Journal of Veterinary Science & Medical Diagnosis

Research article

A SCITECHNOL JOURNAL

Diagnosis of Echinococcosis in Naturally Infected Dogs using Latex Agglutination Test and Anti-Dog Echinococcus Antibody (IgG) Direct ELISA Test

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Received date: 05 August, 2021, Manuscript No. JVSMD-21-38784;

Editor assigned date: 01 August, 2022, Pre QC No. JVSMD-21-38784 (PQ);

Reviewed date: 15 August, 2022, QC No. JVSMD-21-38784;

Revised date: 22 August, 2022, Manuscript No. JVSMD-21-38784 (R);

Published date: 29 August, 2022, DOI: 10.4172/2325-9590.100032

Abstract

Echinococcosis a zoonotic disease caused by the tapeworm transmitted by the definitive host dogs and wild carnivores to the domestic animals (intermediate hots) and humans (aberrant intermediate hosts) either directly or indirectly shed from faeces. Two foremost diagnostic methods broadly used in dogs are purgation with arecoline compounds and necropsy of the small intestine. Alternatively, immunodiagnostic techniques are being used to detect specific antibodies or antigens. In the present study 100 dogs revealed an overall 8 %taeniid eggs, the prevalence of taeniid eggs was much higher in stray dogs (14%) having access to condemned meat/offal living in an around slaughter houses as compared to pet dogs (2%). The overall prevalence of echinococcosis using latex agglutination test as a diagnostic tool in 100 suspected serum samples of dogs screened was found to be 10 per cent with highest prevalence of 18 per cent in stray dogs and least 2 per cent in pet dogs. A total of 90 suspected serum samples were screened for the detection of Echinococcus granulosus antibodies using anti-dog echinococcus antibody (IgG) direct ELISA kit, of which only 13 samples were found positive with a prevalence of 14.44 per cent. Highest prevalence of 26.66 per cent was observed in stray dogs and the lowest 2.22 per cent in pet dogs. Establishing epidemiological figures of echinococcosis and preventing human and livestock infection and also, performing a rapid and on-site diagnosis/screening for infection in definitive hosts would be advantageous, a goal behind this study.

Keywords: Arecoline; Echinococcosis; Immunodiagnostic; Necropsy

Introduction

Echinococcosis a zoonotic disease caused by the tapeworm is transmitted by the definitive host viz., dogs and wild carnivores to the domestic animals (intermediate hots) and humans (aberrant intermediate hosts) either directly or indirectly shed from faece. Due to the sublime condition in India there is potential threat to the humans and domestic animals due to free access of dogs and other canids which contaminate the vegetables and other food items. There are five morphologically distinct species in this genus. Of more significance for establishing epidemiological parameters of echinococcosis and preventing human and livestock infection diagnosis of Echinococcus in definitive host is of prime step . Since there is extreme morphologic similarity, routine coprological techniques cannot differentiate the eggs of Echinococcus from other Taenia species. Two foremost diagnostic methods broadly used in dogs are purgation with arecoline compounds and necropsy of the small intestine. The method of choice being necropsy and is considered as the gold standard but both the methods have many limitations. Meanwhile, arecoline purgation is the method of choice for the diagnosis of echinococcosis in living dogs. These methods being laborious are expensive procedure with biological risk. Alternatively, immunodiagnostic techniques are being used to detect specific antibodies or antigens. Several immunological and serological tests have been developed for the prompt diagnosis of Echinococcus spp.in definitive hosts. Coproantigen detection appears to be prompted in detecting infection in a definitive host with extraordinary specificity and sensitivity and the coproantigen test has the benefit of early recognition of the infection during prepatency, in 4-10 day post-infection. Coproantigens are parasite explicit products in the faeces of the host that are acquiescent to immunological detection and are associated with parasitic metabolism and copro-antigen Enzyme-Linked Immunosorbent Assay (ELISA) has been used in diagnosis of Echninococcosis in samples from live animals. The other reliable method for diagnosis in dogs is by Latex Agglutination Test (LAT) with sensitivity and specificity of 100 and 78.80% respectively and similarly, Shimizu used LAT for the detection of E. multilocularis copro-antigens in the definitive host and found 47 and 61% sensitivity and 94 and 86 % specificity with non-heated and heated faecal samples of wild foxes, respectively. This study is part of epidemiological surveillance of Echinococcosis among sheep population in slaughter house accurate diagnosis of Echinococcus infection in definitive hosts had always been an important component for establishing epidemiological parameters of echinococcosis and preventing human and livestock infection. Furthermore, performing a rapid and on-site diagnosis/screening for infection in definitive hosts would be advantageous. In view of that for diagnosis of the echinococcosis in dogs which harbour the infection using the latex agglutination test and anti-dog echinococcus antibody (IGG) direct ELISA was done [1].

Materials and Methods

Sample collection

As the cycle of transmission of parasite goes from definitive host to intermediate host and *vice-versa* by eating the offals/by-products of later ones. Thus the faecal samples of 100 dogs including 50 pet dogs visiting TVCC, FVSc and AH, SKUAST-J, R.S. Pura, Jammu for routine check-up and 50 stray dogs in and around municipal slaughter

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houses of Jammu were collected during the present study for diagnosis of various parasitic infestations including the tapeworms of dogs. Simultaneously, 100 blood samples of the said animals were also collected. As the study was epidemiological surveillance of echinococcosis in sheep population and correlate with the dog menace it was necessary to know the status of parasite in dog population roaming/in contact with the sheep population thus matter of public health significance. As the dogs are definitive host of this parasite and necropsy is considered as the gold standard for diagnosis but to keep the animal welfare matter into consideration alternatives have to be checked for diagnosis of Echinococcosis in natural harbours [2].

Faecal examination

Faecal samples were first examined microscopically for the presence of various parasitic eggs or proglottids of cestodes and confirmation of eggs was done on the basis of its morphological characteristics.

Qualitative faecal examination

Direct method: A small quantity of faeces was placed on a glass slide, mixed with a drop of water, spread out, covered with a coverslip and examined directly under the microscope (10x).

Concentration methods

Sugar floatation method: 2 gm of faeces was mixed with 20 ml of water in pestle mortar and put in a wide mouthed short bottle. Then a small amount of filtered faeces was taken in a test tube and rest of the test tube was filled with saturated sugar solution. After that a cover slip was placed over the tube avoiding bubbles in between. After 25-30 minutes, the cover slip was drawn upwards gently and placed on a glass slide and examined under microscope for presence or absence of eggs of various parasites of dog having low specific gravity [3].

Sedimentation method: Again 2 gm of faeces was mixed with 20 ml of water in pestle mortar and put in a wide mouthed short bottle. Then a small amount of this filtered faeces was taken in a test tube and left in a test tube rack. After 25-30 min, the supernatant was thrown and sediment was examined under microscope for different parasitic eggs having high specific gravity [4].

Latex agglutination test

The preliminary diagnosis of echinococcosis in serum samples of 100 dogs was done using latex agglutination test as per the technique of Shimizu and for negative control distilled water was used instead of serum sample from dog. The test involved the following different steps:

Serum preparation

The blood from all the dogs was collected in a sterile vial and was kept undisturbed at room temperature for 10-20 min. Thereafter centrifuged the same at 2000-3000 rpm for 20 min when serum separated out. The serum so obtained was pipetted out in a separate sterile vial and kept at refrigeration for further use.

Preparation of faecal supernatant

2-4 g of faecal material collected aseptically from the rectum of each of the suspected animal was collected separately in sterile containers for the preparation of faecal supernatant which is as follows:

Approximately 2 g of faecal sample was mixed with phosphate buffered saline in a 15 ml centrifuge tube in proportion of 1:2 until slurry was formed. The faecal slurry was then centrifuged at 2000 rpm for 20 min at room temperature. Afterwards the supernatant was collected in a 2 ml screw capped tube and stored at -20 °C until use for coproantigen detection.

Preparation of Sensitized Latex Particles with Antigen

Polystyrene latex particles supplied by Sigma-Aldrich were diluted in the concentration of 1:100 with glycine buffered saline. 0.5 ml of the diluted latex was then added to 0.5 ml of coproantigen and the mixture was shaken well for 30 min at room temperature. Thereafter Bovine serum albumin and Tween-20 were added to the mixture to make final concentration of 1%and 0.1%, respectively to eliminate non-specific agglutination reaction. The latex reagent was then stored at 40C until use.

Test Procedure

50 µl of latex reagent was mixed with an equal volume of serum collected from dogs in a cavity glass slide. The slide was then rocked gently with orbital motion for 15 min until signs of agglutination were observed under dark background illumination [5].

Enzyme-linked Immunosorbent Assay

Anti-Dog echinococcus antibody (IgG) Direct ELISA Kit manufactured by Sunlong Biotech Co., Ltd and procured from New India Technologies, Ahmedabad was used in this test to detect specific antibodies in the 100 suspected serum samples collected from dogs suspectedly infected with Echinococcus granulosus infection as per the protocol supplied which is as follows:

Two wells of the micro-ELISA strip plate were kept as negative control, two wells as positive control where as one well empty as blank control. Positive control sera were harvested and sera of 1week old puppy were used as negative control [6].

Negative and positive control in a volume 50 µl were then added to the negative and positive control wells respectively. Then in the sample wells 40 µl of sample dilution buffer and 10 µl of serum sample were added. Samples were loaded onto the bottom with the precaution that it did not touch the well wall. Then the micro-ELISA strip plate was shaken gently well with rotatory movements. Afterwards the micro-ELISA strip plate was kept in the incubator for 30 min at 37 °C after sealing with closure plate membrane. Thereafter concentrated washing buffer was diluted 30 times with distill water for washing procedure. Closure plate membrane was then carefully peeled off, material aspirated from the wells and refilled with the wash solution which was discarded after resting for 30 seconds. Similar washing procedure was repeated for 5 times. Afterwards 50 µl HRP-Conjugate reagent was added to each well except the blank control well and kept in the incubator at 37 °C for 30 minutes. Again washing procedure was repeated for 5 times. Then 50 µl of Chromogen Solution A and 50 µl Chromogen Solution B were added to each well, gently mixed and incubated at 37 °C for 15 minutes. At last 50 µl of stop solution was added to each well to terminate the reaction which was indicated by change of the color in the well from blue to yellow.

Lastly, absorbance (O.D) reading were taken within 15 min after adding stop solution at 450 nm using a Microtiter Plate Reader [7].

The critical value (cut off value) for interpretation of the test is the average value of the positive control

Positive judgement: If the OD value ≥ CUT OFF, the sample is canine echinococcus-IgG positive.

Negative judgement: If the O.D value < CUT OFF, the sample is canine echinococcus-IgG negative.

Comparison of Latex agglutination test and Anti-Dog echinococcus antibody (IgG) Direct ELISA test

Comparison of the two diagnostic tests viz. latex agglutination test and enzyme-linked immunosorbent assay using anti-dog echinococcus antibody (IgG) direct elisa kit was also undertaken in the present study by calculating kappa coefficient. It was Cohen in 1960 who coined the word kappa coefficient which is a measure of agreement between the two tests and gave a formula for its calculation. The calculation is based on the difference between how much agreement is actually present ("observed" agreement) compared to how much agreement would be expected to be present by chance alone ("expected" agreement). The formula for calculation of kappa coefficient is as follows

$$K = \frac{Po - Pe}{1 - Pe}$$

Where Po =The proportion of observation in agreement or observed level of agreement

Pe = The expected proportion in agreement by chance

Interpretation of Kappa

Poor agreement= < 0.20 Fair agreement = 0.20–0.40 Moderate agreement = 0.40–0.60 Good agreement = 0.60–0.80 Very good agreement = 0.80 – 1.00.

Results and Discussion

Diagnosis of Echinococcosis in Naturally Infected Dog

A total of 100 dogs comprising 50 each visiting TVCC, SKUAST-J, RS. Pura for treatment against various parasitic infestation and living around municipal slaughter houses of Jammu respectively suspected suffering naturally with tapeworm were diagnosed initially by faecal examination followed by latex agglutination test (14) and finally by commercially available anti-dog echinococcus antibody (IgG) direct Elisa kit for Echinococcus granulosus infection [8-9].

Diagnosis by Faecal Examination

The prevalence of various gastrointestinal parasitic infestation in dogs was initially based on qualitative faecal examination by direct faecal examination followed by concentration methods. The study on 100 dogs revealed an overall highest prevalence of 14 per cent of strongyles eggs. followed by 12 per cent Toxocara eggs, 8 % taeniid eggs and the minimum 3 per cent of trichurid eggs. However, positive stray dogs outnumbered pet dogs visiting TVCC, RS. Pura for various gastrointestinal parasites including taeniid eggs. Amongst 37 dogs found positive against various parasitic infestations, highest prevalence of 78.37% was observed in stray dogs followed by 21.62 % in pet dogs visiting TVCC, R.S. Pura as depicted in Table 1, Figures 1-4. Further out of a total of 37 positive cases highest prevalence of 72.97%of mono parasitism was observed followed by 27.02% of mixed parasitism as depicted in Table 2. The prevalence of taeniid eggs was much higher in stray dogs (14%) having access to condemned meat/offal living in an around slaughter houses as compared to pet dogs (2%). Previous studies are also in confirmation with the present findings which also recorded higher prevalence of gastrointestinal helminthes including tapeworms in stray as compared to pet dogs. The probable reasons of higher prevalence in stray dogs might be due to free roaming habit, lack of deworming and easy access of stray dogs to left over condemned meat/offal's at the slaughter houses and meat shops as compared to pet dogs. Further the prevalence of mono-parasitism (72.97%) was significantly higher than the mixed infestation (27.02%) in the present research work. This present finding is in agreement with the previous findings.

Type of egg identified	Dogs visiting (*) TVCC, R.S. Pura n=50	Stray dogs (*) n=50	Total (*) Positive n=100
Taeniid spp.	01 (2)	07 (14)	08 (8.00)
Strongyles spp.	02 (4)	12 (24)	14 (14.00)
Toxocara spp.	04 (8)	08 (16)	12 (12.00)
Trichurids spp.	01 (2)	02 (4)	03 (03.00)
GrandTotal	08 (21.62)	29 (78.37)	37 (37.00)

Table 1: Prevalence of various gastrointestinal parasitic infestation. *Figures in parenthesis denote percentage.

Type of dog	Samples screened	Samples positive	Mono parasitism	Mixed parasitism
Stray	50	29	21 (72.41)	08 (27.58)
Pet	50	8	06 (75.00)	02 (25.00)
Total	100	37	27 (72.97)	10 (27.02)

Table 2: Prevalence of mono and mixed gastrointestinal parasitism.

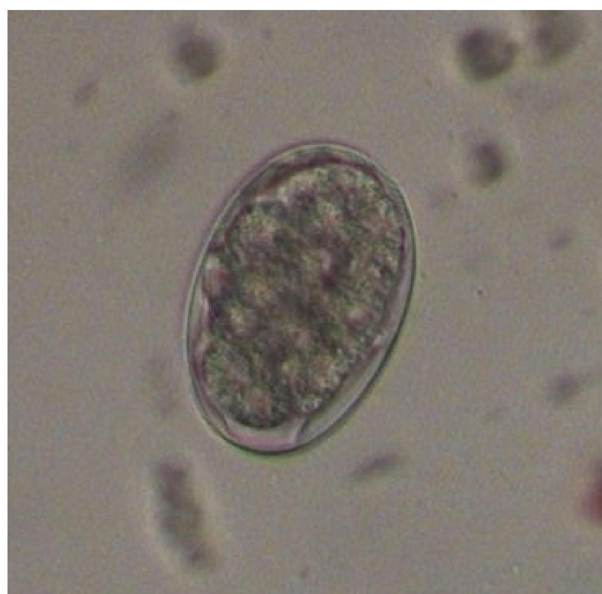

Figure 1: Strongyle egg from faecal sample of dog (X 40).

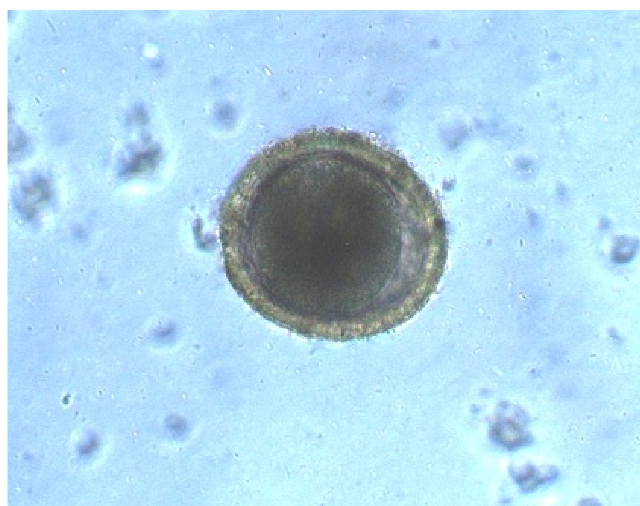

Figure 2: Toxocara egg from faecal sample of dog (X 40).

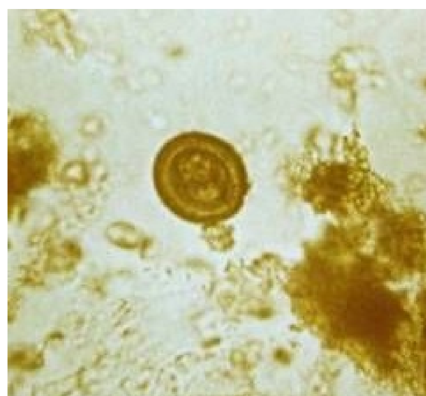

Figure 3: Taeniid egg from faecal sample of dog (X 40).

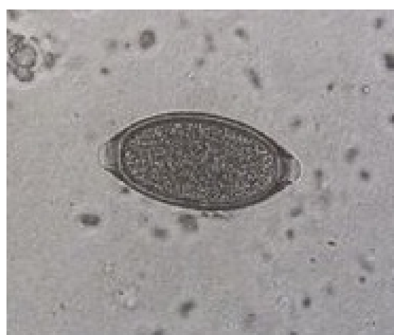

Figure 4: Trichurid egg from faecal sample of dog (X 40).

Diagnosis by Latex Agglutination Test

The overall prevalence of echinococcosis using latex agglutination test as a diagnostic tool in 100 suspected serum samples of dogs screened was found to be 10 per cent with highest prevalence of 18% in stray dogs and least 2%in pet dogs as depicted in Table 3, Figure 5. Szyfres, and Kagan, mentioned LAT is 100% sensitivity in detecting antibodies in the positive hydatid sera and 97% specificity with the other sera. Futhermore, latex agglutination test was also used by various workers for the detection of coproantigens specific to different echinococcus species and were of the opinion that this test has good sensitivity and specificity for diagnosis of echinococcosis in definitive host.

Type of dog	Serum samples screened	Echinococcosis +ve (*)
Stray	50	09 (18.00)
Pet	50	01 (2.00)
Total	100	10 (10.00)

Table 3: Prevalence of Echinococcosis using latex agglutination test.

Figure 5: Depicting latex agglutination test circle 1. Negative control Circle 2. Positive reaction

Diagnosis by Anti-Dog Echinococcus Antibody (Igg) Direct Elisa Kit

A total of 90 suspected serum samples were screened for the detection of Echinococcus granulosus antibodies using anti-dog echinococcus antibody (IgG) direct ELISA kit, of which only 13 samples were found positive with a prevalence of 14.44%. Highest prevalence of 26.66% was observed in stray dogs and the lowest 2.22%in pet dogs.

The sample was considered canine echinococcus (IgG) positive, where in O.D value of the tested sample was ≥ 1.72 cut off value (critical value) which was the average value of the positive control as depicted in Table 4.

Type of dog	Serum samples screened	Echinococcosis +ve
Stray	45	12 (26.66)
Pet	45	01 (2.22)
Total	90	13 (14.44)

Table 4: Prevalence of echinococcosis using anti-dog Echinococcus antibody (IgG) direct ELISA kit.

The present finding is supported by the preceding findings, who also reported prevalence of 12.45%in serum samples of dogs using the same test. The present study, thus established the presence of canine echinococcosis in Jammu throwing a possibility of hydatid disease in human population of Jammu region.

Lastly, on comparing the results of latex agglutination test with anti-dog echinococcus antibody (IgG) direct ELISA kit showed high correlation with a kappa coefficient value of 0.85 which was suggestive of very good agreement of the both the tests.

Comparison of Latex Agglutination Test and Anti-Dog Echinococcus Antibody (IgG) Direct Elisa Test

On comparing Latex agglutination test with anti-dog echinococcus antibody (IgG) direct Elisa test, Kappa coefficient value calculated was observed to be 0.85 which indicate that the two tests undertaken were very good in agreement. Kappa was calculated by the following formula

$$K = \frac{0.9666 - 0.776}{1 - 0.776} = 0.85$$

Conclusion

Diagnosis of echinococcosis in dogs by latex agglutination test and anti-dog echinococcus antibody (IgG) direct ELISA method proved to be in very good agreement on comparing by kappa coefficient value calculation. Hence both the tests are good and reliable tests for disease diagnosis. In addition to this dogs are natural inhabitant in the human society so awareness is needed for the regular deworming and curative therapies needed so as to safeguard the lives of livestock as well as humans. So, mass preventive measures for the canid is need of the hour.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgement

We are thankful to Division of Veterinary Medicine, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology Jammu for providing financial assistance during the study period.

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