature higher than 38°C) corresponding to environmental changes that endanger the survival of *Malassezia pachydermatis*.

Thus, judging by the results obtained in this study, it appears that the *Malassezia pachydermatis* may be considered as a possible source of infection in the auditory canal of cattle; also these surveys seem to confirm the role of *Malassezia pachydermatis*, as an important fungal agent of the external mycotic otitis in cattle.

Under normal conditions, yeasts species can be sporadically isolated from the ear but always in few numbers whereas in chronic situations some yeast species, particularly *M.pachydermatis*, are predominantly found, and its excessive population can lead to inflammation. Moreover *M.pachydermatis*
could produce virulent factors such as chondroitin-sulphatase, hyaluronidase, phospholipase and proteinase, thus resulting in increased inflammation reaction in external ears (Duarte et al., 2003).

Although infection by *M. pachydermatis* represents an important disease in clinical practice involving small animals, especially dogs, the virulence and pathogenesis of the diseases caused by this yeast are still unknown (Guillot and Bond, 1999). The production of enzymes by microorganisms has been related to their pathogenicity. In this respect, the production and secretion of exoenzymes have been established as the main factor of virulence for some bacteria and yeasts (Schaechter et al., 1993). Based on observations made on yeasts of the genus *Candida*, Coutinho and Paula. (2000) demonstrated that samples of *M. pachydermatis* isolated from the external auditory canal and skin of dogs with malasseziosis are also able to produce these enzymes. However, the authors did not perform any experiment to determine the relationship between enzyme production and pathogenicity (Coutinho. 2005).

In addition, members of the genus *Malassezia* degrade substrates, principally the lipids present in cerumen facilitating the nutrition to nematodes as well as bacteria present at the site of the infection, and that may be stimulating the over production of the cerumen and therefore increasing the amount of over all nutrients. Virulent factors, presence of the antigen, the environmental temperature and humidity may also constitute a stimulus for increased secretions and inflammation.
Conclusions and Recommendations

Conclusions

From the results obtained in this study, it appears that

- Two samples yielded yeast growth representing 1.3% of the total samples. These isolates were identified as *Malassezia pachydermatis*

- *Malassezia pachydermatis* may be considered as a possible source of infection in the auditory canal of cattle; also these surveys seem to confirm the role of *Malassezia pachydermatis*, as an important fungal agent of the external mycotic otitis in cattle.

- Positive Gram stains of cerumen with dominant population of yeasts were considered as a possible source of infection.

- High occurrence of positive culture was observed in adults than calves.

- High occurrence of positive culture was observed in females rather than in males; hormonal differences could be responsible for the higher prevalence of the yeast in females studied.
Recommendations

Based on the results of study the following is recommended

- Further works should focus on Zebu and other breeds to estimate the epidemiology of *Malassezia pachydermatis*; to allow comparisons of the results obtained from various studies.
- Further works should focus on seasons are necessary to evaluate the occurrence of *Malassezia pachydermatis* in cattle during different seasons.


Weidman, F.W (1925). Exofaliative dermatitis in Indian rhinoceros with a
description of a new yeast species. (Cited by Salkin, I. F.,


ATaxonomic Study, Third ed. ( NJW Kreger-van Rji ed.),


Zargari, A., Midgley, G., Back, O., Johansson, S.G. and Scheynius, A.
(2003). IgE-reactivity to seven Malassezia species. Allergy
58:306–311.