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ORIGINAL ARTICLE

Phenotypic and molecular characterization of virulence-associated enzymes in *Malassezia* spp. isolated from companion animals in northern Iran

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ABSTRACT

Background and Objectives: *Malassezia* spp. are lipid-dependent yeasts that colonize the skin of humans and animals and may act as opportunistic pathogens. This study aimed to characterize virulence-associated enzymes and their encoding genes in *Malassezia* spp. isolated from companion animals in northern Iran.

Materials and Methods: A total of 300 clinical samples were collected from dogs and cats with dermatological or otic lesions. Species identification was performed using ITS-based PCR. Multiplex PCR was used to detect LIP, PLB, and KER genes. Enzymatic activity was evaluated using the Pz index. Statistical analyses included Chi-square and Spearman's correlation tests ($p < 0.05$).

Results: Eighty isolates were identified as *Malassezia pachydermatis* (87.5%) and *Malassezia nana* (12.5%). LIP, PLB, and KER genes were detected in 90%, 62.5%, and 50% of isolates, respectively. Strong enzymatic activity ($Pz \leq 0.69$) was observed in 41.2% of isolates. A significant association was found between the presence of the PLB gene and phospholipase activity.

Conclusion: *Malassezia pachydermatis* was the predominant species. Virulence-associated genes were associated with higher enzymatic activity, supporting their role in pathogenic potential.

Keywords: *Malassezia pachydermatis*; *Malassezia nana*; Virulence factors; Lipase; Phospholipase; Keratinase

INTRODUCTION

Malassezia species are lipophilic yeasts that are part of the normal skin microbiota in humans and animals. They are particularly abundant in sebaceous areas, such as the external ear canal, muzzle, and interdigital spaces of dogs and cats (1, 2). Under normal conditions, these yeasts function as commensals; however, factors such as high humidity, excessive sebum secretion, allergic dermatitis, or immunosuppression can transform them into opportunistic pathogens, resulting in otitis externa and dermatitis (3).

Among the 18 recognized *Malassezia* species,

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Malassezia pachydermatis (*M. pachydermatis*) is the predominant species isolated from dogs, while *Malassezia nana* (*M. nana*) occurs mainly in cats. Although *M. pachydermatis* was once considered non-lipid-dependent, genomic studies have revealed several genes involved in lipid metabolism, confirming its lipophilic adaptability and ecological flexibility (4).

Pathogenicity in *Malassezia* is mainly linked to the production of extracellular hydrolytic enzymes, including lipases, phospholipases, and keratinases. These enzymes play a crucial role in enabling adhesion, tissue invasion, and the utilization of host lipids. The activity of these enzymes has been recognized as a significant virulence factor in *Malassezia* isolates found in animals with dermatitis and otitis (5). Despite the presence of corresponding virulence genes, the relationship between phenotypic enzyme activity and these genes is still poorly understood, especially in isolates from Iran. Most previous studies have focused on either enzymatic assays or molecular identification individually, instead of combining both methods (6).

Due to the rising incidence of *Malassezia*-related skin infections in companion animals in Iran, there is an increasing need for local data on species distribution and virulence characteristics. This study aimed to identify the *Malassezia* species isolated from dogs and cats in northern Iran and to assess their enzymatic activities as well as the presence of virulence-associated genes (LIP, PLB, and KER). By integrating molecular and enzymatic analyses, this research provides valuable insights into the pathogenic potential of these yeasts, which can enhance diagnostic and therapeutic strategies in veterinary dermatology (7).

MATERIALS AND METHODS

Sample collection and fungal isolation. A total of 300 clinical samples were collected from dogs and cats exhibiting dermatological or otic lesions at four veterinary centers in northern Iran: Gilan Pet, Rafa Hospital, Lahijan Pet, and Fereshtegan Clinic. The samples included 190 ear swabs, 50 skin scrapings, and 60 hair samples.

The samples were examined microscopically using Gram and methylene blue staining to identify yeast-like cells. They were then cultured on modified Dixon agar (Oxoid, UK) containing chloramphenicol (0.05 g/L) and cycloheximide (0.5 g/L). The plates were incubated at 32°C for 3 to 5 days (8, 9).

DNA extraction and molecular identification. Genomic DNA was extracted using the manual phenol–chloroform method. Briefly, fresh yeast colonies were suspended in a lysis buffer containing SDS and proteinase K, incubated at 56°C, and extracted with phenol–chloroform–isoamyl alcohol. The DNA was precipitated with ethanol, washed with 70% ethanol, air-dried, and finally resuspended in TE buffer. The purity and concentration of the extracted DNA were assessed spectrophotometrically (A260/A280). Species-specific PCR assays were performed to identify *M. pachydermatis* and *M. nana* isolates using primer sets targeting the ITS region of rDNA. The specific primers for *M. pachydermatis* were Mp-F (5′-GATTTGCTGCGTTCTTCATC-3′) and Mp-R (5′-AGCTGGCAGTTGAGGTCATT-3′), which amplify a 483 bp fragment. For *M. nana*, primers Mn-F (5′-CGCTGCGTTCTTCATCGAT-3′) and Mn-R (5′-GCTGGCAGTTGAGGTCATTG-3′) were used, yielding a 350 bp product. Each PCR reaction was carried out in a final volume of 25 µL containing 12.5 µL of 2× PCR Master Mix (Thermo Fisher Scientific, USA), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and nuclease-free water up to the final volume. Amplification was performed in a thermal cycler (Eppendorf Master cycler, Germany) under the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s; with a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and observed under UV illumination (10).

Detection of virulence-associated genes. Genomic DNA was extracted from *Malassezia* isolates using a standardized fungal DNA extraction protocol. Briefly, yeast cells were harvested from fresh cultures and subjected to enzymatic and/or mechanical lysis to ensure efficient disruption of the lipid-rich cell wall. DNA purity and concentration were assessed using spectrophotometric analysis and agarose gel electrophoresis.

Multiplex PCR was performed for simultaneous detection of lipase (LIP), phospholipase (PLB), keratinase (KER), and ACT1 genes. Primer sequences and expected amplicon sizes are presented in Table 1.

Table 1. Primers used for multiplex PCR detection of virulence-associated genes

Gene	Primer	Sequence (5′–3′)	Expected amplicon size (bp)
LIP	LipF	ACCCAAACATTTGCTTCGTTC	520
	LipR	TCAATTATCAATGGTCGCGA	
PLB	PhosF	AACTGGTGGATTTGCTGACC	459
	PhosR	CTTTACGGGTCCAAGGTGAA	
KER	KerF	ACGTCATGCTCAGATTGCAG	380
	KerR	GACTTCCGCGAAGAACAAAG	
ACT1	ActF	CTCTCCTTGTACGCCTCTGG	200
	ActR	TTGACAAGATGCTCCGTCAG	

PCR reactions were carried out in a final volume of 25 µL containing 12.5 µL of 2× PCR Master Mix, 1 µL of each primer, 2 µL of template DNA, and nuclease-free water. Amplification conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels (11).

Enzymatic activity assays (Pz determination). Phospholipase, lipase, and keratinase activities were evaluated using the methods described by Rizzo et al. (2024). Colonies were cultivated on specific media—egg yolk, Tween 80, or keratin azure agar—and incubated at 32°C for 7 days. The Pz index was determined as the ratio of the colony diameter to the total diameter (which includes the colony and the precipitation zone). Lower Pz values indicate higher enzymatic activity. The levels of enzymatic activity were classified as follows: strong (Pz ≤ 0.69), moderate (0.70–0.89), weak (≥ 0.90), or negative (1.0).

Statistical analysis. Data were analyzed using SPSS v26.0 (IBM Corp., USA). Frequencies, mean ± SD, and 95% confidence intervals (Wilson method) were calculated. Associations between gene presence, enzymatic activity, and host factors (age, sex, species) were tested using Chi-square or Fisher's exact tests. Spearman's correlation coefficient assessed the relationship between gene presence and enzyme activity (12).

Ethical considerations. The samples used in this study were obtained during routine veterinary diagnostic procedures performed on dogs and cats presenting with dermatological or otic lesions. No animals were subjected to experimental manipulation, invasive procedures, or additional sampling exclusively for research purposes. The study was conducted using clinical specimens collected as part of standard veterinary care. All information was handled confidentially, and the study complied with accepted principles of veterinary clinical research.

RESULTS

Isolation and identification of *Malassezia* species. Out of 300 clinical samples, 135 (45%) yielded fungal growth. Eighty isolates were identified as *Malassezia* spp., including *M. pachydermatis* (87.5%) and *M. nana* (12.5%). Most isolates originated from ear swabs of dogs and cats, while a smaller number were obtained from skin samples (Table 2).

PCR amplification of the ITS1 region confirmed two species: *Malassezia pachydermatis* (n = 70; 483 bp) and *Malassezia nana* (n = 10; 350 bp). Representative electrophoresis bands are shown in Fig. 1. Other fungal isolates included *Trichophyton mentagrophytes* (n = 50) and *Candida tropicalis* (n = 5).

Host-related distribution (age and sex). The distribution of isolates by host age and sex is summarized in Table 3.

The highest number of isolates was observed in animals aged 1–5 years (57.5%), followed by those under 1 year (21.2%) and over 5 years (11.3%). There was no significant difference in isolation frequency between male and female animals ($p>0.05$). Both dogs and cats exhibited a similar age pattern, with the majority of isolates recovered from animals in the 1–5-year age group.

Table 2. Distribution of isolates by species, host, and sample site

Host	Sample site	*M. pachydermatis* (n=70)	*M. nana* (n=10)	Total (n=80)
Dog	Ear swab	35	0	35
Dog	Skin scraping	15	0	15
Cat	Ear swab	10	0	10
Cat	Skin scraping	10	10	20
Total		70 (87.5%)	10 (12.5%)	80 (100%)

*No *Malassezia* isolates were recovered from hair samples of cats

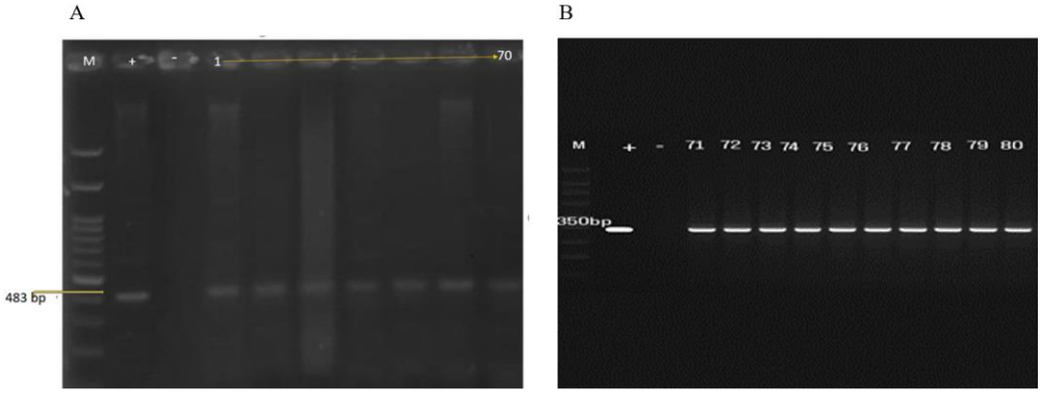

Fig. 1. PCR amplification of the ITS1 region for identification of *Malassezia* species.
(A) *M. pachydermatis* isolates showing a 483 bp amplicon; (B) *M. nana* isolates with a 350 bp amplicon. Lane M: DNA ladder (100 bp); Lane +: positive control; Lane −: negative control; Lanes 1–80: representative clinical isolates from dogs and cats.

Table 3. Distribution of *Malassezia* isolates by age and sex of host

Species	Age (years)	Male Dog	Female Dog	Male Cat	Female Cat	Total
M. pachydermatis	<1	5	5	3	3	16
M. pachydermatis	1–5	18	12	8	8	46
M. pachydermatis	>5	3	3	1	1	8
M. nana	<1	0	1	0	0	1
M. nana	1–5	3	2	2	2	9
M. nana	>5	0	0	0	0	0
Total		29	23	14	14	80

Detection of virulence-associated genes. Multiplex PCR assays revealed that 90% (72/80) of isolates carried the LIP gene, 62.5% (50/80) the PLB gene, and 50% (40/80) the KER gene, while all isolates contained the ACT housekeeping gene. The frequency of all three virulence genes was higher in *M. pachydermatis* than in *M. nana* ($p < 0.05$). Representative gel electrophoresis showing gene amplification is presented in Fig. 2.

Enzymatic activity (Pz value determination). Phenotypic assays revealed that 90% of isolates exhibited lipase activity, 62.5% phospholipase, and 50% keratinase activity. Mean Pz values were 0.78 ± 0.04 for lipase, 0.84 ± 0.05 for phospholipase, and 0.87 ± 0.06 for keratinase. Strong enzymatic activity ($Pz \leq 0.69$) occurred in 41.2% of isolates, moderate activity (0.70–0.89) in 45%, and weak activity (>0.90) in 13.8% (Table 4).

Association between virulence genes, enzyme activity, and host age. When isolates were categorized by host age (<1 year, 1–5 years, >5 years), both gene frequency and enzymatic activity were highest in

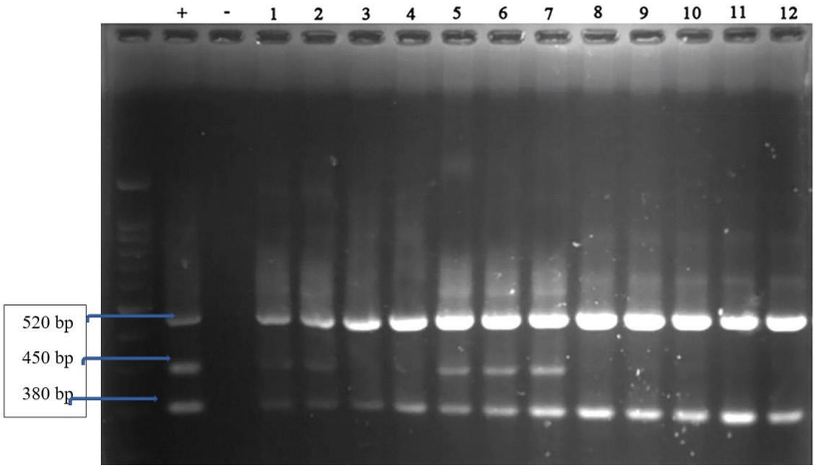

Fig. 2. Multiplex PCR amplification of virulence-associated genes in *Malassezia* isolates.
Lanes + and − represent positive and negative controls, respectively; Lanes 1–12 show amplification of LIP (520 bp), PLB (459 bp), KER (380 bp), and ACT (200 bp) genes among representative isolates.

Table 4. Phenotypic enzyme activity (Pz value determination) among *Malassezia* isolates

Enzyme	Positive isolates (n, %)	Mean Pz ± SD	Strong (≤0.69)	Moderate (0.70–0.89)	Weak (≥0.90)	Negative (1.0)
Phospholipase	50 (62.5%)	0.76 ± 0.08	20	15	10	5
Lipase	72 (90%)	0.64 ± 0.05	35	20	10	5
Keratinase	40 (50%)	0.82 ± 0.09	12	15	8	5

*Phenotypic enzymatic activity (Pz index) among *Malassezia* isolates. Lower Pz values indicate stronger enzyme activity. Lipase activity was dominant among all isolates.

the 1–5-year group. The phospholipase gene (PLB) showed a statistically significant association with enzyme activity ($\chi^2 = 6.42$, $p = 0.04$), while lipase and keratinase did not ($p > 0.05$). No correlation was found between the presence of the gene and host sex ($p > 0.05$). A summary of Pz distribution by age group is presented in Fig. 3.

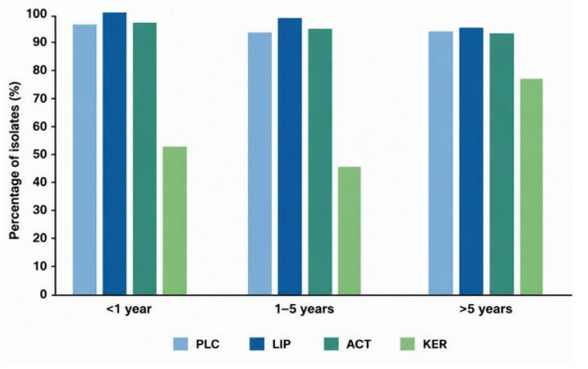

Fig. 3. Distribution of virulence gene detection (LIP, PLB, KER, ACT1) among *Malassezia* isolates from animals of different age groups (<1 year, 1–5 years, >5 years). The frequency of all virulence genes was highest in the 1–5-year group.

Correlation between gene presence and enzymatic activity. Spearman's correlation confirmed significant negative correlations between Pz values and corresponding virulence genes. As Pz values decrease with stronger activity, this indicates a direct relationship between gene presence and enzymatic expression. Table 5 summarizes both the enzymatic activity (Pz values) and the molecular detection of corresponding virulence genes.

DISCUSSION

The current study found a high prevalence of *M. pachydermatis* among clinical isolates from companion animals in northern Iran. This finding is consistent with previous reports identifying this species as the most common cutaneous yeast in dogs and cats worldwide. Its ability to thrive in seborrheic and lipid-rich environments, along with its metabolic flexibility, likely contributes to its success in veterinary dermatology settings (1, 2).

Table 5. Phenotypic enzyme activity (Pz index) and correlation with virulence gene presence among *Malassezia* isolates

Enzyme / Gene	Positive isolates (n, %)	Mean Pz ± SD	Strong (≤0.69)	Moderate (0.70–0.89)	Weak (≥0.90)	Negative (1.0)	Gene detected (n, %)	Spearman's ρ	p-value
Lipase (LIP)	72 (90%)	0.64 ± 0.05	35	20	10	5	72 (90%)	−0.81	0.001*
Phospholipase (PLB)	50 (62.5%)	0.76 ± 0.08	20	15	10	5	50 (62.5%)	−0.74	0.008*
Keratinase (KER)	40 (50%)	0.82 ± 0.09	12	15	8	5	40 (50%)	−0.52	0.042*
ACT1 (control)	80 (100%)	—	—	—	—	—	80 (100%)	—	—

*Combined summary of phenotypic enzyme activities (Pz index) and molecular detection of corresponding virulence genes among 80 *Malassezia* isolates. Lower Pz values indicate stronger enzyme activity. Significant negative correlations ($\rho < 0$, $p < 0.05$) indicate that the presence of virulence genes was associated with higher enzymatic activity.

M. nana was primarily found in feline samples, reinforcing previous molecular studies that identified this species as part of the normal and opportunistic skin microbiota in cats. The higher frequency of *M. nana* observed in this study compared to some European reports may indicate regional differences in climate, host management practices, and diagnostic sensitivity (13).

A key finding of this study was the high prevalence of genes encoding lipase (LIP) and phospholipase (PLB) among the isolates. This suggests that lipid hydrolysis plays a central role in the colonization and persistence of *Malassezia* spp. on the skin of the host. Lipases are known to break down host triglycerides into free fatty acids, which serve both nutritional and adhesive functions. This process facilitates the survival of the yeast in sebaceous microenvironments. Phospholipases play a role in disrupting membranes and damaging host tissues, which leads to inflammatory responses related to otitis externa and dermatitis (14-16).

A significant association was observed between the presence of the PLB gene and phospholipase enzymatic activity. This supports the idea that genetic factors may partially influence phenotypic expression. However, it's important to note that simply detecting a gene does not guarantee that it is actively transcribed. Additionally, factors such as environmental lipid availability and the host's immune status likely regulate enzyme expression (16). Therefore, our findings should be interpreted as a correlation between genetic potential and enzymatic phenotype rather than direct evidence of gene regulation.

The combination of multiplex PCR and phenotypic enzyme assays enhances the reliability of virulence profiling in clinical isolates. Previous studies have usually assessed enzymatic activity or molecular detection separately, which limits the comparative interpretation of virulence potential (9). Our integrated approach offers a more thorough framework for evaluating pathogenic potential in veterinary isolates.

The study observed age-related differences, showing that animals aged 1 to 5 years had higher enzyme activity and greater gene prevalence. This may be linked to increased sebaceous gland activity during adulthood, which provides more lipids for the growth of *Malassezia*. Overall, both studies confirm the central role of *M. pachydermatis* in canine-associated *Malassezia* populations. The lack of significant sex-related differences further supports the idea that hormonal influence on *Malassezia* colonization is limited when compared to environmental and physiological factors.

The significant negative correlation between Pz values and the presence of virulence genes indicates that fungal isolates containing the LIP, PLB, and KER genes tend to show stronger enzymatic activity. This suggests a functional relationship between genetic content and enzymatic phenotype. A strong relationship between virulence-associated genes and enzymatic activity has been reported previously in *Malassezia* species. Studies evaluating lipase, phospholipase and keratinase production demonstrated that isolates carrying virulence-associated genes frequently exhibit increased enzymatic activity, suggesting a functional contribution of these genes to pathogenicity (15).

However, further transcriptomic studies are needed to confirm the regulatory pathways involved. Recent advancements in fungal pathogenesis research highlight the importance of integrating genomic, transcriptomic, and proteomic approaches to fully understand host-fungus interactions (17).

From a clinical standpoint, the observed enzy-

matic profiles may offer important diagnostic and therapeutic insights. Increased levels of lipase and phospholipase activity have been linked to heightened inflammatory responses in dermatitis in dogs and cats. Consequently, these enzymes could serve as surrogate markers for pathogenicity in veterinary practice (18, 19). Enzymatic activity alone should not be considered a definitive predictor of disease severity without taking into account the host's immune status and microbiome composition. A strong relationship between virulence-associated genes and enzymatic activity has been previously demonstrated in *Malassezia* species. These findings are consistent with the current study, where isolates containing LIP and PLB genes exhibited significantly greater enzymatic activity. This supports the hypothesis that genetic potential contributes to the observed virulence phenotype in *M. pachydermatis* from companion animals (15, 20).

One limitation of this study is the lack of sequencing-based confirmation for species identification, which limits our ability to resolve phylogenetic relationships and interpret strains at a detailed level. While species-specific PCR provided reliable identification, incorporating sequencing methods such as ITS (Internal Transcribed Spacer) or multilocus phylogenetic analysis would improve taxonomic accuracy and offer deeper insights into genetic diversity (21). Additionally, gene expression was not evaluated at the transcriptional level. Therefore, future studies should include techniques like qPCR or RNA-seq to better understand the regulatory mechanisms behind the production of virulence factors (17).

In conclusion, *M. pachydermatis* is the dominant species affecting companion animals in northern Iran. The presence of virulence-associated enzymes, particularly lipases and phospholipases, appears to be crucial for its pathogenic potential. Utilizing both molecular detection methods and phenotypic assays provides a strong framework for assessing the virulence of *Malassezia*. Future research that incorporates genomic and transcriptomic analyses across various geographic regions will be vital for understanding the evolutionary and clinical aspects of *Malassezia* infections in veterinary medicine.

CONCLUSION

This study demonstrates the predominance of *M. pachydermatis* and highlights the importance of lipolytic and phospholipase enzymes in its pathogenicity. The correlation between gene presence and enzymatic activity confirms the genetic basis of virulence. Combining molecular identification with enzyme assays provides a rapid, reliable approach for assessing pathogenic potential in veterinary dermatology.

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