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Research Article

Evaluation of different therapeutic protocols for canine malasseziosis

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Abstract

Dogs presented with clinical signs suggestive of malasseziosis were screened by cytological examination and included in the study group. The main objective of the study was to evaluate the efficacy of different therapeutic protocols against malasseziosis in dogs. Upon cytological examination, thirty out of 204 dogs were found positive for malasseziosis. All the animals were subjected to detailed anamnesis and clinical examination with special emphasis on the integumentary system. On examination, clinical signs reported were alopecia, scaly lesions, foul odour, hyperpigmentation, erythema, pruritus, ear affections and lichenification. Skin and ear swabs from suspected clinical cases were cultured on Modified Dixon's Agar (MDA) for the isolation of *Malassezia* spp. The isolates obtained were characterized based on the colony characteristics and microscopic morphology by Gram staining. Total Deoxyribonucleic acid (DNA) was extracted from the pure cultures of the isolates and subjected to confirmation by Polymerase Chain Reaction (PCR) targeting large subunit ribosomal RNA gene. Twenty four dogs positive for malasseziosis were randomly selected and divided into four groups for comparing standard antifungal therapeutic protocols with oral itraconazole and fluconazole and topical application of neem oil and miconazole. Therapeutic responses in each group were recorded at two-week interval using Clinical Lesion Score, Visual Analog Score for pruritus and *Malassezia* Index score. The present study concluded that oral itraconazole and topically neem oil effectively managed canine malasseziosis based on clinical (*Malassezia* Index score) and cytological (Impression smear examination) improvement.

Key words: Malasseziosis, PCR, SDA

Highlights

- On haematological examination of dogs with malasseziosis, a significant increase of TLC and a significant decrease of Hb and VPRC values were found.
- Polymerase chain reaction targeting the large subunit ribosomal RNA gene was found effective for the identification of *Malassezia* spp.
- Oral itraconazole and topically neem oil was found as effective treatments using CLS, VASP and MI score.

INTRODUCTION

Malassezia spp. are non-mycelial saprophytic lipophilic yeasts of the normal cutaneous or mucosal microbiota of many warm-blooded vertebrates. These yeasts may become opportunistic pathogens with changes in the micro-environment or defence mechanisms of the skin and with an altered immune system. *Malassezia* organisms cause dermatitis through inflammatory or hypersensitivity reactions by the host to yeast antigens or products (Mauldin *et al*., 1997). *Malassezia* yeasts also activate complement and trigger the release of inflammatory mediators, which can alter the cutaneous microenvironment, thereby leading to inflammation and pruritus (Jackson and Marsella, 2012). Diagnosis of *Malassezia* sp. is based on cytology, culture and molecular techniques (Nuttall and Halliwell, 2001). Polymerase chain reaction (PCR) can be used as an effective diagnostic tool for *Malassezia*

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species identification (Guillot *et al*., 2000).

Treatment of malasseziosis in dogs includes the use of topical therapy and systemic therapy. For localized lesions, topical antifungal agents are usually used and are more effective in short-coated animals. In chronic or generalized cases, systemic therapy with azole antifungals is effective. Azoles impair ergosterol synthesis in the fungal cell membrane by inhibiting cytochrome P450 sterol C14 alpha-demethylation (Koch *et al*., 2012). Salazar *et al.* (2015) reported that terpenoids in neem (*Azadirachta indica)* extracts (both the neem leaves and seed oil extracts) had either fungistatic or fungicidal power on several pathogenic fungi. This article reports the evaluation of different therapeutic protocols for the management of malasseziosis in dogs using clinical and cytological examinations.

MATERIALS AND METHODS

Dogs of different breed, age and sex groups presented with clinical signs of alopecia, pruritus, erythema, hyperpigmentation, lichenification, foul odour and otitis externa were screened by cytology for malasseziosis and were included in the study group. Haematological parameters including Haemoglobin (Hb), Volume of Packed Red Cells (VPRC), Total Erythrocyte Count (TEC), Total Leucocyte Count (TLC), Differential Leucocyte Count (DLC), Platelet Count (PLT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were estimated. A three-part fully automated haematological analyzer (Mindray BC-2800Vet) was used to analyze haematological parameters. Biochemical parameters like alanine aminotransferase, alkaline phosphatase, total protein, albumin, globulin and total bilirubin were estimated using a semi-automatic biochemical analyzer (MISPAVIVA 2578-10/17). Commercially available standard diagnostic kits from Agappe Diagnostics Pvt. Ltd., Kochi were used to estimate the above biochemical parameters. Independent t-test was done to compare haematological and serum biochemical parameters between study and control animals. Methylene blue staining was done for the cytological examination of skin and ear swabs. Cultural examination was done by inoculating the samples on MDA and incubating at 37°C up to seven days (Rathnapriya *et al*., 2016). DNA extraction from the pure cultures of the isolates was done using the standard phenol-chloroform method. Polymerase chain reaction targeting the large subunit ribosomal RNA gene of *Malassezia* spp. was carried out (Guillot *et al*., 2000). Twenty four dogs found positive for malasseziosis were enrolled and randomized into one of the four treatment groups. Each group was consisted of six Malassezia positive dogs. (Table 1). The treatment duration in each group was continued for four weeks. Necessary supportive therapy including antimicrobials in case of associated pyoderma, antihistaminics, herbal immunostimulants and essential fatty acids were given to animals under the treatment groups. Six apparently healthy dogs were selected as the control group.

A *Malassezia* Index (MI) score was assigned to all dogs in the treatment group on day 1, day 14 and day 28 for evaluating the therapeutic efficacy of the drugs using the following equation (Olivry *et al*., 2003; Rosales *et al.*, 2005):

MI score = Clinical Lesion Score (CLS) +
[Visual Analog Score for Pruritus (VASP) X 1.8]

Table 1. Antifungal therapeutic protocols

Group	Drug	Details of administration
I	Itraconazole	5-10 mg/kg body weight o.d., orally
II	Neem (*Azadirachta indica*) oil	Topical, b.i.d.
III	Fluconazole	5-10 mg/kg body weight o.d., orally
IV	2 per cent Miconazole ointment	Topical, b.i.d.

The CLS was assessed by summing the scores of all four features for each of the six sites (Table 2). The maximum CLS was 72. The degree of pruritus in the infected dogs was evaluated using Visual Analog Score for Pruritus (VASP) (Rosales *et al.*, 2005). The VASP was calculated on a scale with zero indicating no pruritus and 10, constant pruritus. The VASP was then multiplied by 1.8. The maximum VASP was 18, and the maximum MI score was 90. Per cent reduction for CLS, VASP and MI score for each treatment group was calculated. A reduction greater than 50 per cent for each of these parameters was considered clinically significant (Olivry *et al.*, 2002).

Data were entered in a Microsoft Excel spreadsheet and imported into IBM-SPSS 24 software for statistical analysis and graphing.

RESULTS

A total of 204 dogs presented with dermatological problems were screened for malasseziosis. Upon screening, thirty dogs were found positive for malasseziosis by microscopy and were selected as the study group. Among the 30 *Malassezia* positive dogs, 24 dogs were randomly selected for comparing standard therapeutic protocols. Clinical parameters were found to be within the normal range upon general clinical examination with a mean respiratory rate of 27.23±1.03/ min, pulse rate of 92±0.61/ min and temperature of 102.17±0.15°F. Haematological examination revealed a significant increase of Total Leucocyte Count (TLC) and significant decrease of haemoglobin (Hb) and Volume of Packed Red Cells (VPRC) values in dogs with malasseziosis upon comparison with the control group (Table 3). Serum biochemical study revealed a significant decrease in total proteins and globulin values and a significant decrease in albumin values and a significant increase of alkaline phosphatase (ALP) and a highly significant increase of total bilirubin in the study group when compared to the control group (Table 4).

Impression smear examination revealed the presence of blue coloured and footprint shaped budding yeast cells in all the positive cases (Fig. 1). Twenty four isolates suggestive of *Malassezia* spp. were obtained on MDA. The colonies were cream to buff coloured with a smooth, convex surface (Fig. 2). Gram's staining of colonies revealed dark blue coloured footprint shaped organism (Fig. 3). All isolates were positive in the PCR targeting LSU region of *Malassezia* spp. (Fig. 4).

Clinical response to therapy was monitored using CLS, VASP and MI score on 14th and 28th day of treatment. The results of CLS, VASP and MIS between periods and groups are depicted in Tables 5, 6 and 7. The pair-wise comparison revealed a significant decrease in the CLS values between 1st and 14th day in all the groups. Similarly, there is a significant decrease in the CLS values between 14th and 28th day in all the groups. Pair-wise comparison in the VASP values revealed a significant decrease between the 14th day and the 1st day in group I. There is also a significant decrease between the 1st and 28th day in groups II and III. Pair-wise comparison in the MI score values

Table 2. Clinical Lesion Score (Rosales *et al.*, 2005)

Lesion site / Lesion	Ventral neck	Axilla	Perineum	Feet	Left ear	Right ear
Erythema						
Greasy scale/exudate						
Hyperpigmentation						
Lichenification						

Score: 0 – Absent, 1 – Mild, 2 – Moderate, 3 - Severe

Table 3. Comparison of haematology of dogs with malasseziosis and control animals

Sl. No.	CBC values	Mean ± SE			
		Study group n=24	Control group n=6	t-value	P-value
1	Hb (g/dL)	13.62±0.43	16.03±0.70	2.57*	0.016
2	VPRC (%)	41.09±1.28	48.18±1.89	2.58*	0.015
3	TEC (x 10^6/cmm)	6.31±0.17	6.87±0.26	1.56^{ns}	0.131
4	TLC (x 10^3/cmm)	21.48±1.37	11.85±0.66	3.45**	0.002
5	Platelet count (x10^3/µL)	360.50±3.31	274.67±25.28	2.50*	0.025
6	MCV (fL)	65.27±1.24	70.20±0.6	3.58**	0.001
7	MCH (g/dL)	21.53±0.33	23.23±0.22	4.25**	< 0.001
8	MCHC (pg)	33.15±0.39	33.20±0.20	0.06^{ns}	0.950
9	N (%)	80.47±1.43	76.75±1.66	1.24^{ns}	0.226
10	L (%)	14.28±1.38	16.97±1.79	0.92^{ns}	0.366
11	M (%)	3.30±0.19	4.55±0.44	2.87**	0.008
12	E (%)	1.96±0.14	1.73±0.38	0.68	0.500

** Significant at 0.01 level (P<0.01); * Significant at 0.05 level (P<0.05)

Table 4. Comparison of serum biochemical values of dogs with malasseziosis and control animals

Sl. No.	Parameters	Mean ± SE			
		Study group n=24	Control group n=6	t-value	P-value
1	Alanine Aminotransferase ALT (U/L)	25.63±3.56	25.16±2.13	0.064^{ns}	0.950
2	Alkaline Phosphatase ALP (U/L)	105.78±11.2	77.16±7.19	2.150*	0.041
3	Total Protein TP (g/dL)	5.11±0.12	6.11±0.12	3.867**	0.001
4	Albumin (g/dL)	2.40±0.11	2.88±0.08	2.182*	0.038
5	Globulin (g/dL)	2.72±0.16	3.23±0.05	3.072**	0.005
6	Total Bilirubin (mg/dL)	0.90±0.05	0.58±0.07	3.123**	0.004

** Significant at 0.01 level (P <0.01); * Significant at 0.05 level (P <0.05)

also revealed a significant decrease between 1st and 14th day in all the groups. Similarly, there is a significant decrease in the MI score values between 14th and 28th day in all the groups. Table 5 to Table 7 represents the results of comparison between groups and between periods. Between groups comparison was made by using KruskalWalli's ANOVA followed by Mann Whitney U test for pair-wise comparison. Between periods comparison for each group was done using Friedman's test followed by Wilcoxon signed-rank test for pair-wise comparison.

An excellent clinical response along with quick recovery was noticed in all the six dogs treated with oral itraconazole by 14th day itself. Those animals treated with oral fluconazole, topical neem oil and miconazole also exhibited clinically significant improvement by 28th day of treatment. In the present study, orally itraconazole and topically neem oil was found to be effective for treating canine malasseziosis

Table 5. Comparison of CLS between treatment periods

Group	1st Day	14th day	28th day	Mean % reduction		c^2-value (P-value)
				14th day	28th day	
Group I	14.8, 14.5[a]	6.7, 7[b]	0, 0[c]	54.73	100	12.00**
n=6	(11, 18)	(4, 9)	(0, 0)			(0.002)
Group II	17.8, 17[a]	11.3, 11[b]	1.3, 1[c]	36.52	92.70	12.00**
n=6	(15, 23)	(10, 14)	(0, 4)			(0.002)
Group III	17.8, 16.5[a]	8.8, 8.5[b]	1, 1[c]	50.56	94.40	12.00**
n=6	(14, 25)	(6, 13)	(0, 2)			(0.002)
Group IV	14.8, 14[a]	8.7, 7[b]	1.3, 1.5[c]	41.22	91.22	12.00**
n=6	(11, 22)	(6, 20)	(0, 3)			(0.002)
χ^2-value	4.411	11.910**	5.428			
(P-value)	(0.220)	(0.008)	(0.143)			

** Significant at 0.01 level (P<0.01); * Significant at 0.05 level (P<0.05); Values in same row bearing same superscript do not differ significantly

Table 6. Comparison of VASP between treatment periods

Group	1st Day	14th day	28th day	Mean % reduction		χ^2-value (P-value)
				14th day	28th day	
Group I	5.8, 5[a]	0, 0[b]	0, 0[c]	100	100	12.00**
n=6	(5, 10)	(0, 0)	(0, 0)			(0.002)
Group II	5, 5[a]	3.3, 5[ab]	0, 0[b]	34	100	8.00*
n=6	(0, 10)	(0, 5)	(0, 0)			(0.018)
Group III	6.7, 5[a]	2.5, 2.5[ab]	0,0[b]	62.70	100	9.579**
n=6	(5, 10)	(0, 5)	(0, 0)			(0.008)
Group IV	2.5, 0	1.7, 0	0, 0	32	100	3.714[ns]
n=6	(0, 10)	(0, 5)	(0, 0)			(0.156)
F-value	5.367	4.313	-			
(P-value)	(0.147)	(0.230)				

** Significant at 0.01 level (P<0.01); * Significant at 0.05 level (P<0.05); Values in same row bearing same superscript do not differ significantly

using Clinical Lesion Score, Visual Analog Score for Pruritus, and *Malassezia* Index score.

DISCUSSION

Malassezia spp.was commonly isolated from the face, neck, legs, ears and digits of the affected animals (Kumar *et al*., 2011). These yeasts may become opportunistic pathogens causing dermatitis (Figueredo *et al*., 2012). Increased risk of *Malassezia* dermatitis was reported in dog breeds like West Highland White Terriers, English Setters, Shih Tzus, Basset Hounds, American Cocker Spaniels, Boxers, Dachshunds, Poodles and Australian Silky Terriers (Guillot and Bond, 2020). Atopy, seborrhea, hyperadrenocorticism and hypothyroidism were the main predisposing factors in *Malassezia* infections (Reagan *et al*., 2019). The pathogenesis of the genus *Malassezia* depends mainly on the production

Table 7. Comparison of MIS between treatment periods

Group	1st Day	14th day	28th day	Mean % reduction		c^2-value (P-value)
				14th day	28th day	
Group I	25.3, 23.5[a]	6.7, 7[b]	0, 0[c]	73.52	100	12.00**
n=6	(20, 36)	(4, 9)	(0,0)			(0.002)
Group II	26.8, 26[a]	15.8, 15.5[b]	1.3, 1[c]	41.04	95.15	12.00**
n=6	(16, 41)	(10, 23)	(0, 4)			(0.002)
Group III	29.8, 25.5[a]	13.3, 14[b]	1, 1[c]	55.37	96.64	12.00**
n=6	(23, 43)	(6, 20)	(0, 2)			(0.002)
Group IV	16.3, 15.5[a]	11.7, 7[b]	1.3, 1.5[c]	28.22	92.02	10.33**
n=6	(11, 22)	(6, 29)	(0, 3)			(0.006)
F-value	11.418**	10.240*	5.428			
(P-value)	(0.010)	(0.017)	(0.143)			

** Significant at 0.01 level (P<0.01); * Significant at 0.05 level (P<0.05); Values in same row bearing same superscript do not differ significantly

of lipases that plays a role in the invasion, colonization, persistence and proliferation within host tissues (Lautert *et al*., 2011; Petrokilidou *et al*., 2019). Clinically, *Malassezia* dermatitis in canines was characterized by erythematous lesions and greasy exudation in skin folds and toe webs. In chronic cases, the major clinical signs included traumatic alopecia, hyperpigmentation, lichenification and prominent malodor (Bond, 2010). Hromada *et al*. (2005) observed a significant increase in total leukocyte count in dogs infected with *Malassezia* yeasts that might be due to inflammatory changes in the skin. Takahira (2009) reported mild to moderate non-regenerative anaemia in dogs with fungal, bacterial, viral and protozoal diseases. PCR method for *Malassezia* species identification can be used in epidemiological surveys and routine practice (Affes *et al*., 2009). Identification of underlying predisposing factors is necessary to reduce the chance of recurrence of *Malassezia* infection. If predisposing factors cannot be controlled or recurrence is common, administration of routine topical therapy or pulse oral medications can be done (Scott *et al*., 2001). The current study concludes that orally itraconazole and topically neem oil can be used effectively to treat canine malasseziosis. After oral administration, itraconazole is distributed to the epidermis through passive uptake by keratinocytes in the basal layer. Tissue levels of itraconazole are found to be 3–20 folds higher than plasma concentration, and it persists in the stratum corneum for three weeks after stopping therapy and thus prevents recurrence of infection. This explains a reservoir effect of itraconazole over other antifungal therapies (Srivastava and Kothiwala, 2017). Clinical Lesion Score, Visual Analog Score for Pruritus and Malassezia Index score analysis can be used effectively for assessing therapeutic response.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: AKD: Data collection, analysis and interpretation; PVD, CR: Supervised the research work; KSS: Contributed to the implementation of the research.

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