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Identification of dermatophytes and comparative efficacy of topical antifungals for treating canine dermatophytosis

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Abstract: Dermatophytosis is a potentially zoonotic superficial skin infection caused by keratinophilic fungi. Identification of dermatophytes aids in understanding the epidemiology of infections and finding out better treatment modalities. The study aimed to identify dermatophytes in dogs and compare the effectiveness of three different topical drugs for treating dermatophytosis. Molecular identification of dermatophyte isolates obtained on culturing was done by restriction fragment length polymorphism of the amplicon obtained by polymerase chain reaction of the Internal Transcribed Spacer (ITS) region of the recombinant DNA. Thirty of the positive cases were divided into three groups, Group I was treated with shampoo containing 2% each of miconazole chlorhexidine, Group 2 with shampoo containing 4% chlorhexidine, and the third group with lime sulphur dip. Clinical response and fungal colony counts were regularly monitored to assess improvement. Among a total of 30 dermatophyte isolates, 14 *Microsporum canis*, 8 *Trichophyton rubrum*, 6 *M. gypseum,* and 2 *Epidermophyton floccosum* isolates were identified. Increased occurrence of *T. rubrum* indicates frequent transmission of dermatophytes from humans to dogs. Shampoo containing 4% chlorhexidine for an average duration of 99 days resulted in a faster cure, compared to the other topical preparations.

Key words: Dermatophytosis, topical therapy, chlorhexidine, lime-sulphur dip

1. Introduction

Dogs have a long history of companionship with humans which has led to the coevolution of several diseases between them. Dermatological conditions are commonly encountered in small animal medicine, as the skin, being the body's first line of defence, is exposed to various environmental adversities. Dermatophytosis, which refers to the infection of the hair, claw, or stratum corneum of the skin by keratinophilic fungi, is one such condition [1]. In companion animals, dermatophytosis is characterized by a superficial infection of keratinized skin structures. The fungi produce keratinases that break down the keratin protein complex, allowing them to penetrate deeper into the stratum corneum of the skin, triggering an inflammatory response [2]. As the fungi move away from the inflamed area, the central part of the lesion heals, while the periphery becomes affected, leading to characteristic ringworm lesions.

The identification of dermatophytes is important for designing and evaluating treatment strategies. Conventional methods of identification, based on colony characteristics, conidial morphology, and biochemical tests, can classify them up to the genus level. However, these tests may not provide sufficient resolution for the accurate identification of specific pathogens. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) targeting the internal transcribed sequence adjacent to the 5.8S rDNA of dermatophytes has gained attention in this regard. The polymorphism of this region has been utilized to discriminate between different species of dermatophytes [3]. PCR-RFLP is a faster, more accurate, and more sensitive method compared to traditional phenotypic methods for species identification of dermatophytes [4].

Treatment of dermatophytosis in pets aims to expedite recovery, prevent the spread of infection, and reduce the risk of transmission to humans and other animals. Due to the increasing prevalence of antifungal resistance, it is crucial to employ appropriate and responsive treatment protocols that eliminate the pathogen nonspecifically,

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minimizing the risk of the development of antifungal resistance. Comparing the efficacy of commercially available antifungal shampoos containing miconazole-chlorhexidine (2%), 4%chlorhexidine, and lime sulphur dip in treating dermatophytosis in dogs can help in identifying alternative modes of therapy for this condition. The study aimed to identify the dermatophyte species causing dermatophytosis in dogs and compare the efficacy of nonspecific antifungals for its treatment.

2. Materials and methods

The study was conducted during the period 2021–23. Dogs presented at the Teaching Veterinary Clinical Complex and Peripheral Veterinary Clinic formed the subject of the study.

2.1 Isolation of dermatophytes

Condition of skin and hair coat, as well as the nature and distribution of lesions and clinical signs, were recorded. Lesions were cleaned using 70% isopropanol, and the area was allowed to dry. Skin scrapings, hair plucks, and scales were collected from the periphery of the lesions in a sterile container. The samples were collected from lesions of different parts of the body, pooled, and transported to the lab. The sample was inoculated onto a Dermatophyte Test Medium (DTM, Himedia, India) and incubated at ambient temperature. Plates were examined daily for the presence of fungal colony growth for three weeks. Plates were discarded if no growth was obtained by four weeks. Presumptive identification of the dermatophyte was done based on colony characteristics and microscopic examination of stained fungal colonies using Chicago Sky Blue 6 B.

A section of the fungal colony was tweezed out using a pair of dissection needles and placed on a glass slide. A drop of Chicago Sky Blue stain was added, and a coverslip was placed. The stain was allowed to react for 5 min and observed under high power using the objective lens [5].

2.2 Molecular identification of dermatophyte isolates

The total DNA from the fungal colony was extracted using the phenol-chloroform method. The purity and yield of the DNA samples were estimated using the Nanodrop 2000 UV-Vis spectrophotometer. The Internal Transcribed Spacer (ITS) rDNA region was amplified using the ITS1 and ITS4 primer pairs (ITS1 Forward- 5 '-TCCGTAGGTGAACCTGCGG-3', ITS4 Reverse- 5'-TCCTCCGCTTATTGATATGC-3 ') [6].

Total DNA from *Microsporum canis* isolate MC1 with an already sequenced ITS region (NCBI Accession No. OOQ940468) was used as the positive control, and nuclease-free water was used as the negative control. The PCR products were subjected to gel electrophoresis in a 1.2% agarose gel in 0.5X TBE buffer, and the gel was examined using a gel documentation system under UV light to confirm the size of the product.

The PCR amplicons were subjected to RFLP using the restriction enzyme *Mva 1* for species-level identification. For each microgram of DNA, 0.1 units of *Mva 1* enzyme was used. A 20 µL RFLP mix containing 1 µL of *Mva 1* Enzyme, 2 µL of 10X Buffer R, 5 µL of PCR product, and 12 µL of nuclease-free water was prepared. The digested products were subjected to gel electrophoresis in a 3% agarose gel in 0.5 X TBE buffer, documented, and analyzed. The species-level identification was made based on the number and size of fragments obtained. The amplicon obtained by PCR of the *M. canis* isolate MC1 was used as the positive control, while nuclease-free water was used as the negative control.

2.3 Response to treatment

A total of 30 confirmed cases of dermatophytosis, which gave positive cultures in DTM, were selected and randomly grouped into three groups, with ten animals each, for evaluation of treatment. Animals in Group I were treated with a commercially available shampoo containing 2%miconazole and 2% chlorhexidine. In Group I, the shampoo, formulated to create a lather, was applied uniformly to the entire body, ensuring coverage of affected areas. The lather was allowed to remain in contact with the skin for 5 min to facilitate optimal drug absorption. Subsequently, the shampoo was thoroughly rinsed off. This treatment regimen was administered once every three days until a mycological cure was confirmed. Group II animals were treated topically with a commercially available shampoo containing 4% chlorhexidine. Similar to Group I, the shampoo was applied to create a lather, covering the entire body, including affected areas. The lather was left in contact with the skin for 5 min before thorough rinsing. This treatment was administered once every three days until a mycological cure was confirmed. Animals in Group III were treated with a commercially available lime sulphur dip. The lime sulphur dip was applied uniformly to the entire body, allowed to act for 30 min, and then washed off. Cases were reviewed every two weeks, and photographic records were obtained to assess clinical improvement. Skin scrapings were taken at every visit, and subjected to fungal culture. The mycological response was assessed by the number of dermatophyte colonies obtained [7]. Treatment was administered once every three days. Cases were reviewed every two weeks. Skin samples were taken at each review and cultured in DTM. Treatment continued until two consecutive cultures in DTM gave no fungal growth. The efficacy of different treatments was evaluated based on the disappearance of lesions and clinical signs, regrowth of hair, and two consecutive negative fungal cultures. Results were analyzed using the Chi-square test.

2.4 Ethical considerations

The animal study was reviewed and approved by the Faculty research committee vide Order No.KVASU/DAR/Acad/A3/30187/2021 Dt. 15.03.2022 of Kerala Veterinary

and Animal Sciences University. Experiments were done on clinical specimens submitted to our laboratory by registered veterinarians. The clinical samples used for diagnosis were collected by the veterinarians after getting oral consent from the pet owners. Treatments advised were as per standard prevailing practices in the country.

3. Results

3.1. Isolation of dermatophyte

Skin scrapings were collected from 114 dogs with scaly lesions, localized or generalized alopecia, pruritus, and inflammation. Growth was observed in DTM among 30 of 114 samples (26.32%). Morphological studies of the stained isolates revealed that out of the 30 dermatophytes, 20 were *Microsporum* spp. eight were *Trichophyton* spp. and two were *Epidermophyton* spp. isolates. Micromorphological features of the isolates are provided in Figure 1.

3.2 Identification of dermatophyte isolates

Upon PCR targeting the ITS of rDNA, 14 of the *Microsporum* spp. isolates yielded amplicons of approximately 737 bp, while six of them yielded amplicons of approximately 666 bp. All eight isolates of *Trichophyton* spp. yielded amplicons of approximately 692 bp, and the two *Epidermophyton* spp. isolates yielded amplicons of approximately 780 bp (as shown in Figure 2).

Restriction Fragment Length Polymorphism (RFLP) of the amplicons was performed using *Mva 1*, and restriction patterns were observed in all 30 amplicons. Based on the amplicon size and fragment size, the isolates were identified. The 14 isolates of *Microsporum* spp. that produced 737 bp amplicons yielded three fragments of 441, 165, and 103 bp. These isolates were identified as *M. canis* (as shown in Figure 3). The remaining six Microsporum isolates produced amplicons of 666 bp and yielded three fragments of 289, 179, and 146 bp. They were identified as *M. gypseum* (*Nannizia gypsea*) (as shown in Figure 4). The eight isolates of *Trichophyton* spp. that yielded amplicons of 692 bp produced three fragments of 368, 164, and 95 bp, and were identified as *T. rubrum* (as shown in Figure 5). Two isolates of *Epidermophyton* spp. that yielded amplicons of 780 bp produced three fragments of 361, 231, and 169 bp, and were identified as *E. floccosum* (as shown in Figure 6).

3.3 Response to treatment

All dogs in each group recovered during treatment. Recurrence of dermatophytosis was reported in two cases in Group I after six months of recovery. Animals treated with a miconazole-chlorhexidine shampoo combination showed clinical recovery within 27.7 days (as shown in Figure 7).

All dogs treated with a 4% chlorhexidine solution showed clinical recovery, and skin lesions were absent within an average of 19 days. Two consecutive culture-negative samples, indicating mycological cure, were obtained within an average of 99 days as shown in Figure 8). Group III, treated with lime sulphur dip, showed clinical recovery within an average of 21 days, and mycological cure was achieved within an average of 112 days of treatment (as shown in Figure 9). Details regarding the response to treatment are summarized in the Table.

3.4. Statistical analysis

Chi-square analysis revealed that the number of days taken for clinical recovery varied significantly between groups. Treatment with 4% chlorhexidine resulted in a significantly faster clinical response compared to lime sulphur dip or miconazole- chlorhexidine combination. Mycological cure was also significantly faster in chlorhexidine treated animals compared to miconazole-chlorhexidine group, but the difference was not statistically significant compared to lime sulphur dip (as shown in Table).

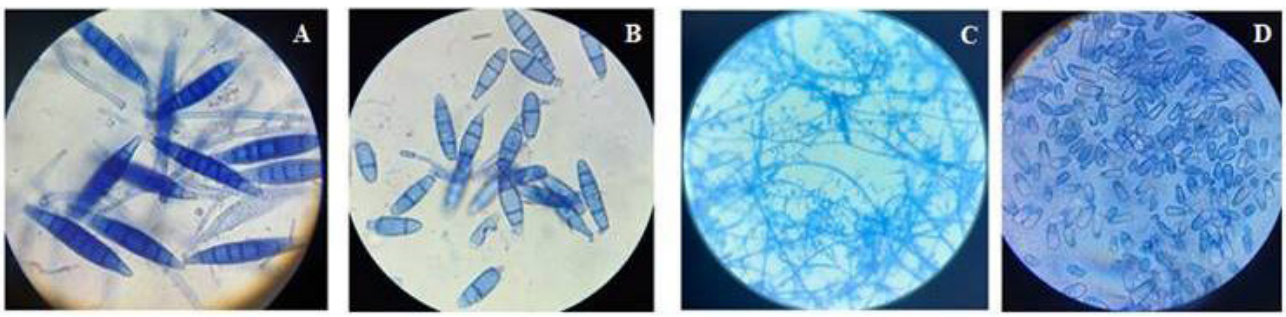

Figure 1. Dermatophytes isolated from skin samples of infected dogs.
A*: Microsporum canis*
B*: Microsporum gypseum*
C*: Trichophyton rubrum*
D*: Epidermophyton floccosum*

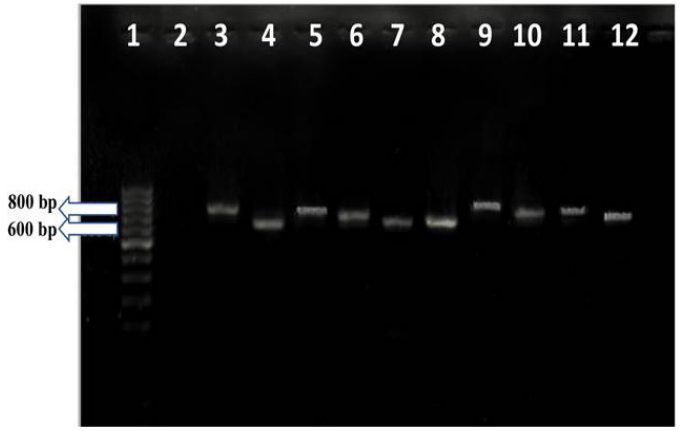

Figure 2. PCR amplicons of dermatophyte isolates. PCR targeting the ITS of rDNA of total DNA extracted from dermatophyte isolates produced amplicons of size varying from 666 to 780 base pairs.
Lane 1: 100bp Ladder
Lane 2: Negative Control
Lane 3: Positive Control
Lane 4–12: Isolates

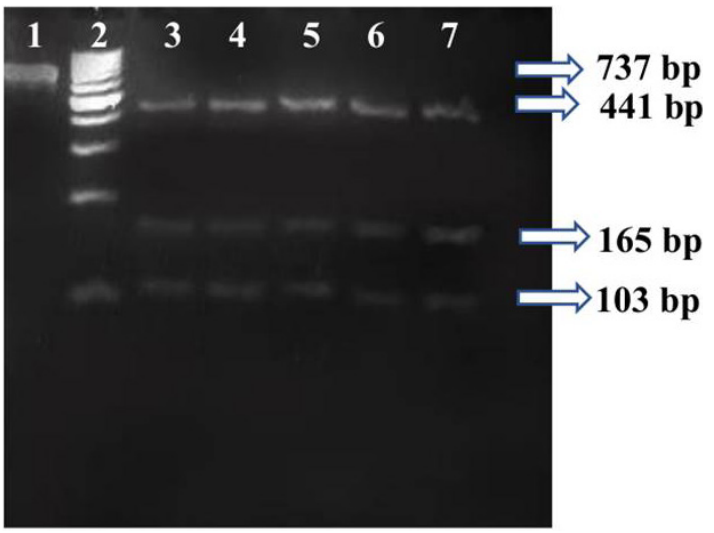

Figure 3. RFLP band patterns generated by Mva1 digestion of the PCR product from ITS of rDNA from M. canis isolates. Restriction digestion of PCR product using Mva1 enzyme produced three fragments of 441, 165, and 103 base pair-sized products.
Lane 1: Unrestricted amplicon
Lane 2: 100bp Ladder
Lane 3–7: Restriction fragments

4. Discussion

Based on their growth pattern in the dermatophyte test medium, dermatophytes were detected in 26.23% of cases with dermatological lesions. A similar study reported the occurrence of dermatophytosis in 25% of dogs in Gujarat state of India [8]. *Microsporum* spp. was identified as the commonest aetiology (20 out of thirty isolates) of canine dermatophytosis in the present work. However, the identification of species isolated could not be determined based solely on the morphology. The PCR targeting the ITS region of ribosomal DNA gave amplicons of varying sizes ranging from 666 bp to 780 bp, indicating multiple species involved within the same genus. Overall, the findings are in agreement with earlier reports that describe

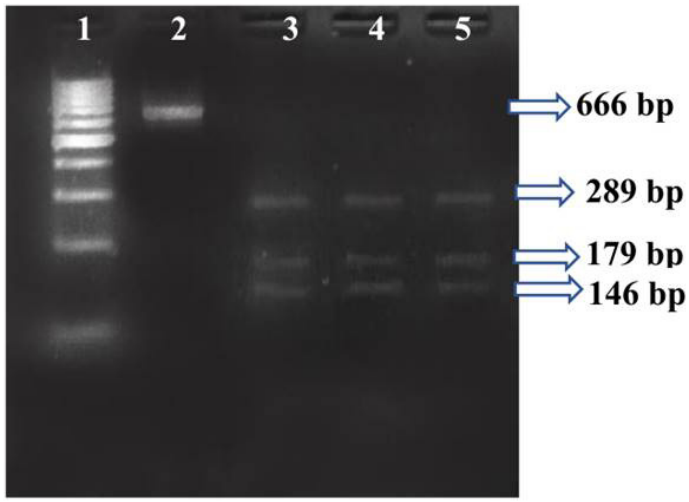

Figure 4. RFLP band patterns generated by *Mva1* digestion of the PCR product from ITS of rDNA from *M. gypseum* (*N. gypsea*). Restriction digestion of 666 base pair PCR product using Mva1 enzyme produced three fragments of 289, 179, and 146 base pair sized products.
Lane 1: 100bp Ladder
Lane 2: Unrestricted amplicon
Lane 3,4,5: Restriction fragments

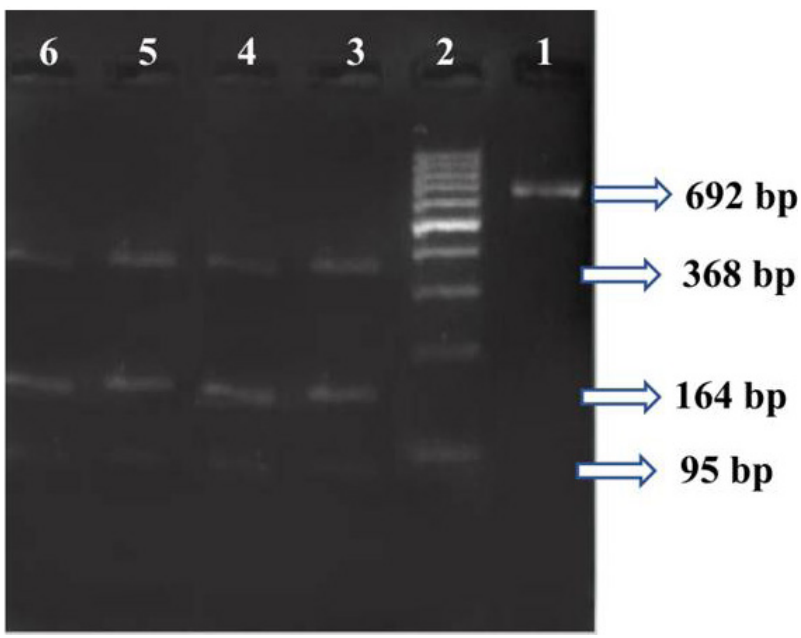

Figure 5. RFLP band patterns generated by *Mva1* digestion of the PCR product from ITS of rDNA from *T. rubrum*. Restriction digestion of 692 base pair PCR product using *Mva1* enzyme produced three fragments of 368, 164, and 95 base pair sized products.
Lane 1: Unrestricted amplicon
Lane 2: 100bp Ladder
Lane 3–6: Restriction fragments

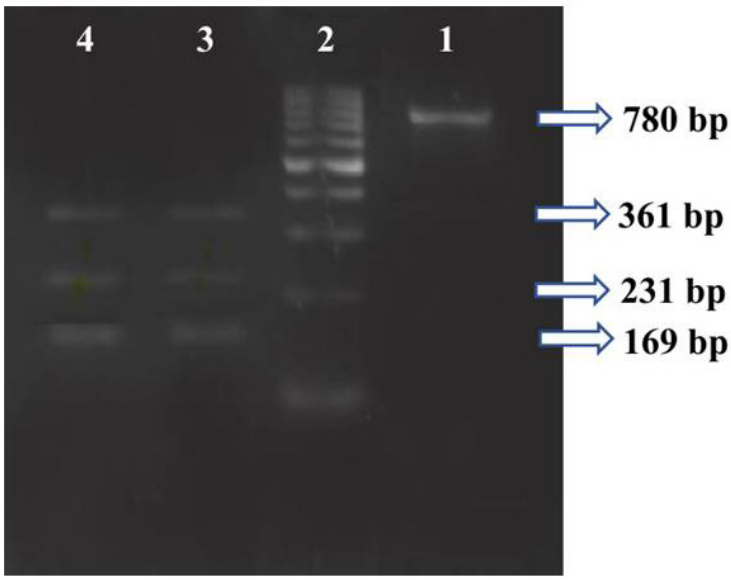

Figure 6. RFLP band patterns generated by *Mva1* digestion of the PCR product from ITS of rDNA from *E. floccosum*. Restriction digestion of 780 base pair PCR product using *Mva1* enzyme produced three fragments of 361, 231, and 169 base pair sized products.
Lane 1: Unrestricted amplicon
Lane 2: 100bp. Ladder
Lane 3,4: Restriction fragments

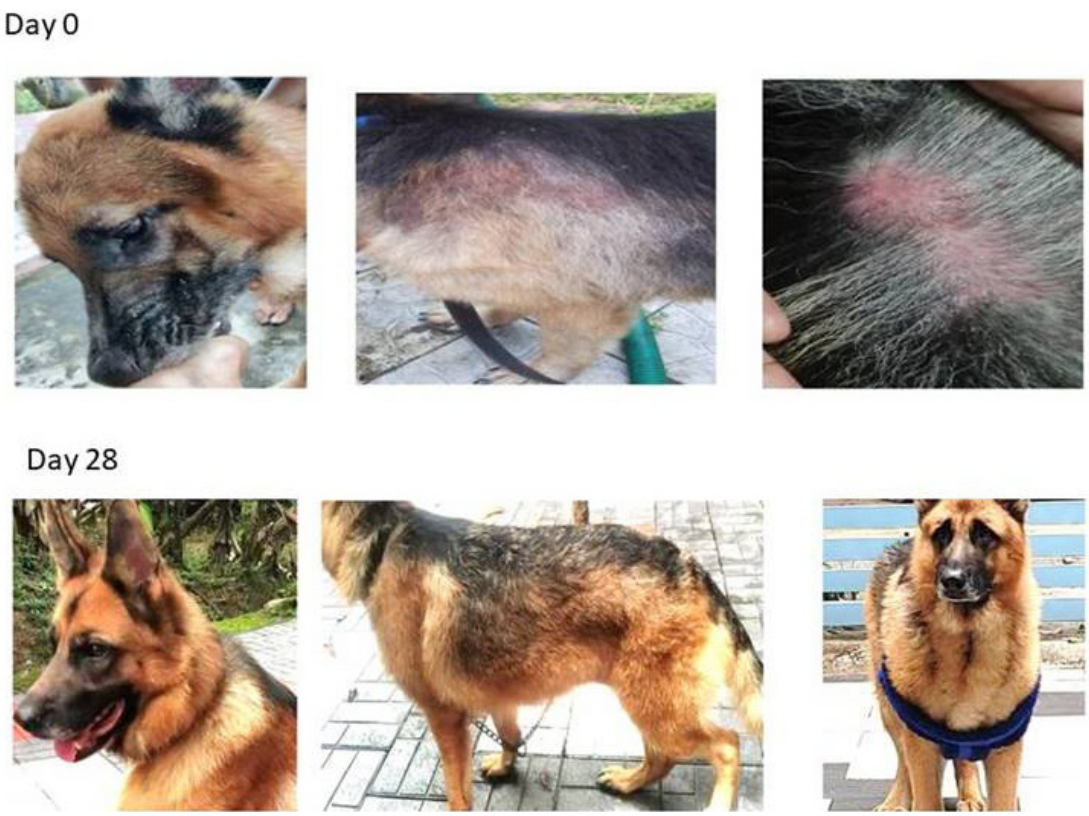

Figure 7. Case of dermatophytosis treated with chlorhexidine-miconazole shampoo.

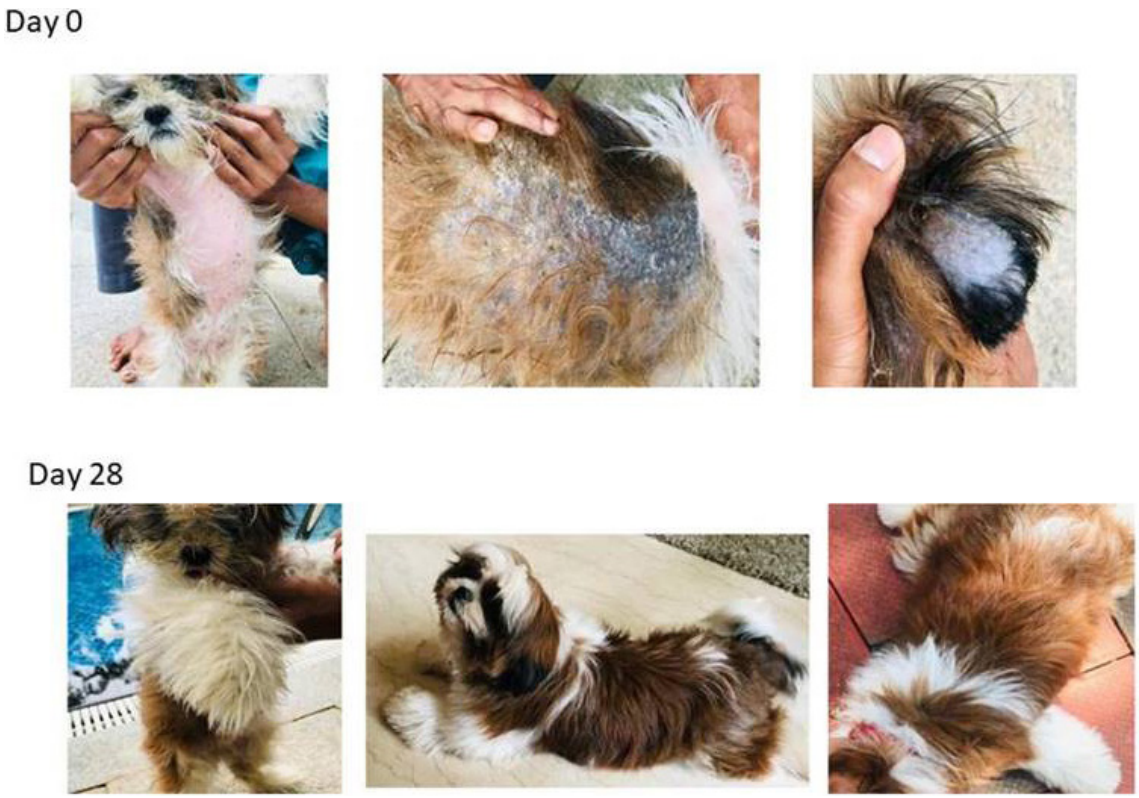

Figure 8. Case of dermatophytosis treated with 4% chlorhexidine shampoo.

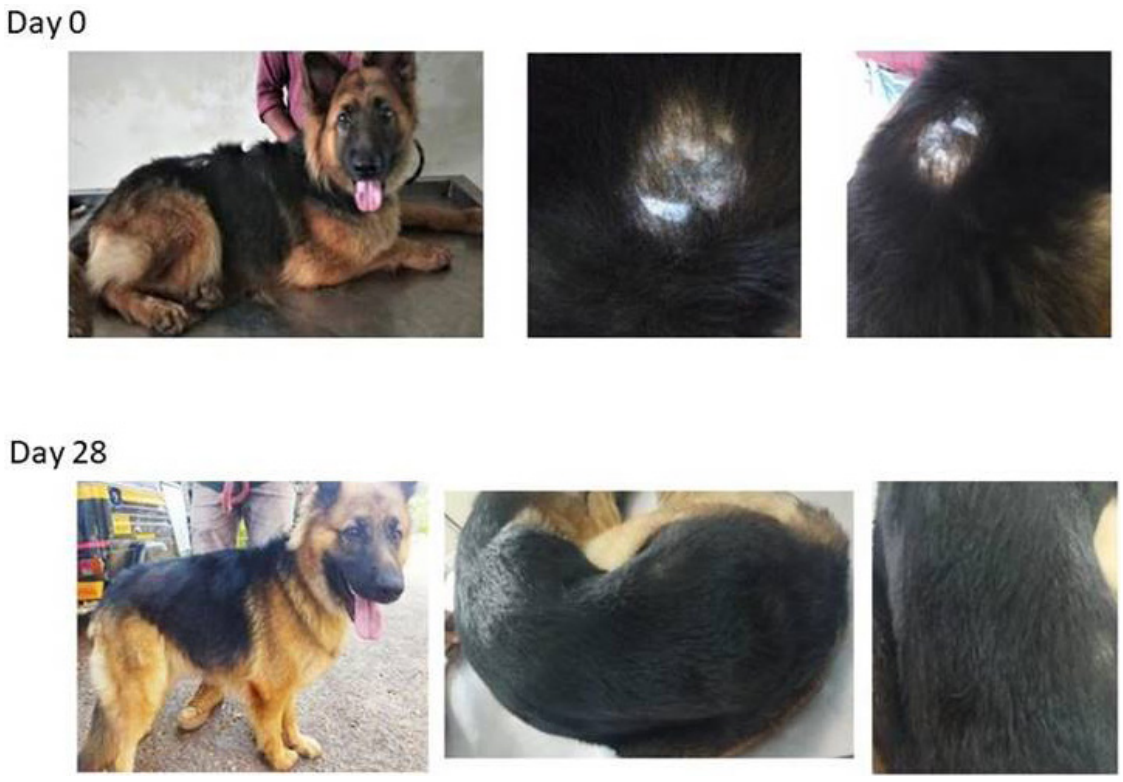

Figure 9. Case of dermatophytosis treated with Lime sulphur dip.

Table. Response to treatment.

Treatment Group	Group I (n = 10)	Group II (n = 10)	Group III (n = 10)	F value	p-value
Treatment Given	2% Miconazole + 2% Chlorhexidine	4% chlorhexidine	Lime sulphur dip		
Absence of clinical signs (Mean + SE)	27.7 +1.03c	19 + 0.52a	21.8 + 0.63b	34.20*	<0.001
Mycological cure (Mean + SE)	122 + 6.11a	11 + 6.4b	112 + 6.46ab	3.322ns	0.051

ns: Nonsignificant * Statistically significant
Means having different superscript differ significantly

traditional diagnostic approaches to be time-consuming as dermatophyte polymorphism might make morphological identification difficult [9,10]. Hence, molecular methods like PCR aid in better identification of dermatophytes [11].

The species- level identification of the isolates was made based on the RFLP pattern of the amplicons using *Mva 1* [6]. Thus, 14 isolates of *Microsporum* spp. were identified as *M. canis* and the remaining six were identified as *M. gypseum* (*Nannizia gypsea*). The eight isolates of *Trichophyton* spp. were identified as *T. rubrum*. Presence of eight isolates of *T. rubrum* in the present study was considered interesting, as the pathogen is considered to be associated primarily with humans rather than dogs [12]. Increased occurrence of the isolates in dogs could be because of increased transmission of *T. rubrum* from humans to their canine companions. *Trichophyton rubrum* is reported to be one of the most common dermatophyte aetiologies among Indians [13]. Two isolates of *Epidermophyton* spp. were identified as *E. floccosum*. *Mva 1*-based identification of dermatophytes offered efficient and accurate identification compared to conventional microscopy [14]. The use of *Mva 1* to differentiate dermatophytes has been widely tested. The enzyme was reportedly superior to other restriction enzymes [15]. Conventional microscopic techniques are time-consuming, cumbersome, and can be equivocal concerning biochemical tests. However, RFLP assays of ITS genes were reportedly in close agreement with the newer taxonomical classification of dermatophytes [16]. The RFLP-based diagnosis could clearly differentiate the two species of Microsporum and easily establish the specific identity of *Epidermophyton floccosum* and *Trichophyton rubrum* [17]. The technique immensely helps in the early identification of the dermatophyte involved and could determine better treatment and control options for canine dermatophytosis.

It was observed that mycological and clinical cure could be achieved in all the thirty cases of dermatophytosis. However, the recurrence of the disease in two cases of Group I could be due to the persistence of risk factors such as environmental, climatic, hygienic, and management practices, which could aid the survival of fungi in the environment [18, 19]. Even though clinical recovery following therapy has been reported with miconazole-chlorhexidine combinations [7], the present study observed that two consecutive culture-negative skin samples, indicating mycological cure took an average of 122 days. Faster clinical recovery was observed in cases treated with 4% chlorhexidine shampoo within an average of 19 days and mycological cure was obtained by 99 days on average. Treatment with lime sulphur dip needed an average of 21 and 112 days, respectively for the clinical and mycological cures. The period for a mycological cure was significant. Even though the clinical signs disappeared after about one month of treatment, mycological cure took almost twice the time. Stopping the treatment at the point of clinical recovery can be risky, as it can lead to recurrence and possible resistance against antifungals [20]. It was observed that the use of chlorhexidine twice a week resulted in a faster clinical and mycological cure. Chlorhexidine is well known for its germicidal action and has been widely used as a topical antiseptic. It was found to have the highest biocidal activity against dermatophytes [21].

Chi-square analysis revealed that treatment with 4% chlorhexidine resulted in significantly faster clinical as well as mycological response compared to lime sulphur dip or miconazole- chlorhexidine combination. The improved activity of chlorhexidine and lime sulphur dip could be because of the better residual activity of the drugs compared to miconazole [22]. Notwithstanding that the study is limited by relatively small sample size, topical treatment with nonspecific biocides such as chlorhexidine and lime sulphur dip for cutaneous fungal infections was found to be better and will greatly reduce the cost of treatment as well as help in reducing the dependence on specific antifungals and thereby minimise the risk of development of antifungal resistance.

Conflict of interest statement

None of the authors have any conflict of interest to report.

Informed consent

Since treatment provided and samples collected from the animal subjects as a part of routine clinical examinations, ethical approval was not required. However, verbal consent from the owners was taken prior to sample collection and utmost care was taken to ensure the comfort and well-being of the animal subjects while handling them.

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