

DIFFERENTIAL MOLECULAR DETECTION OF DERMATOPHYTES IN DOGS USING COMMON PAIR OF OLIGOS IN A UNIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT: Dermatophytes are common pathogenic fungi of fundamental importance in small animal practice and are given due consideration due to their zoonotic potential. A number of techniques are available to diagnose dermatophytosis and differentiate among the genera that cause it, but most of these techniques are either laborious, time consuming or lack cost effectiveness. The current study was designed to develop and validate a rapid and accurate diagnostic assay to detect two important genera of dermatophytes in dogs, *Microsporum* and *Trichophyton* differentially by a uniplex PCR reaction. 18S ribosomal RNA gene of both the genera was used to design common forward and reverse primers. Genome specific product sizes were detected in clinically positive samples. Out of 52 clinically positive dogs based on clinical signs, direct microscopy of skin or hair and wood's lamp technique, 46 samples (88.46%) were positive for dermatophytosis through the uniplex PCR out of which 38 samples (82.6%) were positive for *Microsporum*, 6 samples (13.04%) for *Trichophyton* and two samples (4.34%) were positive for mixed infection of *Microsporum* and *Trichophyton*. The sensitivity of the test was 88.46%. The uniplex PCR was also applied to 20 clinically negative samples. Four clinically negative samples were identified as false positives and 16 samples were true negatives by the uniplex PCR. The specificity of the test was 80.0%. It was concluded that uniplex PCR can be used for accurate diagnosis of dermatophytes in dogs.

Keywords: Dermatophytosis, *Microsporum*, *Trichophyton*, Uniplex PCR and Common oligo.

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INTRODUCTION

Dermatophytosis is a highly contagious cutaneous disease of pets with zoonotic potential (Pal and Dave, 2013). The etiological agents of the dermatophytosis can be categorized into one of the three genera: *Microsporum*, *Epidermophyton* and *Trichophyton* and about 20-25% of the world human population is affected with dermatophytosis (Havlickova *et al.*, 2008). Infection may occur regardless of the breed of the pets, however, Dalmatian, Poodle, Jack Russell Terrier, Manchester terrier, Yorkshire terrier and Persian cats may be at increased risk for generalized Dermatophytosis. Effective and in-time treatment of dermatophytosis is of utmost importance due to concern of pet's health and zoonosis. Different antifungal drugs are being used for treatment of dermatophytosis with target specifications. Terbinafine has been found to be most effective drug in case of infection with *Trichophyton* and *Epidermophyton* (Badali *et al.*, 2015) while Itraconazole has been thought to be drug of choice for *Microsporum* infections (Tosti *et al.*, 1996). The use

of correct drug, however, depends upon an efficient diagnosis system.

Routine diagnosis of Dermatophytes includes slide microscopy, wood's lamp and fungal cultures (Cafarchia *et al.*, 2004). The drawbacks of these methods include non-reliability, inaccuracy and delay in isolation and identification through culture letting animals suffer from disease and consequent complications especially immuno-suppression (Hall *et al.*, 2004). Molecular approaches are more reliable and accurate than detection based upon phenotypic characteristics (Graser *et al.*, 2000). A number of molecular assays have been developed to differentially diagnose dermatophytes in clinical infections (Mehlig *et al.*, 2014; Kac, 2000) but commercially available assays that are cost effective with limited time consumption are rare. Keeping in view the need of private clinical practice, the current study was designed to validate a rapid and cost effective PCR based assay using a common pair of oligos for reliable differential diagnosis of two important genera of dermatophytes.

MATERIALS AND METHODS

Experimental design: A total of fifty two (n=52) clinically positive dog skin scraps for dermatophytosis based on clinical signs, microscopic examination of skin or hair and wood's lamp technique were identified as prospective samples for this research. The samples were collected in a one-year study period starting from 25th December 2014 from veterinary practice, Centre for Animal Health, Lahore. Another twenty (n=20) randomly selected clinically negative samples for ringworm based on clinical signs, microscopic examination and wood's lamp technique were also included in the study during the time period and skin scraps of these dogs were collected.

Sample collection and DNA extraction: Genomic DNA of fungi was extracted from samples by organic Phenol Chloroform Iso-amyl alcohol (PCI) extraction method. Reference DNA sequences of hyper variable region 18-S rRNA gene of *Trichophyton* (Acc. No. AB220045.1, AB166666.1 and AB094674.1) and *Microsporum* (Acc. No. AB193672.1, AB 193687.1 and AB663231.1) were taken from NCBI (<http://www.ncbi.nlm.nih.gov>). Aligned sequences allowed designing of a common pair of positive sense and negative sense primers capable of amplifying genomic products of 351bp and 366bp for *Trichophyton* and *Microsporum* respectively, on in-silico PCR (Table 1, Table 2 and Table 3).

Table-1: Primer sequences and their properties

Primer	Sequence	Annealing (°C)	Product (bp)		Primer positions
			Microsporum	Tricophyton	
VDLDr-F	CAACAACGGATCTCTTGGTTCC	59.52	366	351	345-366
VDLDr-R	TACCTGATCCGAGGTCAACC	58.52			731-712

Table-2: Annealing positions of reverse primer VDLDr-R on reference DNA sequences of hyper variable region 18-S rRNA gene of Microsporum and Trichophyton

Species/Abbrv	Δ	Group Name	- T G T A T T A T T C - G G T T A C C C - - - G G T C A G G - A G G G A T A -
1. Microsporum ferrugineum		AB663231.1	C T C T G C A A T T T A G G T T G A C C T C G G A T C A G G T A G G G A T A C
2. Microsporum incurvatum1		AB193672.1	C T C T A C A - T C T A G G T T G A C C T C G G A T C A G G T A G G G A T A C
3. Microsporum incurvatum2		AB193687.1	- T A T A C T T A T C A G G T T G A C C T C G G A T C A G G T A G G G A T A C
4. Trichophyton tonsurans1		AB220045.1	- T A T A C T T A T C A G G T T G A C C T C G G A T C A G G T A G G G A T A C
5. Trichophyton tonsurans2		AB166666.1	- T A T A C T T A T C A G G T T G A C C T C G G A T C A G G T A G G G A T A C
6. Trichophyton tonsurans3		AB094674.1	- T A T A C T T A T C A G G T T G A C C T C G G A T C A G G T A G G G A T A C

Table-3: Annealing positions of forward primer VDLDr-F on reference DNA sequences of hyper variable region 18-S rRNA gene of Microsporum and Trichophyton.

Species/Abbrv	Δ	Group Name	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
1. Microsporum ferrugineum		AB663231.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
2. Microsporum incurvatum1		AB193672.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
3. Microsporum incurvatum2		AB193687.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
4. Trichophyton tonsurans1		AB220045.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
5. Trichophyton tonsurans2		AB166666.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G
6. Trichophyton tonsurans3		AB094674.1	T A A A A C T T T C A A C A A C G G A T C T C T T G G T T C C G G C A T C G

Thermal cycling conditions and recipe for amplification reactions were optimized using gradient PCR option in a Bio-rad I Cycler. Amplification reaction for all clinical samples along with positive and negative controls were carried out to produce multiple copies of the target regions of dermatophytes with final reaction volume of 25 µL: 2 µL (10ng per µL) of DNA sample, 1 µL of 10pM forward and reverse primers (each), 3 µL of 10mM dNTPs, 2 µL of 10X reaction buffer, 2 µL of 25mM Magnesium Chloride, 3U of DNA Taq Polymerase

and 13 µL double distilled deionized water. The reaction mixture was initially denatured at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 25 seconds, and extension at 72°C for 30 seconds. This was followed by a final extension step for 10 min at 72°C in a thermal cycler. The PCR products were genotyped on 5% Agarose Gel by electrophoresis at 100 volts for 45 minutes to separate specific PCR products based upon their sizes.

Validation of Uniplex PCR: The uniplex PCR was validated on the 52 skin scrap samples initially diagnosed through clinical signs, microscopic examination and wood's lamp technique and 20 randomly selected clinically negative samples. Epidemiological estimates of the test like sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), false positive and false negative rule and likelihood ratio were calculated using a 2x2 contingency table.

RESULTS AND DISCUSSION

Extraction of genomic DNA from skin scrap samples were of good quantity as determined by absorbance readings ratio at 260/280 nm. The ratio was 1.76 ± 0.13 and the DNA concentration was above 100ng/μL. All the positive clinical samples and positive controls of the uniplex PCR produced results of 351bp for *Trichophyton* and 366bp for *Microsporum*. Most of the samples were infected with single type of dermatophyte genus; however, some clinical samples produced results of mixed infection by producing characteristic bands of *Microsporum* and *Trichophyton* (Figure 1). Fifty two clinically positive samples were tested for dermatophytosis by uniplex PCR out of which 46 samples (88.46%) were found positive. Samples found infected with genus *Microsporum* were 38 (82.6%) while 6 (13.04%) of the positive samples were detected positive for genus *Trichophyton*. 2 (4.34%) of the positive samples produced results for mix infection by both genus *Microsporum* and genus *Trichophyton*. The sensitivity of the test was 88.46%.

4 false positives and 16 true negatives were also identified by the uniplex PCR in 20 clinically negative dermatophytes samples. All the false positives were of *Microsporum* genera. The specificity of the test

calculated was 80%. Positive predictive value was 92% and negative predictive value was 72%. False positive rate or Type I error was calculated to be 20% and false negative rate or Type II error was calculated to be 11.54%. Positive likelihood ratio was equal to 4.4 and negative likelihood ratio was 0.15. Apparent prevalence based on N=72 was 69.44% and true prevalence was 72.22% (Table 2).

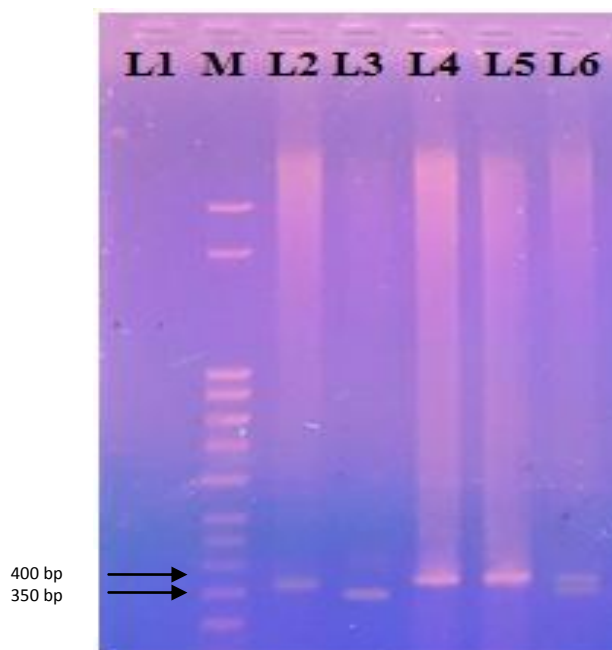


Figure-1: Agarose gel based genotyping of PCR products. Negative Control (L1), 50 bp DNA Marker (M), *Trichophyton* positive samples 366 bp (L2, L4, L5), *Microsporum* positive sample 351 bp (L3) and mixed infection by *Microsporum* and *Trichophyton* (L6).

Table-2: A 2x2 contingency table for evaluating the performance of uniplex PCR assay. (Dogs having ringworm disease = 52, without ringworm disease = 20 based on clinical signs, Microscopic examination of skin scrap and Wood's Lamp technique, n=72).

		Ringworm Disease (Clinical signs, Microscopic examination of skin scrap and Wood's Lamp Technique)		
		Disease	No Disease	
Uniplex PCR	Positive	46 (True Positive)	4 (False Positive)	Positive Predictive Value = 92%
	Negative	6 (False Negative)	16 (True Negative)	Negative Predictive value = 72%
		Sensitivity = 88.46%	Specificity = 80%	N = 72

Dermatophytosis is one of the prominent zoonotic infections having high potential to be transferred from pets to human. Timely differential diagnosis is necessary to establish effective treatment strategy to

avoid complications of infection in both animals and human. Present study defined and validated a simple diagnostic assay to detect two important genera of dermatophytes. It has been investigated that diagnosis of

dermatophytosis based upon wood's lamp test and clinical findings provide an accuracy of almost 50% (Graser *et al.*, 2012) which is far less than the specificity and reliability of any molecular assay based protocol. Use of molecular assays for detection of dermatophytes in initial step and individual dermatophyte genera in following steps, is a common tool to diagnose dermatophytosis in clinical samples. Two pairs of oligos have been employed to detect dermatophytes in general and then specifically *Trichophyton* species using second pair of oligos (Spiliopoulou *et al.*, 2015). Though authentic, such approach is time consuming, labour intensive and cost ineffective. Present study, however, targeted this lapse by validating differential diagnostic techniques between two dermatophyte genera in a single reaction to reach exact diagnosis in time.

Application of real time PCR is also proposed for detection of dermatophytes. Probe based real time PCR technique is used to diagnose different pathogenic forms of fungi in human at a time (Vollmer *et al.*, 2008). The oligos and probes designed in the study complemented all types of pathogenic fungi without discrimination of genera of species in results. The present assay in comparison provides a suitable alternative technique for detection of dermatophytes differentially without adding up the cost factor of real-time PCR hence making this strategy cost economical in commercial market. Direct RFLP analysis is another molecular tool to differentiate pathogenic fungi to differentiate between dermatophytes and other fungal species (Machouart *et al.*, 2001). They adopted the said procedure considering the issue of cross reactivity of oligos between dermatophytes and other pathogenic fungi such as *Scytalidium spp.* in qualitative PCR technique. However, in current investigation, extensive alignment analysis between three genera and their species were performed to design primers avoiding cross complementation with other dermatophytic and non-dermatophytic fungi.

A multiplex PCR technique based on the pairs of oligos targeting ITS-2, 18-S rDNA and 28-S rDNA regions to differentiate between dermatophytic genera has also been validated (Kim *et al.*, 2011). The involvement of amplification of three genomic regions and their consequent complex analysis presents drawback of this technique if this is to be employed as rapid diagnostic assay in clinical samples. This problem was overcome in current investigation by reducing involvement of number of genomic regions in analysis. Only hyper variable region of 18-S rDNA gene was selected to target for amplification with common pair of oligos not only to diagnose dermatophytosis but also to differentiate between two common genera of dermatophytes based upon amplicon sizes, hence reducing the assay labour and complexity.

The 2x2 contingency table estimates of the present uniplex PCR were also dynamic to assess the

performance of the assay. The sensitivity of 88.46% indicated the ability of the test to correctly identify the ringworm cases (n=52) as diseased and specificity of 80% showed the ability of test to identify the ringworm negative samples (n=20) as free of disease. The negative predictive value of 72% was calculated indicating that uniplex PCR is 72 % efficient in identifying the uniplex PCR negatives as free of ringworm. The positive predictive value of 92 % indicated that about 92% of uniplex PCR positives are actually positive for ringworm. The false negative rate was 11.54% and false positive rate was 20%. The estimates indicated that the uniplex PCR is efficient in diagnosing disease.

Conclusion: This study led to establishment of a rapid, effective, reliable and cost effective assay based upon molecular technique to diagnose and differentiate between two different genera of dermatophytes. Validation of differential diagnosis between dermatophytes can greatly aid clinicians to treat cases of animals with specific drugs hence reducing the risk of zoonotic transmission.

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