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Research Article

DIAGNOSIS OF CANINE DEMODICOSIS BY INDIRECT- ELISA IN HYDERABAD OF TELANGANA STATE

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Abstract- Canine demodicosis is an inflammatory, non pruritic and non contagious condition is characterized by the presence of numerous mites on the skin. Skin scraping, trichoscopy and skin biopsy are the common diagnostic methods. The existence of subclinical and asymptomatic infection in the skin makes it difficult for veterinarians to diagnose the infection. Hence the present study was aimed to develop a laboratory standardized Indirect ELISA for diagnosis and seroprevalence of canine demodicosis in and around Hyderabad. *Demodex canis* antigen concentration was optimized at 0.5 µg per well of ELISA nunc plate against 1:50 anti- *Demodex* positive sera and 1:5000 dilution of Rabbit anti-dog IgG HRPO conjugate. Out of 88 samples collected from animal birth control centers 17 were positive (19.31 %) and out of 72 samples from pet clinics 22 were positive (30.55 %) with Indirect ELISA. The overall sensitivity of test was 70.58% and specificity was 76.92%.

Keywords- ELISA, Canine Demodicosis, Demodicosis.

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Introduction

Dog was the first carnivore to be domesticated and had been utilized for hunting, patrolling in policemen services, in wars and as companion. Being the largest part of body organ called skin, which protects from external injuries and gives a charming look to the dog. Cutaneous ectoparasitosis or mange is one of the important skin manifestations of dogs and in some instances they may cause a nuisance, debilitation and prove to be life threatening, it is caused by *Sarcoptes scabei* and *Demodex canis*.

Canine demodicosis also known as red mange or follicular mange recognized as localized, generalized, squamous and pustular form most prevalent in short haired breeds of puppies. Natural infestation is apparently acquired neonatal through contact with the nursing bitch when the adult mites invade the hair follicles [1]. This condition is an inflammatory, non pruritic and non contagious characterized by the presence of numerous mites on the dermis of dog. Various diagnostic methods were included *viz.* skin scraping, trichoscopy and skin biopsy, among these skin scraping is the most common method and considered as the gold standard for diagnosis. The existence of subclinical and asymptomatic infection in the skin makes it difficult to the petowners and veterinarians to diagnose the infection. Scarcity of experimental studies on humoral response against *Demodex* mites [2] the present study was prompted to explore on development of an immunological diagnostic technique to caution the pet owners in advance about the disease condition.

Materials and Methods

Collection of skin scrapings

Dogs showing clinical signs like alopecia, itching, erythema, papules, pustules were selected. The lesion area was cleaned by using 70 % alcohol. Affected skin fold was squeezed between the fingers to extrude the mites from the follicles and sebaceous glands before the scraping is done. Deep skin scrapings were collected by using rounded scalpel blade no 10 by scrapping the blade back and forth over the skin until capillary bleeding is evident. 160 canine serum samples were collected, out of which 88 were stray dogs and 72 were from pet dogs collected from different hospitals in Hyderabad for evaluation of laboratory standardized ELISA using Crude somatic antigen of *Demodex canis*.

Collection of reference sera

The serum collected from dogs with a history of dermatological aberrations (teaching veterinary hospital complex) which were found to be positive for demodicosis by skin scraping test were used as a positive control sera in ELISA. Similarly, the serum collected from the day old pups which is free from any kind of dermatological lesions and whose health is apparently normal was used as negative control in ELISA. The sera was preserved by adding 1:10,000 merthiolate suspension, aliquot and stored at -20 ^{0}C until further use.

Preparation of Crude Somatic Antigen

The infected hair with cheesy material was washed several times in sterile PBS (pH 7.4) and all the fur material was removed absolutely until the supernatant fluid became clear and then examined to ensure that it was rich in mites, then transferred to eppendorf tubes containing PBS (pH 7.4) and stored at -20 °C until further use. Pooled mites were washed five times in the Phosphate Buffer Saline (PBS) and finally were homogenized in 1 ml of cool 0.15 M PBS (pH-7.4) containing two to three drops of 1mM phenyl methyl sulfonyl fluoride (PMSF) maintained at 4 ^{0}C in a glass tissue homogenizer. 1ml volume of homogenate is obtained, to which 7ml of cool 0.15 M PBS (pH-7.4) was added to make up volume to 8 ml in a 15 ml centrifuge tube. Homogenized mites were disintegrated by sonication (Sonics vibracell) at 20 watts, of 5 cycles each of 30 sec duration with four intervals of 5 sec each on ice bath as per the method described by [3]. The disintegrated contents were extracted at 4 ^{0}C overnight and then centrifuged

at 4 ^{0}C at 15,000 g for 30 minutes [4] and the resultant supernatant was collected as soluble antigen and stored at -20 ^{0}C by adding 1mM PMSF. The protein concentration of the mite antigen in the supernatant was determined by using a nano drop lite spectrophotometer (Thermo scientific) and this crude somatic antigen was aliquoted and stored at -20 ^{0}C for further use in the assay.

Standardization of ELISA &Optimization of anti-dog IgG HRPO conjugate

Checker board titration was performed to determine the optimal working dilution of antidog IgG HRPO conjugate (Sigma-aldrich), optimal concentration of *Demodex canis* antigen and the optimal working dilution of dog *Demodex canis* positive and negative serum as per the procedure described by [5].

To determine the working dilution of antidog IgG HRPO conjugate, 50 µl of normal dog serum free from any dermatological conditions diluted at 1:100 in coating buffer (0.2M carbonate bicarbonate buffer- pH – 9.6) was added to 96 well ELISA plate and incubated at 37 °C for 1 hour. The ELISA plate was washed thrice with (PBS-T), then blocked with blocking buffer with 1% BSA to block the non-specific reactive sites and incubated at 37 °C for 1 hr. Then the plate was washed with washing buffer thrice. Test conjugate dilutions from 1:1000, 1:2500, 1:5000, 1:10000, 1:15000, 1:20000 were prepared in blocking buffer (PBS-T-BSA) and 50 µl of each dilution was added to the wells and incubated at 37 °C for 1 h. After washing for three times each for 5 minutes, 50 µl of freshly prepared substrate solution containing OPD with 30% H_2O_2 was added to each well and kept at room temperature for 10 min. Finally the reaction was stopped by adding 50 µl of 1M H_2SO_4 to each well and the absorbance was recorded by using automated ELISA plate reader (Multiskan Lab systems) at 492 nm.

Determination of optimal concentration of *Demodexcanis* antigen

To determine the optimal concentration of *Demodex canis* antigen and dog positive serum, 50 µl of different dilutions of *Demodex canis* antigen (4µg, 2µg ,1µg, 0.5µg, 0.25µg, 0.125µg, 0.0625µg) in coating buffer (0.2M carbonate bicarbonate buffer- pH – 9.6) was added to 96 well flat bottom ELISA plate these plates were incubated at 4°C overnight and washed thrice with PBST and blocked with 1% BSA and incubated at 37 °C for 1 hr titrated with different dilutions of positive dog serum starting from 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600 incubated at 37 °C for 1 hr and then washed thrice. Add 50 µl of previously determined optimal dilution of laboratory standardized conjugate anti-dog rabbit HRPO conjugate diluted in blocking buffer was added to all the wells in the plate and incubated at 37 °C for 1 hour.

Determination of optimal working dilution of dog serum

To determine the optimal working dilution of *Demodex canis* dog positive serum, 50 µl of Laboratory standardized *Demodex canis* antigen diluted in coating buffer was added to ELISA plate incubated overnight at 4°C for coating of antigen and blocked with 1% BSA and incubated at 37 °C for 1 hr. Repeat washing step and titrated with different dilutions of positive dog serum (1:50, 1:100, 1: 200, 1: 400, 1: 800) and incubated at 37 °C for 1 h and then washed thrice with PBST.

Determination of cut-off value

Ten negative serum samples were obtained from the day old pups, which were free from any kind of dermatological lesions and apparently normal health were used to determine the cut off. The mean and standard deviation of ten negative sera samples were calculated. The performance of the serological assay is to be validated by adding 2 times the standard deviation to the mean OD values of the negative controls [6]. Sera samples with OD value above this cut off point was considered positive.

Protocol: The 96 well ELISA plate was coated with 50µl containing 0.5µg of Crude Somatic Antigen of *Demodex canis*. The plate was incubated at 4 °C overnight and washed thrice with PBS Tween-20 buffer. Add 75 µl of blocking buffer (1% BSA with PBS Tween-20) and then plates were incubated at 37 °C for 1 hr, test sera (1:50 dilution) was added to the wells and incubated for 1 h at 37 °C. The plates were washed three times with washing buffer and 50 µl of 1:5000 diluted anti-dog rabbit HRPO conjugate was added and incubated at 1 h at 37 °Crepeat washing thrice with PBST and then 50 µl of freshly prepared substrate solution containing OPD with 30% H_2O_2 was added to each well and kept at room temperature for 10 min. Finally, the reaction was stopped by adding 50 µl of 1M H_2SO_4 to each well and the absorbance was recorded by using automated ELISA plate reader (Multiskan Lab systems) at 492 nm.

Sensitivity and Specificity of ELISA

The sensitivity was measured by the following formula

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \times 100$$

The specificity thus arrived was measured by the formula as follows

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative + False positive}} \times 100$$

Results:

The protein concentration of crude somatic antigen was 0.448 mg/ml respectively. The working strength of anti-dog rabbit HRPO conjugate, *Demodex canis* antigen and serum concentration were found to 1:5000, 0.5 µg/well and 1:50 respectively by checkerboard titration yielded a mean OD of 0.354 with a standard deviation (SD) of 0.113. The cut-off value was adjusted to 0.58 (Mean + 2SD) and the samples which gave OD above this value were considered as positive for *Demodex canis*. Out of 160 sera samples screened 39 were positive indicating 24.37% infection with Crude Somatic Antigen of *Demodex canis.* Out of 88 stray dogs 17 were positive and out of 72 pet dogs 22 were detected as positive with ELISA. The sensitivity of ELISA was determined as 70.58% and specificity was 76.92%.

Discussion

ELISA is the most widely used immunological technique in measuring concentrations of antibody, antigen and protein [7]. In the present study the method of collection of skin scrapings was similar with [8] but squeezing of skin fold between fingers over demodectic lesion before scraping yielded more number of mites. These harvested *Demodex* spp. Appeared with cigar shape body divided into three distinct portions gnathosoma, podosoma and opisthosoma, the gnathosoma was bearing mouth parts consisting of paired palps and an unpaired hypostome. Podosoma bearing four pairs of stumpy legs. Long and slender opisthosoma shows transverse striations on dorsal and ventral surfaces was observed similar to reported by [9].

The protein concentration of crude somatic antigen obtained in the present study was 0.448 mg/ml which was less compared to protein concentration 70 µg/100µl obtained [3] study. [10] Yang prepared sodium and urea soluble antigen by Sephadex G100 with a protein concentration is 1.4mg/ml for sodium soluble antigen and l.lmg/ml for urea soluble antigen. The protein concentration of crude somatic antigen obtained in the present study was 0.448 mg/ml which was less compared to [10] and [3] study, this might be due to presence of more number of mites in their collection during extraction of proteins and severity of the demodicosis depends on the immune status of the animal. The protein concentration of crude somatic antigen 0.448 mg/ml obtained in the present study was higher when compared to 6.8 ng/µl observations obtained by [2]. The difference might be due to harvesting protocol and separation of each single mite by visualizing under optical microscope (10X), following this 1200 mites were collected and then disrupted in liquid nitrogen. The protein concentration is directly proportional to severity of the condition and method of harvesting. Similarly, [2] evaluated juvenile generalized demodicosis dogs and elicited humoral response against different crude proteins of *Demodex canis* in the western blot. The protein concentration in the present study was 0.5 µg per well obtained by checker board titration as per protocol of [5] and antigen concentration of 1 µg was lesser and sera (1:50) and conjugate (1:5000) dilutions were higher as compared to the findings of [11].

In the present study, Out of 88 samples collected from animal birth control centers 17 were positive (19.31 %) and out of 72 samples from pet clinics 22 were positive

(30.55 %) with Indirect ELISA. The overall sensitivity of the present assay was 70.58% (12 out of 17 true positive) which might be due to clinical cases showing localized demodicosis or primitive stages of infection which could not induce sufficient amount of antibodies for binding to antigen in the test. The overall specificity of ELISA was 76.92% (10 out of 13 negative sera), it may be due to crude nature of the *Demodex* antigen and interference of the maternal antibodies which are present in the serum in the given assay. It was observed that Rabbit anti-dog IgG HRPO conjugate was reacting to *Demodex canis* antigen even in the absence of anti *Demodex canis* positive sera might be due to presence of some amount of dermal collagen present in the antigen. Hence, the parasitological diagnosis by harvesting mites from skin scrapings was found to be inadequate as a gold standard for determination of sensitivity of the ELISA. Hence an improved technique for purification of mites is required for using in serodiagnostic tests. This was the first attempt in the country for diagnosis of canine demodicosis by serological assay like ELISA to detect the presence of the antibodies in dog serum exposed to *Demodex* antigens.

He [2] reported the mechanisms of mite colonization and factors triggering mite population, hence, immunogenic protein of *Demodex canis* is to be identified then there is also a chance for development of vaccine against canine demodicosis. Though Demodicosis can be detected by simple skin scraping test, which will be similar to detection of helminthic infection by faecal examination, but to caution the pet owner in advance till it gets chronic leading to morbid condition and unaesthetic appearance.

Conclusion

It is concluded that demodectic mange in dogs can be easily confused with many dermatological conditions. Traditionally skin scraping is the only reliable and gold standard diagnostic method available, hence the results of present study are relevant as it was able to detect the presence of serum IgG antibodies against *Demodex canis* by standardized ELISA and if further characterization and purification of immunodominant *Demodex canis* antigen is done then the sensitivity and specificity of the assay can further be improved.

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Author Contributions:

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Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations

µg: Microgram
µl: Microlitre
^{0} C: Degree centigrade
BSA: Bovine Serum Albumin
ELISA: Enzyme Linked Immuno Sorbent Assay
H_2SO_4: Sulphuric acid
HRPO: Horse radish peroxidase enzyme
IgG: Immunoglobulin G
ml: mili litre
OD: Optical Density
OPD: Ortho phenylene diamine
PBS: Phosphate buffered saline
PBST: Phosphate buffered saline with Tween 20
PMSF: Phenyl Methyl Sulfonyl Fluoride
SD: Standard deviation
viz: Namely

Conflict of Interest: None declared

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