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Original Article

Prevalence of Canine Monocytic Ehrlichiosis in Canine Population Across India

Kukreti [1], K., Das [1], M.K., Rastogi [1], A.K., Dubey [1], R., Pandey [2,*], L.K., Sharma[3], P.

1. Biotechnology Section, Central Military Veterinary Laboratory, Remount Veterinary Corps, Meerut Cantt, Uttar Pradesh, 250001, India
2. Department of Biotechnology, St. Aloysius College, Jabalpur, India
3. Department of Microbiology and Immunology, Seoul National University, Seoul, South Korea

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Corresponding Author: lkpandey16@gmail.com

ABSTRACT

Canine ehrlichiosis is a very important emerging disease in India. This study is the first attempt screening a large number of canines in India for the detection of canine monocytic ehrlichiosis. In the present study, 510 blood samples of dogs were screened for the presence of *Ehrlichia canis* and other variants of *Anaplasmataceae* family by serological and molecular methods. Out of the 510 serum samples, 293 (57.5%) cases were found positive for the presence of *E. canis* antibodies through enzyme-linked immunosorbent assay (ELISA). Furthermore, and 45 (8.8%) and 1 (0.2%) specimens were positive for *E. canis* and *A. platys*, respectively, based on the polymerase chain reaction (PCR). In the clinical samples of *E. canis*, the minimum detection limit for PCR was 9 ng. In the immunofluorescence assay (IFA), the positive blood samples showed comparable results with those obtained from the commercially available dot ELISA kit (giving equivalent IFA titer). The results of sequencing were compared with other reported isolates in various regions of the world, and a phylogenetic relationship was established. The 16S rRNA region that was amplified and sequenced for *E. canis* and *A. platys* was highly conserved and so was another Vir B9 region.

Keywords: *Ehrlichiosis Canis*, *Anaplasma platys*, Polymerase chain reaction, ELISA, India

Étude de la prévalence de l'infection par l'érythème monocytaire canin chez des populations canines en Inde

Résumé: L'ehrlichiose canine est une maladie émergente très importante en Inde. C'est la première fois en Inde qu'un si grand nombre de populations canines ont été examinées pour la détection de l'ehrlichiose monocytaire canine. Dans cette étude, environ 510 échantillons de sang de chiens ont été examinés pour la présence d'*Ehrlichia canis* et d'autres variantes de la famille *Anaplasmataceae* par des méthodes sérologiques et moléculaires. Sur 510 échantillons de sérum, 293 (57,5% (étaientpositifs et présentaientdes anticorps contre *E . canis* en ELISA ,alors que seulement 45 (8.8%) échantillons étaient positifspour *E . canis* et 1 (0,2%) pour *A. platys* en PCR. Dans les échantillons cliniques d'*E . canis* ,la limite de détection minimale pour la PCR était de 9 ng .Dans le test IFA, les échantillons de sang positifs traités ont montré des résultats comparables avec le kit ELISA dot disponible dans le commerce donnant un titre d'IFA équivalent. Les résultats du séquençage ont été comparés avec d'autres isolats signalés dans diverses régions du monde et une relation phylogénétique a été établie. La région de l'ARNr 16S qui a été amplifiée et séquencée pour *E . canis* et *A. platys* s'est avérée être hautement conservée, tout comme une autre région Vir B9.

Mots-clés: *Ehrlichiosis canis*, *Anaplasma platys*, Polymerase Chain Reaction, ELISA, Inde

INTRODUCTION

Canine monocytic ehrlichiosis, which is a tick-borne infection, is caused by rickettsia *Ehrlichia canis* and was initially reported in Algeria, North Africa (Donatien, 1935). However, currently, this disease has a global distribution mainly in tropical and subtropical regions. The brown ticks *(Rhipicephalus sanguineus*) and *Ixodus* ticks are carriers for the organism causing ehrlichiosis. *Amblyomma* and *Dermacenter* ticks are also shown to be involved in the transmission of this disease (Goldman, 1998). The acute phase of ehrlichiosis occurs within 1-3 weeks and emerges with such clinical signs as listlessness, swollen lymph nodes, anorexia, fever, as well as eye and nasal discharge. The chronic phase entails such symptoms as widespread haemorrhage, increased mononuclear cell infiltration of organs, nosebleed or other abnormal bleeding, and weight loss, along with the acute phase symptoms. In both phases, the damage in the body is associated with destruction and decreased production of all blood cells. The development of subclinical infection is more common. In this regard, dogs remain as the carrier of the disease, and this phase persists for as long as 4-5 years. Therefore, it is extremely important to diagnose ehrlichiosis at an early stage to improve the prognosis of the treated dogs. The members of *Anaplasmataceae* family are regarded as the agents of many veterinary diseases worldwide, and are also emerging in zoonotic forms. For instance, *A. platys* was reported to infect the circulating platelets among dogs in Japan causing infectious cyclic thrombocytopenia (Inokum, 2000). *A. platys* causes a cyclical risckettsiaemia of 10-day interval with thrombocytopenia of reducing severity with development of immunity. Polymerase chain reaction (PCR) has been found to be a sensitive and specific test for the diagnosis of both acute (McBride, 1996) and persistent subclinical phases (Harrus, 1998). This technique can properly confirm the initial diagnosis of ehrlichiosis if used along with serology (Neer, 2002). With this background in mind, the present study aimed to compare the accuracy of molecular methods with that of the serological methods to enhance the quick and accurate diagnosis of these infections. In addition, sample sequencing was performed to further confirm the specificity of these methods.

MATERIALS AND METHODS

Sampling. The whole blood samples of the dogs showing ehrlichiosis symptoms were obtained from various military veterinary hospitals in different regions across India. Furthermore, the ticks were obtained from the Veterinary Hospitals of Meerut Cantonment, Uttar Pradesh, India.

Serological tests. A total of 510 blood samples were obtained from the dogs to detect the presence of *E. canis* antibodies utilizing commercially available Immuno Comb Canine Ehrlichia Antibody Test Kit (Biogal Galed Labs). The laboratory standardized protocols were developed for the estimation of antibodies against *E. canis* from the canine serum samples using canine ehrlichiosis immunofluorescence assay (IFA) kit (VMRD Pullman WA USA).

Preparation of DNA. DNA extraction from canine whole blood and tissue samples was accomplished using Qiagen Dneasy Blood and Tissue Kit following manufacturer's protocol. The engorged ticks were rinsed in distilled water, and then dried and triturated in a sterile tube. Subsequently, DNA was extracted and stored at 4 °C for immediate use and at -20 °C for longer storage.

Polymerase Chain reaction. The nested PCR of 16S r-RNA gene of *E. canis* was performed in two separate tubes in 25 µl reaction mixture, containing 0.4 µM of each primer amplifying 16S rRNA region, 1X Taq buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates, and 0.1 U of Taq DNA Polymerase (Sigma-Aldrich). The amplification reaction was carried out in Mastercycler$^{@}$ep realplex (USA). The cycling conditions included initial denaturation for 5 mins at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 61 °C (annealing), and 1 min at 72°C, followed by a final extension of 10 min at

72 °C. The outer primer pairs, namely EC1 (Table 1) and EC2, and inner primer pairs, namely EC3 and EC4 (Wen et al., 1997), had similar PCR conditions, except that the template was reduced to one-half the concentration for the inner primer pairs. The PCR amplification for *A. platys* was carried out in a final volume of 25 µl, containing 0.4 µM of each primer, 1X Taq buffer, 1 mM $MgCl_2$, 240 µM of each deoxynucleoside triphosphates, and 0.1 U of Taq DNA Polymerase (Sigma-Aldrich). The amplification reaction was carried out with the initial denaturation of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 30 sec at 62 °C (annealing), and 30 sec at 72 °C, followed by a final extension of 10 min at 72 °C. The primers were EPLAT5 and ERB2 amplifying 16S r-RNA gene of *A. platys* (Sparagano et al., 2003). Another confirmatory PCR amplification for *E. canis* amplifying *Vir B9* gene was performed in a final volume of 25 µl, containing 0.4 µM of each primer, 1X buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates, and 0.1 U of Fas Taq DNA Polymerase (Chromous). The amplification reaction was carried out in Mastercycler@ep (eppendorf) using Ehr1401F and Ehr1780R primers (Kledmanee et al., 2009). The cycling conditions entailed the initial denaturation of 2 min at 94 °C, followed by 35 cycles of 10 sec at 94 °C, 20 sec at 59°C (annealing), and 10 sec at 72 °C, followed by a final extension of 5 min at 72 °C. For the negative control whole blood samples obtained from the earlier *E. canis*-negative tested dogs and for the positive control reference DNA of *E. canis* Oklahoma strain obtained from the University of Texas, Austin, Texas, and stored whole blood samples of earlier positive tested dogs for *E. canis* has been used.

Amplicon detection. Amplified DNA was run in 2% agarose gel at 90 V and stained with ethidium bromide (0.5 µg/µl) for 1 h. After staining, the products were visualised in Alpha imager@ EP UV transilluminator (Alpha Innotech, USA).

Minimum detection limit. The PCR amplification sensitivity was determined by using a plasmid control and DNA dilutions of known concentration in PCR. The concentration of DNA was calculated by Nanodrop ND 1000 spectrophotometer (Germany).

Sequencing. Samples found positive with PCR assays were amplified for the Vir B9 region with forward and reverse primers of A17-EC5 and 817R-EC6, respectively (Iqbal et al., 1994). Then, they were sent for sequencing to Bioserve Technologies Sequencing Facility Hyderabad, Secunderabad, India. The evolutionary history was ascertained by the UPGMA method (Sneath et al., 1998).

RESULTS

The serum samples of the dogs were subjected to enzyme-linked immunosorbent assay (ELISA) and IFA for detecting the presence of *Ehrlichia* antibodies. Out of the 510 serum samples, 293 (57.5%) cases (Table 2) were found positive for the presence of *E. canis*

Table 1. List of primers used in this study

S. No	Name of the primer	Primer Sequence (5' to 3')	Size of the primer (in bp)
1	EC1	AGAACGAACGCTGGCGGCAAGCC	23
2	EC2	CGTATTACCGCGGCTGCTGGC	21
3	EC3	TATAGGATCCGTCATTATCTTCCCTAT	27
4	EC4	CAATTATTTATAGCCTCTGGCTATAGGAA	29
5	EPLAT5	TTTGTCGTAGCTTGCTATGAT	21
6	ERB2	CTCCGGACTCTAGTCTG	17
7	Ehr1401F	CCATAAGCATAGCTGATACCTGTTACAA	28
8	Ehr1780R	TGGATAATAAAACCGTACTATGTATGCTAG	30
9	A17-EC5	GTTTGATCCTGGCTCAG	17
10	817R-EC6	GAGTTTTAGTCTTGCGAC	18

antibodies antibodies through ELISA. In the IFA, the processed positive canine blood samples showed comparable results with the findings obtained by the commercially available dot ELISA kit.

Table 2. Data on seroprevalence studies for *E. canis* antibodies using commercially available dot ELISA KIT[a] for all over India

Region	Total Sample[b]	Positive	%Positive
Northern India	210	152	72.4
Eastern India	89	21	23.6
Central India	120	67	55.8
Western India	91	53	58.2
Total	510	293	57.5

[a] Immuno Comb Canine Ehrlichia Antibody Test Kit (Biogal Galed labs). [b]Serum samples

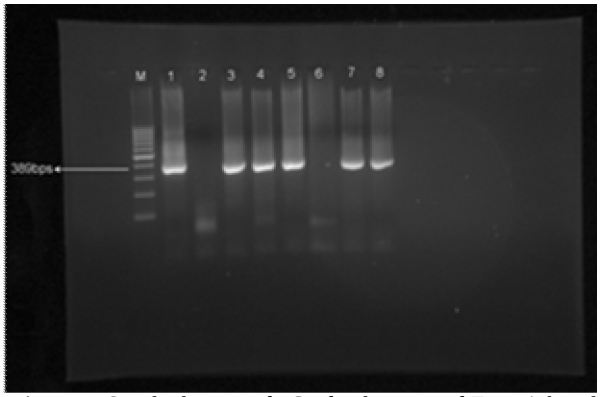

Figure 1. Standardization of PCR for detection of *E. canis* based 16 S rRNA gene.

Lane M: 100bp DNA Ladder, Lane 1: PCR product from reference sample, Lane 2 & 6: Negetive sample, Lane 3-5 & 7: PCR product of a positive sample, Lane 8: positive control.

Out of the 510 samples tested by PCR, 45 and 1 specimens were positive for *E. canis* and *A. platys*, respectively. During the primary reaction of the nested PCR using EC1 and EC2 primers, amplified partial 16S r-RNA gene (i.e., a 478 bp amplicon) was observed in 46 samples, out of which 45 and 1 cases were found positive for *E. canis* and *A. platys*, respectively, in their specific tests. During the secondary reaction with EC3 and EC4 primers, a 389 bp (Figure 1) amplicon was observed in 45 samples confirming the presence of *E. canis*. All *E. canis*-positive samples were positive again giving an amplicon size of about 380 bp, when they were amplified using EhrF and EhrR primers specific to *Vir B9* gene. In addition, 10 cases of the negative samples were randomly tested with *VirB9* protocol and found negative, proving the specificity of the test. One of the samples, which showed a band of 478 bp in primary reaction using EC1 and EC2 primers, but did not show amplification with EC3 and EC4, was amplified using EPLAT5 and ERB2 primers and demonstrated a positive band of about 556 bp. All the ticks' DNA tested by the above PCRs were negative.

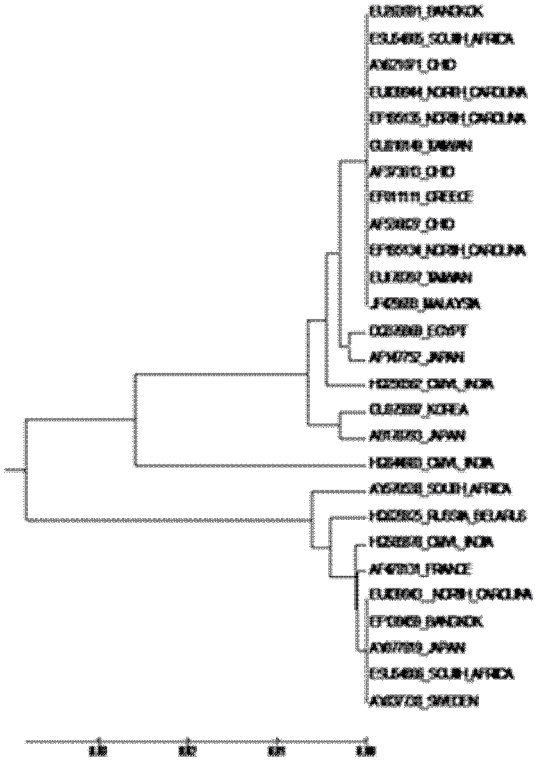

Figure 2. UPGMA-rooted tree obtained with mega align software from Vir B9 gene sequences from data banks. Designations for sequences determined in this study are follows HQ290362, HQ844983, and HQ585878.

All the negative controls showed no bands; however, very low molecular bands were observed in the negative controls, positive controls, and samples, which were presumed to be primer dimers. The positive samples and controls showed no such non-specific

bands, and the observed amplicons were distinct and specific. *Ehrlichia* reference strain DNA was diluted to the concentration of 0.0014 ng/µl, and thereby PCR could detect up to 0.014 ng of DNA. The clinical sample of DNA was diluted till 0.045 ng/µl; therefore, PCR could detect up to 9 ng of DNA. In the *Vir B9* gene screening of *E. canis*, we matched our sequence of JF706287 with the Mexican (AY205342 and AF546158), Venezuelan (AY205343), Hawaiian (AY205341), Californian (AY205340), and Arizonian (AY205339) isolates, revealing 98% identicality to all of the sequences. Our *A. platys* sequence, namely HQ585878, was 99% similar to *A. platys* sequences obtained from the USA (EU439943), Bangkok (EF139459), Japan (AY077619), France (AF478131), South Africa (ESU54806), and Sweden (AY837738). Furthermore, it was found 98% analogous to the South African isolate (AY570538) and Russian-Belarusian isolate (HQ629925). In 16S rRNA screening for *E. canis* of our isolate, namely HQ290362, we found 98% similarity to the Malaysian (JF429693), Taiwanese (EU178797), American (EF195134, EF195135, AF536827, and AF373613), and Greek isolates (EF011111 and AF147752), as well as complete sequence of *Ehrlichia chaffensis*. In addition, it was found 97% identical to the Egyptian (DQ379969) and Korean (GU075697) isolate, and 96% similar to the Japanese isolate (AB178793). However, our isolate, namely HQ844983, was only 94% comparable with the Taiwanese (GU810149), Brazilian (EF195135), American (EU439944 and AY621071), Thai (EU263991), and South African (ESU54805) isolates (Figure 2).

DISCUSSION

Canine monocytic ehrlichiosis is a potentially fatal disease caused by rickettsia *Ehrlichia canis*. Chronic ehrlichiosis may result in leukaemia and lymphosarcoma. Therefore, it is of utmost importance to diagnose this deadly canine disease as early and accurately as possible. The detection of this disease has been successfully accomplished through IFA assay. Our IFA results were comparable to those obtained by the commercial dot ELISA kit, showing equivalent IFA titres. However, this test has its own limitations, canines may show negative test in acute phase due to delay by their immune systems in forming antibodies. They may also show negative to low titres in chronic stage when their immune system is weakened. Moreover, serologic test results are unable to distinguish between current infection and exposure without infection or a previous infection. The IFA test has been the most applied method for the diagnosis of canine ehrlichiosis since its development in 1972 (Ristic, 1972). Ixodid ticks are well known vectors for *E. canis*; however, we were unable to implicate them as vector for either *E. canis* or *A. platys* because we could not detect their infection by PCR. The PCR has been shown to be very sensitive for the detection of fastidious organisms, such as the members of *Ehrlichia* genus (Iqbal, 1994; Engvall, 1996). Positive and negative controls were also included to ensure the specificity of the reaction. All PCRs showed reproducibility, when re-run for the confirmation of infection. The PCR is a very helpful technique for the detection of infection at the acute phase and reduces the Probability of missing an infection. We were unable to develop a sensitive multiplex PCR assay for *Babesia* species and *E. canis* unlike Kledmanee, (2009), as the bands were very faint. Moreover, many known positives for *Babesia* genus were not detected in spite of manipulating the proportion of primers and concentration of PCR reagents. However, to verify the contradictions of microscopic and serologic findings with PCR results, the amplicons should be sequenced to confirm their validity. Accordingly, we performed this sequencing for a few random samples. In the present study, the PCR-targeted *Vir B9* gene was also promising, and its validity was confirmed by the previously tested samples. This gene was highly conserved like *p30-10* (Felek, 2003) and can be taken as a useful target just in 16S rRNA. The new Fast Taq

assay, which was standardised in this study, was found to be very specific, reproducible, and time-saving. *VirB9* was identical to other sequences in GenBank, with highly conserved region like the 16S rRNA gene sequencing of *E. canis* (Unver, 2001). The difference in the sensitivity of PCR-targeted *Vir B9* gene is attributed to the fact that our reference Texas strain contained pure *E. canis* cultured DNA, whereas the clinical sample contained DNA of various microorganisms and animals (from whom we extracted DNA).

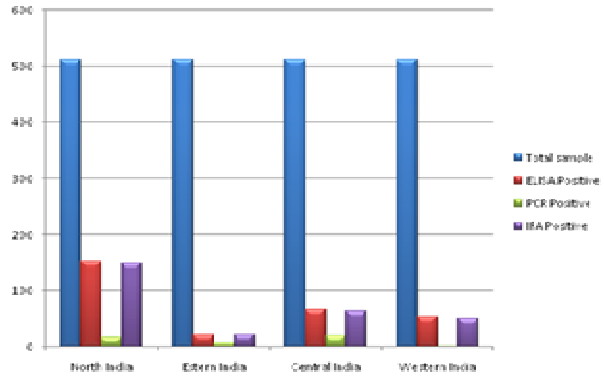

Figure 3. Grafival Presentation for the result of all the three assays performed in thid study.

Furthermore, there were some inhibitors and deoxyribonuclease in the blood and tissue samples as well. We compared the PCR results with the ELISA findings. In the ELISA, 57.5% of the samples were positive, while in the PCR, the rate of positive samples was 8.8% (Table 3). If we compare ELISA with IFA, almost similar results would be obtained (IFA showed 55.3% positivity). The PCR is normally more sensitive than the ELISA. Nevertheless, in our case, we found reverse results. It might be due to the storage condition of the samples for genomic DNA isolation. It is very difficult to maintain the integrity of the DNA in the samples collected in the field condition. Nonetheless, we obtained very good number of positive samples in PCR. The graphical depiction of all three assays is presented in Figure 3. The nucleotide sequences matched with the sequences of other *E. canis* isolates in Gen Bank. There were few differences in the percentage of some sequences of our isolates with those of the different isolates of the world. This can be attributed to the geographic and endemic factors. In the present study, quite diverse strains were observed in India.

The divergence can be attributed to the evolutionary processes and varied geographical distribution. Moreover, some of the sequences with which we compared our isolate were partial sequences of 16S rRNA gene, like ours, which can be a partly different region amplified using different primers because in other sequences we matched our sequences with complete full-length 16S rRNA gene.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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This study was financially supported by the project titled “Seroprevalence and molecular biological

Table 3. Comparative data on seroprevalence studies for *E. canis* antibodies and polymerase chain reaction for samples across India

Region	Total sample	ELISA Positive	% Positive in ELISA	PCR Positive	% Positive in PCR	IFA positive	% Positive in IFA
Northern India	210	152	72.4	17	8	148	70.4
Eastern India	89	21	23.6	7	7.8	21	23.6
Central India	120	67	55.8	20	16.7	64	53.3
Western India	91	53	58.2	1	1	49	53.8
Total	510	293	57.5	45	8.8	282	55.3

approach for diagnosis of canine monocytic ehrlichiosis in various regions of India" funded by the Department Of Biotechnology, Ministry of Science and Technology Government of India, New Delhi, India.

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