Comparative Enzyme Studies of *Microsporum canis* and *Microsporum cookei* in Relation to their Pathogenicity and Diversity.

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ABSTRACT

Infections by dermatophytes can be contracted from animals, humans, soil or contaminated fomites. In the genus *Microsporum*, some species e.g. *M. canis* are commonly associated with cats and dogs which act as an important reservoir for human infections. Others, e.g. *M. cookei* are nonpathogenic and found in the soil. The present studies have investigated the incidence of these ecologically contrasting species on cats, dogs and in the soil, their enzyme expression, and enzyme types as identified by proteinase inhibitors, gelatin/SDS-PAGE and multilocus enzyme electrophoresis, and have led to an investigation of their phenotypic variation. The primary aim was to attempt to detect differences in enzyme production which might be related to mechanisms of pathogenicity of *M. canis*.

Isolation procedures employed were the hairbrush technique for small animals and the keratin-baiting technique for soil with samples being cultured on SDA containing antibiotics. Soil samples revealed 19 fungal genera, three being of keratinolytic fungi, representing 50% of total isolations. *Trichophyton* species were the most common (39% samples) but *M. cookei* was isolated from 6.8%.

Fungi isolated from cats and dogs represented 20 genera, with the predominant isolates being keratinolytic fungi (51.9% of total samples). Cats were the major carriers of keratinolytic fungi (*Chrysosporium*, *Microsporum* and *Trichophyton*). *M. canis* was frequently isolated (18.5% of cats) and its distribution had a seasonal variation, with a peak appearing in May-June.

All isolates of *M. canis* were of the "-" mating type. *M. cookei* isolates were of both the "+" and "-" mating types, but "+" types were predominant.

Biochemical assays showed that *M. canis* produced higher proteinase and keratinase activities in shake cultures than in stationary cultures. Elastase activity was greater in stationary cultures. *M. cookei*'s proteinase and keratinase activities were lower but again greater in shake cultures. There was no detectable keratinase activity in stationary cultures of *M. cookei*, and no significant difference in elastinolytic activity

between shake and stationary cultures. Growth in shake culture produces the "pseudo-parasitic" morphology which mimics that found in infection, therefore, the differing enzyme expression of the two *Microsporum* species may be a reflection of their differing ecological roles.

Characterisation of the enzymes with chemical inhibitors revealed that *M. canis* and *M. cookei* produced serine proteinases, but only *M. canis* produced cysteine and probably aspartic and metallo-proteinases. The serine and cysteine proteinases are considered likely to be of particular significance in the pathogenesis of *M. canis* infections.

Using substrate copolymerised gel electrophoresis (gelatin\SDS-PAGE), shake and stationary cultures were again compared for enzyme expression. Among the six different M_r proteinases (122 KDa, 64 KDa, 62 KDa, 44 KDa, 36 KDa, and 28 KDa) expressed by *M. canis*, three (122 KDa, 62 KDa and 28 KDa) were found to be more highly expressed in shake cultures. In contrast, *M. cookei* isolates expressed seven different proteinases (67 KDa, 64 KDa, 63 KDa, 62 KDa, 54 KDa, 52 KDa, and 42 KDa), of which two (67 KDa, 64 KDa) were expressed only in stationary cultures and one (52 KDa) although expressed in shake cultures was more highly expressed in stationary cultures. Possibly the high and low M_r proteinases expressed by *M. canis* are more important in its pathogenicity than the middle range proteinases also detected in *M. cookei*.

Multilocus enzyme electrophoresis using starch gels and examining eight enzymes, showed *M. canis* to be phenotypically more diverse than *M. cookei* as measured by the normalised Shannon-Wiener diversity statistic. *M. canis* showed a substantial within population variability (84.9%) by geographical region, with a moderate level (21.7%) of interpopulation differentiation. Cluster analysis confirmed this diversity and also revealed a possible grouping of isolates from clinical infections, and based on the accumulated data of these studies, EST phenotype 9 although present in a few carrier isolates was commonly associated with isolates from clinical cases and perhaps deserves further investigation.

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TABLE OF CONTENTS

TABLE OF CONTENTS Contd.

Page

TABLE OF CONTENTS Contd.

Page

TABLE OF CONTENTS Contd.

Page

TABLE OF CONTENTS Contd.

Page

TABLE OF CONTENTS Contd.

Page

LIST OF TABLES

Page

LIST OF TABLES Contd.

Page

LIST OF TABLES Contd.

Page

LIST OF TABLES Contd.

Page

LIST OF FIGURES.

Page

LIST OF FIGURES Contd.

Page

LIST OF FIGURES Contd.

Page

LIST OF FIGURES Contd.

Page

LIST OF FIGURES Contd.

Page

LIST OF PLATES

LIST OF ABBREVIATIONS

3A9EC = 3-amino-9-ethylcarbazole
BPB = Bromophenol blue
CAT = Catalase
DDW = Double distilled water
DFM = Dimethyl formamide
DW = Distilled water
E-64 = L-trans-epoxysuccinyl leucylamido (4-guanidino)-butane
EC = Enzyme Commission
EST = Esterase
Fast blue B = o-dianisidine dihydrochloride
G6P = Glucose-6-phosphate dehydrogenase
G6PDH = Glucose-6-phosphate dehydrogenase
GPI = Glucose-6-phosphate isomerase
IAA = Iodoacetic acid
ITM = Prince Leopold Institute for Tropical Medicine
IUBNC = International Union of Biochemistry
LAP = Leucine aminopeptidase
MDH = Malate Dehydrogenase
M_r = Molecular weight
MTT = Methyl thiazolyl tetrazolium
NAD = β-Nicotinamide adenine dinucleotide
NADP = Nicotinamide adenine dinucleotide phosphate
NBT = Nitro blue tetrazolium
PAGE = Polyacrylamide gel electrophoresis
*p*CMB = *p*-Chloromecuric acid
α_1-P = α-1-proteinase
PEPS = Pepstatin
PEP = Peptidase
PER = Peroxidase
PMS = Phenazine methosulphate
PMSF = phenylmethylsulfonyl fluoride

LIST OF ABBREVIATIONS Contd.

PT = 1,10-phenanthroline
SDS = Sodium dodecyl sulfate
SDW = Sterile distilled water
SGE = Starch gel electrophoresis
SPCA = Society for the Prevention of Cruelty to Animals
TCA = Trichloroacetic acid
TEMED = Tetramethyl ethylenediamine
TRIS = Tris[hydroxymethyl]aminomethane

PREFACE

Medical mycology can be said to have originated with the demonstration by Agostino Bassi in 1835 of the relationship between a disease of silkworms known as muscardine and its causal agent, a fungus, *Beauvaria bassiana* (Utz, 1981). This disease had threatened to destroy the silk industry in France and Italy (Ajello, 1977). But after Bassi's discoveries, other early work was concentrated on the superficial fungal diseases of man.

Robert Remak in 1837 observed spores and hyphae in crusts recovered from a child suffering from favus and later published accounts of the fungus in hair shafts obtained from the patient (Howard, 1983). He also successfully reproduced the disease by self-inoculation. In 1845, he cultivated and named the aetiological agent, *Achorion schöenleinii*, during his work in his mentor Professor Schöenlein's clinic. The fungus is now more commonly known as *Trichophyton schöenleinii*.

Although Remak was the first to associate a microorganism with human disease, the studies of David Gruby made a greater contribution to medical mycology (Ajello, 1974; Howard, 1983). Between 1841 and 1844, Gruby released several publications which described the main pathogens causing ringworm and also independently described the fungal nature of favus (Wilson and Plunkett, 1974). In 1843, he published an account of scalp ringworm caused by a fungus which he named *Microsporum audouini* (on the basis of the *in vivo* growth pattern), in honour of a colleague, Victor Audouin. His work also included studies of endothrix trichophytosis and thrush, a fungal disease of the mouth caused by *Candida albicans*.

Another who contributed a great deal to the development of our knowledge of the ringworm fungi or dermatophytes was Raymond Sabouraud. Sabouraud, in 1892 started issuing numerous reports which culminated in the publication of "Les Teignes" (Sabouraud, 1910). Sabouraud was able, through the techniques of pure culture, which he introduced into medical mycology, to demonstrate the plurality of dermatophytes. He placed these fungi into four genera: *Achorion*,

Epidermophyton, *Microsporum* and *Trichophyton* (Sabouraud, 1910, cited by Ajello, 1968; Seelinger, 1988).

Sabouraud had realised the complex manner in which these fungi grow in culture, with successive cultures taken from the parent stock often showing variation. This capacity for variation can render dermatophytes very difficult to identify and resulted in great difficulties in devising a uniform, internationally accepted classification (Ajello, 1962).

The following years saw incomplete and inaccurate reporting because diagnosis was not based on sound mycological techniques but on small variations in clinical appearance of lesions or slight differences in colonial morphology. The natural history of the fungus was unknown or ignored (Wilson and Plunkett, 1974; Howard, 1983). Due to numerous misleading reports, several hundred "new species" were described and named as human pathogens. Dodge (1935) in his monograph even described 118 dermatophyte species, placed in 9 genera (Baxter and Rush-Munro, 1980b). This confusion, which hindered clinicians in classifying human disease on a mycological basis, forced them to adopt a clinical-anatomical or topographical categorisation.

In 1934, Emmons developed and outlined in extensive detail a strict botanical classification based on accepted rules of nomenclature and using fungal morphology *in vitro*, which avoided classification systems based on clinical appearance. He placed the dermatophyte species into three genera, *Epidermophyton*, *Microsporum* and *Trichophyton*, embracing 18 species (Emmons, 1934). This was generally well accepted by mycologists and clinicians alike (Ainsworth, 1986).

The dermatophytes can be included in an ecological group known as the keratinophilic fungi i.e. fungi with an affinity for keratin. Such fungi may merely use keratin as a surface for growth. In other cases simple mechanical penetration of the substrate may be achieved. But some including the dermatophytes and a number of dermatophyte-like fungi have a marked ability to enzymatically digest keratin and can be termed keratinolytic fungi. The keratinolytic fungi comprise the dermatophytes and certain other fungi such as *Chrysosporium* spp..

The use of Vanbreuseghem's (1952) hair-baiting technique has enabled the detection and isolation of soil-borne (geophilic) non-pathogenic, keratinolytic fungi. Notable amongst these are *Trichophyton (Keratinomyces) ajelloi* (Vanbreuseghem, 1952) Ajello, 1968, *T. terrestre* Durie and Frey, 1957 and *M. cookei* Ajello, 1959. All these geophilic species, with regard to morphology, sexual behaviour (Campbell, 1988) and antigenicity (Mackenzie, 1988) are dermatophytic. The only factor differentiating them from true dermatophytes is their inability to cause disease in man and animals.

The dermatophytes in the broadest sense can be divided into three ecological groups, zoophiles (mammalian and avian hosts); anthropophiles (human hosts) and geophiles, which for the most part degrade keratinous material, e.g. skin, hair, hooves, horns, feathers, in the soil. Of the zoophiles at least ten species are recognised, three of which are of real importance to man, namely; *Microsporum canis*, *Trichophyton mentagrophytes* (and its varieties) and *T. verrucosum.* Of the three, *Microsporum canis* is the most important epizoonotic fungal pathogen, causing a severe public health problem on a world-wide scale.

Besides its impact on human health, there are also social and economic considerations as a result of its infections. For example, ringworm of the scalp is (wrongly) considered to be a social stigma (English, 1972). In New Zealand, the most susceptible age of infection is up to 15 years but the impact of loss of school days on school-going children has not been documented (Mycoses Newsletter, CDCNZ, 1992). In addition to human suffering, the economic cost of medical consultation and drugs for treatment is not known.

M. canis is responsible for a polymorphism of scalp and skin infections in both humans and animals. Its ability to produce enzymes has been implicated in the pathogenesis of skin infections of the host organisms, in countering host defense mechanisms and for providing its nutritional needs. It has been suggested that extracellular enzymes involved in pathogenesis include keratinases, proteinases, elastases, peptidases, aminopeptidases, catalases and peroxidases (Ernst, 1989).

In contrast to *M. canis*, the soil inhabiting dermatophyte, *M. cookei* is of little medical importance. It is morphologically rather similar to *M. canis*

and also has a world-wide distribution. Although it may be isolated from animals, clinical disease is not observed (Rees, 1967). Even though tinea corporis caused by *M. cookei* has been reported in humans (Frey, 1971), the fungus must be considered a non-pathogen.

Accepted therapeutic measures alone do not appear to have altered materially the frequency and course of *M. canis* infection in most communities. An increase in our knowledge of the biological and biochemical properties of a pathogen compared to a nonpathogen could suggest ways of controlling and treating infections. The relatively small number of effective antifungal agents reflects to a large extent the fact that many aspects of fungal physiology and virulence are not well understood (Ernst, 1989). The determination of relative sizes and numbers of, for example, proteinases expressed could suggest possible mechanisms of pathogenicity involving certain of the enzymes and therefore ways of making drugs and/or vaccines for controlling dermatophyte pathogens.

The main purpose of the work to be reported in this thesis was to study and compare aspects of the ecology and biochemical variability of *M. canis*, a pathogen and *M. cookei*, a nonpathogen and to investigate certain enzymes produced by these fungi e.g. keratinases, proteinases and elastases which have been implicated in the pathogenesis of disease. Furthermore, genetic studies of these species are practically non existent. The genetics of the group is not well characterised and there is a lack of suitable methods and scoreable markers for assaying variability in natural populations. Thus an investigation of enzyme marker systems, especially for enzymes implicated in pathogenicity, could be useful in determining phenotypic or genetic relationships among strains.