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Evaluation of Enzymatic Activity of *Microsporum canis* Isolates from Pet Cats by API-ZYM Test

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ABSTRACT

Background: *Microsporum canis* is one of the most common causes of zoonotic dermatophytosis. This study aimed to isolate *M. canis* from suspected cases of dermatophytosis in pet cats and evaluate the enzymatic activities.

Methods: (147) hair and skin samples were collected from pet cats suspected with dermatophytosis. Traditional laboratory methods were used for initial fungal isolation and identification, Confirmed by Conventional polymerase chain reaction. API ZYM test is used to determine the activity of enzymes secreted by *M.canis* isolated which have an important role in the pathogenicity of infection.

Result: *Microsporum canis* was identified in 30/174 (17.2%) of the samples based on conventional mycological approaches and they show positive results by PCR. (30) isolates of *M. canis* were used for detection of their enzymatic activities by API ZYM test. The enzymatic activity profile revealed the highest activities for lipase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase and esterase lipase (C8), each showed the activity percentage in 100%. (24) isolates (80%) with high activity of acid phosphatase, (6) isolates (3.3%) with no activity, followed by valine arylamidase in which five isolates (16.6%) produce this enzyme strongly while (25) isolates (83.3%) produced it moderately. Only one isolate (3.3) with high activity of cystine arylamidase and 29 isolates (96.6%) showed moderate activity. Twenty four isolates (80%) with high activity of acid phosphatase, 5 isolates (16.6%) with moderate activity and one isolate (3.3%) with no activity, α-glucosidase produced in 28 isolates (93.3) with strong activity and two isolates (6.6%) produce this enzyme moderately. It has become necessary to enhance the results of fungal identification by molecular methods like PCR, accompanied by measuring enzymes which considered as an important virulence factor of *M.canis*, contributing it is severity of the infection.

Key words: API ZYM test, Enzymatic activity, *Microsporum canis*, PCR.

INTRODUCTION

Microsporum canis is a crucial filamentous dermatophyte species because it can generate cutaneous zoonosis lesions, frequently in domestic animals and particularly cats (Chupia *et al*., 2022). The diagnosis of dermatophytosis based on clinical findings is unreliable, not just because the dermatological findings are inconsistent (Neves *et al*., 2018), but also because a number of different skin conditions might resemble the characteristic fungal lesion (circular lesions with alopecia) (Pasquetti *et al*., 2017). To identify dermatophytes, several morphological traits and presence of microconidia may be satisfying (Fawzi *et al*., 2023). Diagnosis of *M. canis* by PCR test is important since, detection and species identification is relevant to the choice of treatment and to understand a probable source of infection also it may supply data for epidemiological studies (Sattasathuchana *et al*., 2020; De Meb *et al*., 2022). The enzymatic activity of dermatophytes isolated from companion animals has not been thoroughly studied. However, the enzymatic and antifungal characteristics of dermatophytes are significant contributors to infections in both humans and animals (Aneke *et al*., 2021). Also, the mechanism of infection by pathogenic fungi may vary in severity depending on the host (Pratibha *et al*., 2022).

The role of enzymes is essential in the pathogenesis and survival strategies of dermatophytes, produce keratinases to degrade keratin, a tough protein found in the outer layer of skin, hair and nails (Cafarchia *et al*., 2010). Elastases break down elastin, another protein component of skin and connective tissues, DNases hydrolyze DNA, which can be released from damaged host cells during infection, Collagenases break down collagen, a major structural protein in connective tissues and Lipases hydrolyze lipids (Viani *et al*., 2007). These enzymes are not only important for the virulence and pathogenicity of dermatophytes but also serve as targets for potential antifungal therapies. Understanding their biochemical and molecular characteristics helps in developing strategies

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to combat dermatophyte infections effectively (Alhasan *et al*., 2022).

API ZYM is an efficient enzyme testing method. Its applicability in filamentous fungal identification has been proven (Mohammed *et al*., 2020). Secreted hydrolytic enzymes can aid in the invasion of host tissue, enhancing adhesion by breaking down host surface chemicals or breaking down host immune system cells and molecules to fend off or resist antimicrobial action (Al-abedi *et al*., 2020); Putriningshi *et al*., 2017). The aim of the present study is to identify isolated *M.canis* from suspected dermatophytosis in pet cats confirming it by PCR and evaluate the ferocity of the isolates by studying their enzymatic activities using set hydrolases API ZYM test.

MATERIALS AND METHODS

Ethical approval

All samples were collected from housed cats after getting approval from their owner; euthanized ethically, according to the Animal Welfare Committee in Mosul University- College of Veterinary Medicine No. UM.VET.2023.118 in 23-11-2023.

Sample collection

One hundred and forty-seven hair and skin sample from pet cats with typical clinical signs for dermatophytosis and positive green fluorescence on UV light exposure were collected during the period between December 2023 to March 2024 in Mosul/ Iraq. Wood's lamp examination (Fig 1), all samples are collected by mechanical debridement method (skin scrapping) (Sudad *et al*., 2011) and transported in sterile conditions to the Veterinary Mycological lab. In Mosul university, Where the research is conducted.

Mycological examination

The samples were treated with KOH 10% for 10 min, they viewed under a microscope to note fungal hyphy, then cultured on Sabouraud dextrose agar (SDA) with cycloheximide 0.5 g/ml and chloramphenicol 0.05 g/ml, incubated at 25-30°C for up to 4 weeks (Alhasan *et al*., 2022). The isolated *Microsporum canis* that showed a positive result macroscopically for growth characteristics (roughly wooly colonies with feathery texture) were picked and cultured again on SDA 7days at 3°C. for more purification. The isolates were identified to species level according to the microbiological appearance of conidia.

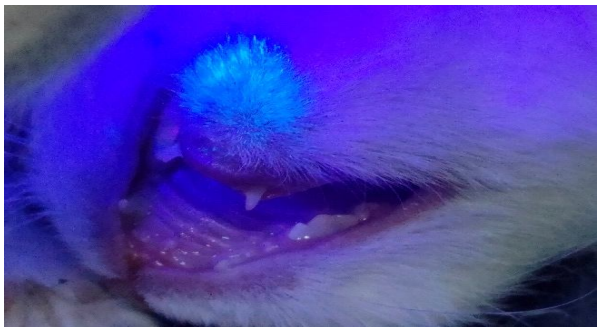

Fig 1: Wood's lamp positive hairs and signs of dermatophytosis lesion in a kitten infected with *Microsporum canis*.

Molecular identification by polymerase chain reaction (PCR)

The identification of suspected *M. canis* isolates was confirmed by amplifying a segment of internal transcribed spacer (ITS) 1 and 4 universal primers with a size of 550 bp for PCR amplicons. *M. canis* isolates were maintained at 4°C for subsequent experiments, The genomic DNA was extracted using Zymo Research DNA MiniPrep kit /USA (Cat No: D6005). ITS Region was detected by conventional PCR Using the forward and reverse primers targeting ITS1 and ITS2 genes provided from IDT Company, Canada (Table 1) with 550 bp amplicon size (Hamied *et al*., 2024).

The rapid fungal DNA extraction and gene detection were performed as described by (Chupia *et al*.,2022). The gene amplification was performed using an Applied Biosystems thermocycler with the following amplification conditions: 95°C for 5 min (initial denaturation) and 35 thermocycler (denaturation for 30 s at 95°C, annealing for 30 s at 52°C for *ITS universal gene* and extension for 1 min at 72°C) followed by final extension for 7 min at 72°C. The PCR product (5 µl) was electrophoresed against 3 µl of DNA marker (100 pb, Promega, USA) for 60 min at 100 volts using 2% agarose gel supplemented with ethidium bromide (0.5 µg/mL), then DNA bands were visualised with a UV illuminator system (Fisher Scientific, UK).

Detection of hydrolytic enzymes by API ZYM Kit

API ZYM test is utilized to profile the enzymatic activities of *M. canis* isolates (BioMerieux, France) API ZYM test strips containing various substrates specific for 19 hydrolytic enzymes evaluating the virulence of the isolated dermatophytes. The suspension of fungal material with 2 ml of distilled water prepared and its turbidity was adjusted to match McFarland tube No. 5, (65) µl of the specimen suspension was dispensed into each capule of the API ZYM test strip. The inoculated API ZYM strips were covered with a plastic lid to prevent exposure to bright light, after incubation (4 hours at 37°C), a drop of ZYM A (tensioactive agent) and ZYM B (diazonium salt) reagents added into each cupule of the API ZYM strip, The inoculated strips were briefly exposed to a powerful light for ten seconds. The color of reactions in each cupule was compared with

Table 1: Sequence of universal primer ITS1 and ITS4.

Gene	Primer	Sequence	Sequence	GC (%)	Size of amplicon
ITS1	Forward	5′ - TCCGTAGGTGAACCTGCGG -3′	60.3	50%	550 bp
ITS2	Reverse	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41%	

standard chart provided (Wawron *et al*., 2011; Hawrra *et al*., 2019).

Scoring

Each enzyme reaction was scored on a scale of 0 to 5: 0: Negative reaction (no color change). 1-4: Intermediate reactions of increasing intensity. 5: Maximum intensity reaction (17).

RESULTS AND DISCUSSION

Identification of isolated *Microsporum canis*

From 174 cat hair and skin samples, 30 of *Microsporum canis* (17.2%, 30/174) were isolated and identified based on the cultural characteristics depending on laboratory process described by (Matloob *et al*., 2019) ,and microscopic examination as shown in (Fig 2) A,these findings were enhanced by (Murmu *et al*., 2017) which include the infected pet cats by dermatophytes as the most predominant host (55.5%) while the most isolated species was *M.canis*.

Molecular identification by amplifying a segment of *ITS universal gene* using specific primers for *Microsporum canis* revealed a single clear amplified DNA band with 550 bp fragment size, as depicted in Fig 3.

Currently, most human infections are caused by microorganisms transmitted from animals, for many reasons, including the increase in the ownership of pets. (Demirbilek *et al*., 2022). Our results relating with the prevalence of dermatophytosis in pet cats showed similarities with percentages of infection caused by *M. canis* of some epidemiological researches reported in other areas like Southern Brazil (10.2%) and Thailand (20.5%) (Copetti *et al*., 2006), also Like many studies conducted on dermatophytes, *M.canis* species was the most prevalent (60.0%) among the other isolated species like *M. gypseum, T. mentagrophytes and T. rubrum* from pet cats and dogs (Murmu *et al*., 2016). While the frequency of *M. canis* was lower than those reported in Iraq (71.6%) (Sattasathuchana *et al*., 2020) which was (27.5%), also in Kurdistan of Iraq (44.36), this variation may be due to the difference in climatic, environmental and economic conditions between different regions in the same country which effect the growth of the pathogenic fungi.

In Brazil similar results found in which the frequency of *Microsporum canis* was (76.9%) (Mohammad and Yassien, 2022. Determining the molecular epidemiology of dermatophytes in pets will be greatly aided by PCR-based detection (Maharana *et al*., 2019).Accurate and clear results were

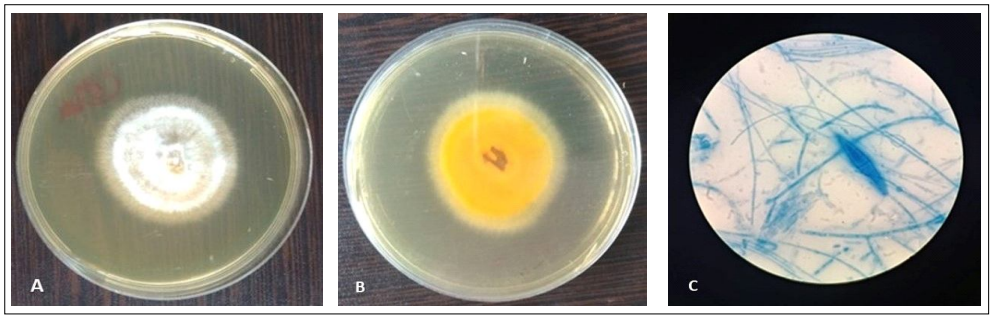

Fig 2: A) *M. canis* colonies, fuzzy to cottony texture and flat to slightly grooved, on the front color varies from white to yellow. B) The backside is orange to deep yellow. C) Microscopic appearance with Lactophenol cotton blue staining confirmed presence of round-shaped microconidia and spindle-shaped, rough, thick-walled macroconidia (400×).

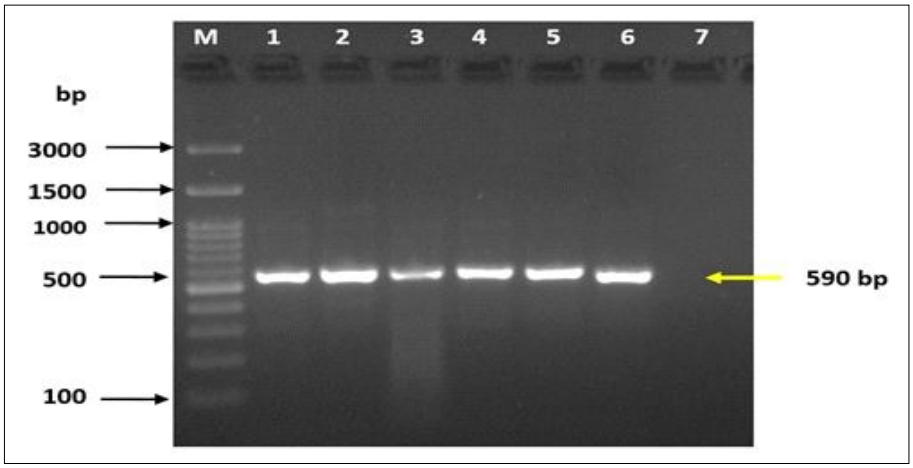

Fig 3: Agarose gel electrophoreses, Lane M=DNA marker 100 bp Ladder, Lanes 1-2-4-5-6= Amplicons for *M.canis* suspected samples, Lane 3=amplicon of *M.canis* negative sample, Lane 6= positive control, Lane 7=negative control.

obtained with PCR for detecting *Microsporum canis*, which gives confirmatory results and enhances traditional methods. PCR is used in this research to support the mycological methods for isolation, thus they were identical. (Chupia *et al*., 2022).

API- ZYM enzymatic activities

Thirty Microsporum canis isolates were used to assess the activity of 19 hydrolases using the API ZYM test. Table 2 list these hydrolases along with naphthol-AS-BI-phosphohydrolase, acid phosphatase, α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The enzymatic activity was measured by observing the color reaction after 5 minutes, with the intensity of the reaction corresponding to nanomoles of hydrolyzed substrate on a 5-point scale: zero (no reaction), 1-5 nanomoles, 2-10 nanomoles, 3-20 nanomoles, 4-30 nanomoles and 5-40 nanomoles as shown in (Fig 4).

The enzymatic activity profile revealed the highest activities for lipase, arylamidase, leucine, naphthol-AS-BI-phosphohydrolase and esterase lipase (C8) each showing the percentage was 100% activity. Twenty four isolates (80%) with high activity of Acid phosphatase with 6 isolates (3.3%) with no activity, followed by Valine arylamidase in which 5 isolates (16.6) produce this enzyme strongly while 25 isolates (83.3%) produced it moderately. One isolates (3.3) with high activity of Cystine arylamidase and 29 (96.6) isolates with moderate activity. Twenty four

Table 2: Evulation of enzymatic activity of *Microsporum canis* (N=30).

Enzyme	Strong activity		Moderate activity		Negative activity	
	No isolates	%	No isolates	%	No isolates	%
Control	-	-	-	-	-	-
Alkaline phosphatase	24	80	-	-	6	20
Esterase (C4)	21	70	2	6.6	7	23.3
Esterase lipase (C8)	30	100	-	-	-	-
Lipase (C14)	30	100	-	-	-	-
Leucine arylamidase	30	100	-	-	-	-
Valine arylamidase	5	16.6	25	83.3	-	-
Cystine arylamidase	1	3.3	-	-	29	96.6
Trypsin	-	-	-	-	-	-
α-chymotrypsin	-	-	-	-	-	-
Acid phosphatase	24	80	5	16.6	1	3.3
Naphthol-AS-BI-phosphohydrolase	30	100	-	-	-	-
α-galactosidase	-	-	-	-	-	-
β-galactosidase	-	-	-	-	-	-
β-glucuronidase	-	-	-	-	-	-
α-glucosidase	28	93.3	2	6.6	-	-
β-glucosidase	-	-	-	-	-	-
N-acetylo-β-glucosyloaminidase	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-

Fig 4: API- ZYM enzymatic activities.

isolates (80%) with high activity of Acid phosphatase and only 5 isolates (16.6%) with moderate activity and 1 isolates (3.3%) with no activity, In case of α-glucosidase 28 isolates (93.3) produce this enzyme with strongly activity and only 2 isolates (6.6%) produce this enzyme moderately.

The observed geographical variations in species distribution discrepancies may stem from several factors, including differences in sample sizes across regions or variations in diagnostic methodologies employed (Jarjees and Issa, 2022). Factors such as environmental conditions, regional veterinary practices and the genetic diversity of local animal populations could also contribute to these discrepancies (Minnat *et al*., 2019). Thus, understanding these nuances is crucial for accurate epidemiological assessments and effective management strategies for fungal infections in animal populations.

Hydrolases API ZYM test showed the activity of enzymes for lipase, arylamidase, leucine, naphthol-AS-BI-phosphohy drolase and esterase lipase (C8) each showed the percentage as 100% activity in all tested *M canis* isolates. Twenty four isolates (80 %) with high activity of Acid phosphatase in six isolates (3.3%) with no activity, followed by Valine arylamidase in 5 isolates (16.6) produce this enzyme strongly while 25 isolates (83.3%) produced it moderately. One isolate (3.3%) with high activity of Cystine arylamidase and 29 (96.6%) isolates with moderate activity. These results were parallel to Hawraa *et al*.,2019. WhileTwenty four isolates (80 %) show high activity of Acid phosphatase and only 5 isolates (16.6%) with moderate activity and one isolates (3.3%) with no activity, In case of α-glucosidase 28 isolates (93.3) produce this enzyme with strongly activity and only two isolates (6.6%) produce this enzyme moderately this may indicate the ferocity of the isolates and the difficulty of controlling it therapeutically.

The current study found differences in enzymatic activity compared to what was reported in the study referenced as (Papini and Mancianti, 1995), who noticed patterns of extracellular enzymatic activity 70 feline *M. canis* isolates for all enzymatic activity, significant intensity fluctuations were found. There was activity for leucine arylamidase in 35 samples (50%) and esterase lipase (C8) in 31 samples (44%), out of 57 samples (81%). Seven (10%) of the samples had valine and cystine arylamidases, while 64 (91%) had acid phosphatase, 60 (86%), alpha-galactosidase in 5, 7%, beta-galactosidase in 6, 8%, alpha-glucosidase in 25, 36%, N-acetyl-beta-glucosaminidase in 41 (58%) and alpha-mannosidase in 51 (73%), were found to have naphthol-AS-BI-phosphohydrolase. The discrepancy between this study and others could be attributed to geographic variances that impact species distribution and, in turn, their capacity to produce enzymes. Studying the pathogens of dermatophytes opens up the horizons for finding alternative treatment solutions, which helps to limit the spread of these transmissible diseases (Nanavare *et al*., 2024).

Dermatophytes cause superficial infections by penetrating the keratinous layer of the epidermis and through hairs and nails. Proteases and lipases are produced by dermatophytes, aiding in their invasion of the host's tissues. The lipid and protein components of lipid cell membranes are structurally disturbed by the enzymes, which impairs or completely destroys the membranes' ability to function (Kobierzycka and Cisto, 2005; Yassein and Zghair, 2020). The interplay of virulence factors which is sometimes represented by a number of enzymes in fungal pathogens plays an important role in pathogenicity and severity of infection . It may also cause transformation of saprophytic fungal agents into life-threatening pathogens.

CONCLUSION

The rates of *M.canis* isolation within skin lesions among pet cats in Vet clinics were determined using traditional and molecular methods which have proven effective in dealing with and diagnosing transmissible pathogens such as dermatophytes., variable hydrolytic enzymes which is known to be putative virulence factors in *M. canis* pathogenesis has been investigated by API ZYM test . API ZYM test proven effective as a good method for detection the hydrolytic enzymes activities and it is an additional procedure to diagnose and classify isolates.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors have disclosed that they do not have any conflicts of interest. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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