Journal of Entomology and Zoology Studies
Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 1931-1935
© 2020 JEZS
Received: 20-01-2020
Accepted: 21-02-2020

Abhishek Dharm Singh
PhD Scholar, Department of Veterinary Public Health, W.B.U.A.F.S, Kolkata, West Bengal, India

Chanchal Debnath
Associate Professor and Head, Department of Veterinary Public Health, W.B.U.A.F.S, Kolkata, West Bengal, India

Ripan Biswas
Assistant Professor, Department of Veterinary Public Health, W.B.U.A.F.S, Kolkata, West Bengal, India

Rahul Barua
Assistant Professor, Department of Veterinary Public Health, W.B.U.A.F.S, Kolkata, West Bengal, India

Corresponding Author:
Abhishek Dharm Singh
PhD Scholar, Department of Veterinary Public Health, W.B.U.A.F.S, Kolkata, West Bengal, India

Analysis of putative virulence-associated factors of *Nannizzia gypsea* isolated from pet dogs

Abhishek Dharm Singh, Chanchal Debnath, Ripan Biswas and Rahul Barua

Abstract
Nannizzia gypsea is a geophilic dermatophyte which has an ability to infect both animals and humans. To invade skin, dermatophytes utilize different enzymes which are associated with virulence that contribute to their pathogenicity. These enzymes are poorly studied in *N. gypsea*. The aim of the present study was to investigate the *in vitro* expression of enzymes like keratinase, catalase, urease, hemolysin and aspartic protease in 17 *N. gypsea* isolates from pet dogs. In addition, thermotolerance was assessed by comparative analysis of fungal growth at 25°C and 35°C. All isolates showed moderate to high enzymatic activity for keratinase, hemolysin and aspartic protease. Regarding catalase activity, all isolates except one were classified as moderate producers. All isolates produced urease and presented growth at both studied temperatures. Our results revealed a risk to pet-owners as a high percentage of isolates produced these enzymes which can lead to virulence and survival in the host.

Keywords: *Nannizzia gypsea*, enzymes, virulence, thermotolerance

1. Introduction
For decades, the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* were recognized as dermatophyte fungi. Recently, a taxonomic review revealed nine holomorph genera within the dermatophytes. Currently, the genus *Nannizzia* has nine species, which can be separated into distinct subclades using a multi-locus genetic analysis [1]. *Nannizzia gypsea* (NANNIZZI) Stockdale is a cosmopolitan fungus distributed world-wide having a particular affinity for keratin and is transmitted to humans and animals through contact with soil [2, 3].
Clinical signs of dermatophytosis are a result of the breakdown of keratinized tissues due to the fungal action, as well as the immune response of the infected host. During the invasion phase some virulence factors are expressed by dermatophytes contributing to tissue penetration, nutrient uptake, and better adaptation to the environment [4]. The production of enzymes like keratinase, lipases, elastase, and phospholipases by dermatophytes allows hydrolysis of several skin components, facilitating fungal invasion and utilization of the host *stratum corneum* as a nutrient for growth [5].
Nevertheless, these virulence-related factors are not properly explored in *N. gypsea*. Several other factors like urease activity which has been characterized as a virulence factor in *Cryptococcus neoformans* has only been explored as a taxonomic phenotype in dermatophytes [6]. Aspartic proteases, associated with adhesion and degradation of immunologic proteins in *Candida* genus are poorly studied in *N. gypsea* [7]. Catalases protect fungi against oxygen reactive species but their role in dermatophytosis is unknown [8]. Hemolysins, enzymes related to iron acquisition and diminishing the immune response have not been well studied in *N. gypsea* [9]. Lastly, thermotolerance was associated to the severity of human and feline sporotrichosis but it was not evaluated in the context of dermatophytosis [10].
The aim of this study was to evaluate the enzymatic expression of keratinase, hemolysin, aspartic protease, urease, and catalase, as well as the thermotolerance of *N. gypsea* isolates obtained from pet dogs from in and around Kolkata, India.

2. Materials and Methods
2.1 Isolates
From September 2018 to December 2019, skin scrapings were collected from pet dogs (n=178) which were clinically suspected of dermatophytosis. The collected samples were inoculated into Dermatophyte Test Medium (DTM) and Sabouraud's Dextrose Agar (SDA)

with chloramphenicol (0.5%) (HiMedia®, India) [2].

2.2 Keratinase, aspartic protease and hemolysin evaluation

The inoculums for the experiments were prepared from isolates grown on Oat Meal agar (HiMedia®, India) at 25 ºC for seven days. Aliquots of 10 µL conidial suspension equivalent to the 0.5 McFarland Standard (≈1×10^6 conidia) were added to the surface of each medium and incubated for seven days at 25 °C and subsequently incubated for more 48 h at 35 °C [11]. Keratinase activity was evaluated using keratin agar medium (0.06% azure keratin, 0.05% $MgSO_4$, 0.01% KH_2PO_4, 0.001% $FeSO_4$, 0.0005% $ZnSO_4$, 0.386% NaH_2PO_4, 0.397% Na_2HPO_4, Agar 1.5%), incubated at 25 ºC for 15 days [12]. Aspartic-Protease evaluation was carried out using Yeast Carbon Base (YCB) medium supplemented with 0.5 g/L azoalbumin and 1.5% Agar [13]. The hemolytic activity was evaluated in a commercial sheep blood agar plate (HiMedia®, India). The colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured by a graduated ruler, and the enzymatic activities were expressed as the P_z value (a/b). The P_z value was scored into four categories: P_z equal to 1.0 indicated no enzymatic activity; P_z between 0.999 and 0.700 indicated weak (low) enzymatic production; P_z between 0.699 and 0.400 corresponded to good (moderate) enzymatic production; and a P_z lower than 0.399 meant excellent (high) enzymatic production [14].

2.3 Catalase activity

Test tubes containing Potato dextrose agar (PDA) medium were used in this assay. The culture medium was inoculated with 30 µL of conidial suspension of *N. gypsea* corresponding to 0.5 McFarland standard and incubated at 25 ºC for seven days and two subsequent days at 35 ºC. Determination of catalase activity was performed using a semiquantitative assay in which 1 ml of a fresh prepared mixture of 10% tween 80 (HiMedia®, India) and 30% hydrogen peroxide (Sigma-Aldrich, now Merck™) was added and the column of bubbles were measure using a ruler after five minutes [15].

2.4 Urease Production

N. gypsea conidial suspension (500µL) equivalent to 0.5 McFarland was mixed with 4.5 ml of Christensen's urea broth (0.1% peptone, 0.5% NaCl, 0.2% KH_2PO_4, 2% urea, 0.1% glucose, 0.0016% phenol red) and incubated at 35 °C. After 14 days, the cultures were centrifuged at 10,000 g and 100 µL of the supernatant were transferred to a flat bottom 96-well polystyrene plate (Tarson®, India). Isolates from *Candida albicans* and *Cryptococcus neoformans* (departmental isolates) were used as negative and positive controls, respectively. The absorbance of both sample and control supernatants was obtained at the 559 nm wavelength using the iMark™ microplate reader (Bio-Rad®, India) [13].

2.5 Thermotolerance determination

Aliquots of 10 µL of a suspension of *N. gypsea* conidia equivalent to 0.5 McFarland were added to the SDA medium. The procedure was performed in two SDA plates per isolate. One plate was incubated at 25 °C and other at 35 °C for seven days. After the incubation period, the diameter of the colonies was measured with a graduated ruler. The percent growth inhibition (%GI) was then calculated using the formula [1-(D35/D25)]×100, where D25 and D35 are the colony diameters at 25 °C and 35 °C, respectively [16]. In this study, the classification of the isolates according to their thermotolerances was settled as follows: (i) isolates with %GI from 0 to 33% were classified as having high thermotolerance, (ii) isolates with %GI from 33.1 to 66% were classified as presenting moderate thermotolerance, and (iii) isolates with %GI from 66.1 to 100% were classified as having low thermotolerance.

3. Results

A total of 17 *N. gypsea* isolates were obtained from a pool of 178 samples which were confirmed through conventional laboratory techniques both macroscopically (Figure 1A) and microscopically (Figure 1B). These isolates were then sequenced by ITS (Internal transcribed spacer) region of rRNA (ribosomal RNA) gene for further confirmation. The gene sequences of all the isolates have been submitted to GenBank database and Accession numbers are: MT328560, MT328561, MT328555, MT328569, MT328566, MT328581, MT328582, MT328583, MT328584, MT328585, MT328587, MT328586, MT328589, MT328591, MT328592, MT328593 and MT328594.

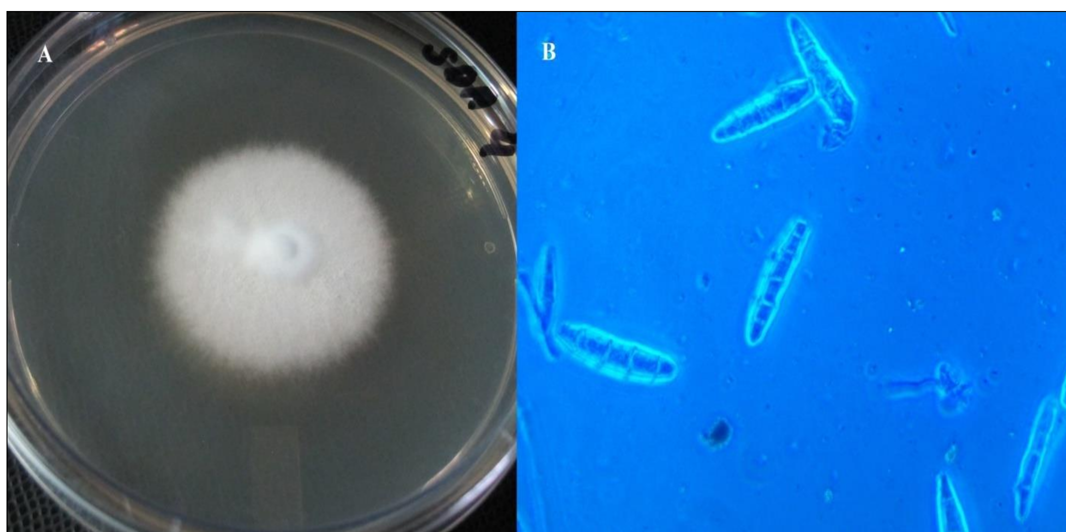

Fig 1: Obverse section of *Nannizzia gypsea* growth on SDA medium (A) and Lactophenol cotton blue staining showing macroconidia under phase contrast microscope (B) (100X)

3.1 Production of extracellular enzymes
All isolates were able to produce keratinase (Figure 2A), aspartic protease (Figure 2B), and hemolysin (Figure 2C). Regarding keratinase, 14 isolates (82.35%) presented moderate enzymatic activity and 3 (17.65%) were classified with high activity. About aspartic protease activity, 9 (52.94%) had moderate activity and 8 (47.06%) high activity. Moderate hemolysin activities were observed in 13 (76.47%) of the isolates analyzed, whereas 4 (23.53%) presented high activity (Figure 3). All isolates also showed enzymatic activity for catalase (Figure 2D). Only 1 isolate presented a column of bubbles higher than 4.5 cm height. Rests were all classified as moderate producers of this enzyme. All *N. gypsea* isolates were able to produce urease and the distribution of the activities was heterogeneous (Figure 2E).

3.2 Thermotolerance
All isolates grew at both studied temperatures and could be classified as presenting low (n=3), medium (n=9), or high (n=5) thermotolerance (Figure 2F).

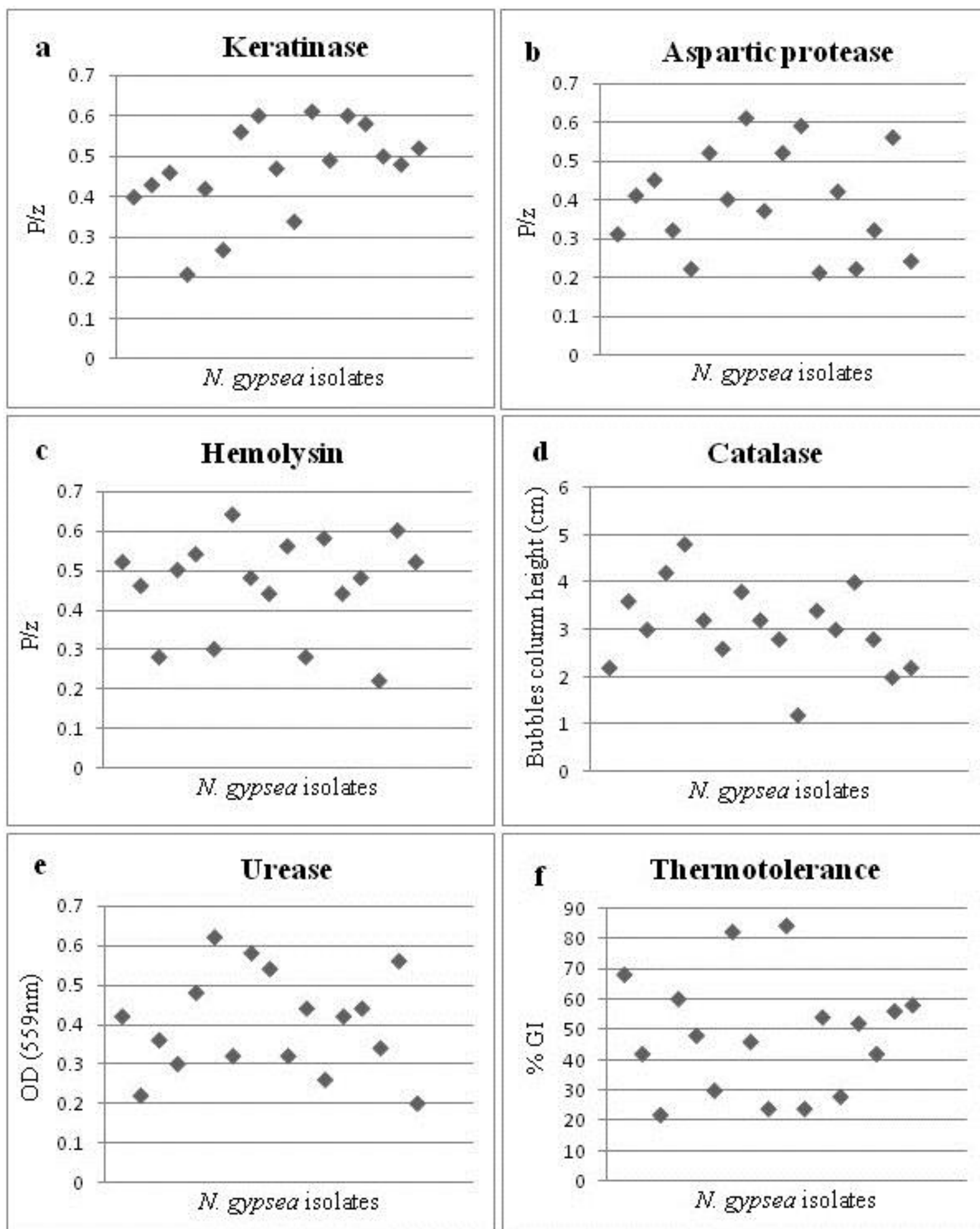

Fig 2: Expression of putative virulence-associated factors by 17 *Nannizzia gypsea* isolates from pet dogs. The level of keratinase (a), aspartic protease (b), hemolysin (c), catalase (d), urease (e) and thermotolerance (f) are presented. Each point represents a single isolate.

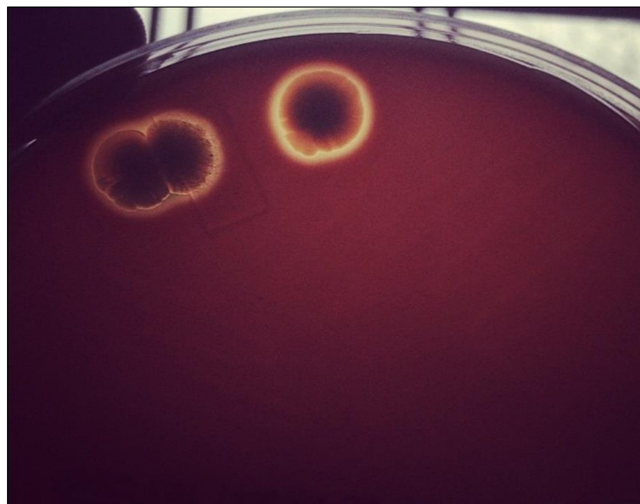

Fig 3: Appearance of hemolysis on Sheep blood agar plate by *Nannizzia gypsea* isolate.

4. Discussion

In the past few years, the number of pet animals especially dogs living with humans in India has increased [17]. While dermatophytosis due to *N. gypsea* in humans is not common, the incidence of infection due to this organism has been gradually increasing as pets like dog and cat can easily transfer them [18]. The pathogenesis of dermatophytosis is associated, among other factors, with the secretion of enzymes that degrade the components of the infected tissue. The spectrum of enzymes secreted by dermatophytes is broad, and the intensity of the enzymatic production differs between the isolates [5]. Our results corroborate this observation with the presentation of some new putative virulence-associated enzymes secreted by *N. gypsea*. A better understanding of the mechanisms underlying an infection can be the rationale for a future development of therapeutic and prophylactic strategies [19]. Keratinase is the major virulence factor of dermatophytes [5]. The present study shows *N. gypsea* as a producer of keratinase, which is in accordance with other data published in the scientific literature [19, 20]. In the present study, after seven days of growth at 25 ºC, the isolates were then incubated at 35 ºC, a temperature closer to that found by the fungus during parasitism. According to one previous study [21], this procedure improves the enzymatic production by dermatophytes. Currently, a growing number of proteases secreted by dermatophytes have been identified by proteomic or genomic techniques, but their role during infection remains under study [21]. It is known that the secretion of proteases by dermatophytes is important for the virulence of these organisms [5]. Also, the ability of dermatophytes to degrade skin proteins may require the use of combination of different types of proteases [4, 5]. According to the results obtained in this study, *N. gypsea* is also able to produce aspartic proteases *in vitro*. During dermatophyte infection the host immune system secretes macrophages, neutrophils and mast cells into skin as an immunological reaction. In the present study a majority of the isolates presented moderate hemolysin activity which could help them in destruction of these cells which is a well known phenomenon in bacteria. Catalase plays an antioxidant function, through the conversion of hydrogen peroxide, which is lethal to the fungus, in water and oxygen, thus allowing the fungus to escape from the phagocytic response of the host which can help in the survival in the host. Since urea is present in physiologic levels in the epidermis of the healthy skin [12], urease production during parasitism may offer nitrogen sources for *N. gypsea* growth. The high thermotolerance of the isolate included in this study is probably related to the ability of this fungus to infect dogs, which have a normal temperature higher than humans. Due to the absence of other studies on the production of catalase and urease, as well as thermotolerance of dermatophytes, it is necessary to verify the expression of these factors by other species, preferably isolated from different hosts. Also, their role on *N. gypsea* pathogenesis remains to be elucidated.

5. Conclusion

N. gypsea which has transitioned itself from saprophytism to parasitism through evolution is responsible for various outbreaks in animals and humans. This in fact can pose a significant public health risk to humans especially immunocompromised and neonates. Also, a proper understanding of putative virulence factors involved in pathogenicity of dermatophytes would greatly assist in the development of potential drug targets and new therapeutic approaches.

6. Acknowledgement

The authors express their sincere gratitude to Indian Council of Agricultural Research [ICAR], New Delhi, India for providing the necessary funds under 'Outreach Programme on Zoonotic Diseases'.

7. Conflict of Interest

The authors declare no conflict of interest.

8. References

1. deHoog GS, Dukik K, Monod M, Packeu A, Stubbe D, Hendrickx M *et al*. Toward a novel multilocus phylogenetic taxonomy for the dermatophytes. Mycopathologia. 2017; 182:5-31.
2. Singh A, Debnath C, Banerjee A, Batabyal K, Roy B, Samanta I. Effects of propylene glycol and magnesium chloride against dermatophytes isolated from companion animals. Indian Journal of Animal Health. 2018; 57:213-218.
3. Dolenc-Voljc M, Gasparic J. Human infections with *Microsporum gypseum* complex (*Nannizzia gypsea*) in Slovenia. Mycopathologia. 2017; 182:1069-1075.
4. Martinez-Rossi NM, Peres NT, Rossi A. Pathogenesis of dermatophytosis: sensing the host tissue. Mycopathologia. 2017; 182:215-227.
5. Elavarashi E, Kindo AJ, Rangarajan S. Enzymatic and non-enzymatic virulence activities of dermatophytes on solid media. Journal of Clinical and Diagnostic Research. 2017; 11:23.
6. Fu MS, Coelho C, DeLeon-Rodriguez CM, Rossi DC, Camacho E, Jung EH *et al*. *Cryptococcus neoformans* urease affects the outcome of intracellular pathogenesis by modulating phagolysosomal pH. PLoS Pathogens. 2018; 14:1-31.
7. Monika S, Zbigniew O. Contribution of aspartic proteases in *Candida* virulence. Protease inhibitors against *Candida* infections. Current Protein and Peptide Science. 2017; 18:1050-1062.
8. Pradhan A, Herrero-de-Dios C, Belmonte R, Budge S, Garcia AL, Kolmogorova A *et al*. Elevated catalase expression in a fungal pathogen is a double-edged sword of iron. PLoS Pathogens. 2017; 13:1-25.
9. Boral H, Metin B, Dogen A, Seyedmousavi S, Ilkit M.

Overview of selected virulence attributes in *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, and *Exophiala dermatitidis*. Fungal Genetics and Biology. 2018; 111:92-107.
10. Boechat JS, Oliveira MME, Almeida-Paes R, Gremiao IDF, Machado ACDS, Oliveira RDVC *et al*. Feline sporotrichosis: associations between clinical-epidemiological profiles and phenotypic-genotypic characteristics of the etiological agents in the Rio de Janeiro epizootic area. Memórias do Instituto Oswaldo Cruz. 2018; 113:185-196.
11. Aktas E, Yıgıt N. Hemolytic activity of dermatophytes species isolated from clinical specimens. Journal De Mycologie Medicale. 2015; 25:25-30.
12. Cafarchia C, Figueredo LA, Coccioli C, Camarda A, Otranto D. Enzymatic activity of Microsporum canis and Trichophyton mentagrophytes from breeding rabbits with and without skin lesions. Mycoses. 2012; 55:45-49.
13. Almeida-Paes R, DeOliveira LC, Oliveira MME, Gutierrez-Galhardo MC, Nosanchuk JD, Zancope-Oliveira RM. Phenotypic characteristics associated with virulence of clinical isolates from the *Sporothrix* complex. BioMed Research International, 2015, 1-10.
14. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia: Journal of Medical and Veterinary Mycology. 1982; 20:7-14.
15. Figueiredo-Carvalho MHG, Ramos LDS, Barbedo LS, Oliveira JCAD, Santos ALSD, Almeida-Paes R *et al*. Relationship between the antifungal susceptibility profile and the production of virulence-related hydrolytic enzymes in Brazilian clinical strains of *Candida glabrata*. Mediators of Inflammation. 2017:1-11.
16. Mesa-Arango AC, delRocio Reyes-Montes M, Perez-Mejia A, Navarro-Barranco H, Souza V, Zuniga G *et al*. Phenotyping and genotyping of *Sporothrix schenckii* isolates according to geographic origin and clinical form of sporotrichosis. Journal of Clinical Microbiology. 2002; 40:3004-3011.
17. Lazzaroni M, Range F, Bernasconi L, Darc L, Holtsch M, Massimei R *et al*. The role of life experience in affecting persistence: A comparative study between free-ranging dogs, pet dogs and captive pack dogs. PloS One. 2019; 14:1-17.
18. Fike JM, Kollipara R, Alkul S, Stetson CL. Case report of onychomycosis and tinea corporis due to *Microsporum gypseum*. Journal of Cutaneous Medicine and Surgery. 2018; 22:94-96.
19. Gnat S, Lagowski D, Nowakiewicz A, Zieba P. Phenotypic characterization of enzymatic activity of clinical dermatophyte isolates from animals with and without skin lesions and humans. Journal of Applied Microbiology. 2018; 125:700-709.
20. Viani FC, Viani PRC, Rivera ING, daSilva EG, Paula CR, Gambale W. Extracellular proteolytic activity and molecular analysis of Microsporum canis strains isolated from symptomatic and asymptomatic cats. Revista Iberoamericana De Micología. 2007; 24:19.
21. Bagut T, Baldo A, Mathy A, Cambier L, Antoine N, Cozma V *et al*. Subtilisin Sub3 is involved in adherence of Microsporum canis to human and animal epidermis. Veterinary Microbiology. 2012; 160:413-419.