Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/x0r79831

DNA EXTRACTION OF GASTROINTESTINAL HELMINTHS EGGS OF FECAL SAMPLES USING DIFFERENT PROTOCOLS

Muhammad Zeeshan[1]*, Dr Tahir Latif[2], Dr Nasir Khan[3], Dr Muhammad Hanif[4], Dr Muhammad Junaid[5], Dr Ikram Ullah[6], Dr Jamshed Khan[7]

[1]*MPhil Biotechnology, Department of Biotechnology, Abasyn University, Peshawar, Pakistan
[2]Assistant Professor Community Medicine, Loralai Medical College (LMC) Loralai, Pakistan
[3]Lecturer/ MO Internal Medicine, Loralai Medical College (LMC), Loralai, Pakistan
[4]Assistant Professor, HOD Paeds Department, Loralai Medical College (LMC), Loralai, Pakistan
[5]Associate Professor of Anatomy, Islam Medical & Dental College, Sialkot, Pakistan
[6]Dental Surgeon, Assistant Professor, Department of Forensic Medicine, Loralai Medical College (LMC), Loralai, Pakistan
[7]Associate Professor, Department of Anatomy, Loralai Medical College (LMC), Loralai, Pakistan

***Corresponding author:** Muhammad Zeeshan
*MPhil Biotechnology, Department of Biotechnology, Abasyn University, Peshawar, Pakistan
Email: mz335854@gmail.com

ABSTRACT

Helminths found in the gastrointestinal tract pose significant challenges in managing andcontrolling animal health issues on farms. To effectively control and prevent theseparasites, it is necessary to have access to epidemiological and structural information about them. Molecular approaches, specifically DNA sequence analysis, are commonly employed for identifying and quantifying different nematode species. However, extracting pure genomic DNA from internal parasites is challenging due to the presence of a hard eggshell, which hinders access to the parasitic DNA and complicates the extraction process. In this study, we assessed the performance of three DNA extraction methods: TRIZOLE method, TRIZOLE with sonication and the commercially available GeneJET Genomic DNA Purification kit. Initially, fecal samples were collected from foursheep and subsequently homogenized and centrifuged. Sugar was added to the samples toform sheather sugar flotation solution, followed by another round of centrifugation to confirm the presence of parasite eggs in all fecal samples through microscopic analysis. Once egg presence was confirmed, DNA extraction was performed using the TRIZOLE method, TRIZOLE with sonication and GeneJET Genomic DNA Purification kit. Gel analysis was conducted to determine the presence or absence of DNA bands in the extracted samples. The results revealed that no DNA bands were observed when using theTRIZOLE method, whereas DNA smears were formed in the case of sonication method. In contrast, clear DNA bands were observed in samples where DNA extraction was performed using the GeneJET Genomic DNA Purification kit. Based on these findings, itcan be concluded that the kit method was more effective for extracting DNA from gastrointestinal nematode eggs compared to the other three methods.

Keywords: TRIOZOLE, GeneJET, Nematode, Fecal, Sonication.

1. INTRODUCTION

Worldwide, sheep productivity and health are seriously threatened by intestinal parasites. According to Shankute et al. (2013), the primary reason for poor health in smallruminants is parasitic diseases. Numerous diseases that these parasites might induce can result in financial losses for the cattle business. Acute and long-term illnesses can result from parasitic infestations. Even when the sheeps seem healthy, these diseases are widespread. Lambs with endoparasite infections in their bodies will still look healthy, eventhough adult sheeps would show clinical symptoms. Endoparasitic infestation in ruminants has been linked to clinical signs as diarrhoea, dehydration, appetite loss, inability to gain weight, and anaemia Gregory and Grandin (2007). To effectively execute control measures,it is imperative to identify and comprehend the nature of these parasites. The purpose of this paper is to examine the subject of gastrointestinal parasites in sheep, with particular attention to the infection process, the financial effects on livestock, diagnostic procedures, difficulties in diagnosis, and the application of PCR-based approaches for precise identification. It also investigates various DNA isolation techniques for enhanced parasiteidentification and characterization.

Sheep parasitic diseases have serious financial ramifications for the agricultural sector. One of the primary causes of the global decline in small ruminant output is helminthiasis. Lone and associates (2012). The financial fallout from lower productivity, including slower growth in body weight, low-quality wool, poorer reproductive outcomes, and higher death rates. Due to their weakened immunity, animals afflicted with gastrointestinal helminths have reduced rates of production and reproduction and are more vulnerable to infection by other infections. The Garedaghi group (2011).Effective management tactics and early detection are crucial, and the costs of treatment, prevention,and control measures will be deliberated. Low milk production, low fertility, diminished labour capacity, involuntary culling, treatment costs, death, and decreased market value areall losses incurred by owners due to sick animals (Regassa et al., 2006, Edosomwan and Shoyemi, 2012). A significant source of work and revenue for low-income farmers in rural regions is livestock production, notably the raising of sheep and goats (Gadahi et al., 2009,Raza et al., 2014).

Identifying gastrointestinal parasites accurately and promptly is essential to puttingthe right treatment and control strategies in place. a summary of the several diagnostic approaches that are frequently used, such as serological assays, coproantigen testing, and faecal inspection. The principal laboratory test for helminth diagnosis is a faecal flotation process that finds the parasites' eggs. Genera of strongylid nematodes generate eggs that are difficult to distinguish from one another due to their identical appearance. They are allgenerally recognised as strongylid or trichostrongylid eggs in faecal samples. The genera of these helminths must be identified using faecal culture. Conboy and Zajac (2012)

There are various difficulties in diagnosing gastrointestinal parasites in sheep. the restrictions and issues that arise during standard diagnostic processes, such as the intermittent shedding of parasites, the low sensitivity of traditional techniques, and challenges in differentiating between species. The mechanisms of resistance to macrocyclic lactones, imidazothiazoles, and benzimidazoles—the three main types of anthelmintic drugs—Kotze and Prichard (2016). Gaining insight into these problems is essential for enhancing the precision of diagnoses and putting into practice efficient parasite control measures.

PCR, or polymerase chain reaction, has become a useful method for characterizing and identifying gastrointestinal parasites in sheep. Concentrate on the fundamentals of PCR-based diagnostics, such as primer design, target gene selection, and amplification methods. We'll talk about the benefits of PCR, including its higher sensitivity and specificity, as well as its possible uses in parasite identification and species detection. ManyPCR-based techniques are now available for the detection of GIN in sheep, however they haven't been compared to microscopy very often (Bott et al., 2009, Learmount et al., 2009).

In PCR-based diagnosis, obtaining high-quality DNA with efficiency is essential. Numerous techniques, such as commercial kits, the TRIZOL method and the TRIZOL withsonication approach, are employed to extract DNA from gastrointestinal parasites in sheep. A phenol and guanidine isothiocyanate mixture called TRIZOL is used to extract proteins,DNA, and RNA from tissues and

cells.(Sacchi and Chomczynski (2006).

To put it briefly, the purpose of the current research is to provide clarification on sheep gastrointestinal parasite detection. Through an examination of subjects like infection, financial consequences, diagnostic approaches, difficulties in diagnosis, PCR-based diagnostics, and DNA isolation procedures, it aims to offer a thorough grasp of this significant field of animal biotechnology study.

LITERATURE REVIEW

Roeber et al. (2013) Through his experiment, he showed how parasitic helminths, orroundworms, of small ruminants and other livestock had a significant global economic impact. The development of useful molecular methods to investigate the epidemiology of these helminths has not advanced very much, despite the significance of the diseases these helminths cause and the discovery of new treatment drugs (anthelmintics). The detection and tracking of anthelmintic resistance in livestock parasites, which is currently a major global concern, as well as parasite control are based on specific diagnostics.

This article's goal is to give a succinct overview of the biology and epidemiology ofgastrointestinal helminths (order Strongylida) from an Australian perspective. It alsohighlights the significance of using sophisticated molecular tools for the precise diagnosis of nematode infections in order to further investigate parasite epidemiology and detect drug resistance in conjunction with traditional methods. It also offers an overview of how genetic, genomic, and bioinformatic technologies may be used to better understand parasites and manage parasitic illnesses.

Win et al. (2020) came to the conclusion in his research that one of the primary economic losses resulting from lower productivity in small ruminants is still intestinal parasite infection. 280 sheep in the Magway and Pwintbyu Townships and 100 goats in the Natmauk Township of Myanmar provided a total of 380 faecal samples. The detection of parasite illnesses was done using faecal flotation and sedimentation techniques. Counts offaecal eggs and oocysts were performed using the McMaster method. In small ruminants, the prevalence of gastrointestinal parasites was 98.4% (374/380). In sheep, gastrointestinalparasites were more common (99.3%) than in goats (96%). 96% of the cases were in Eimeria spp., with Trichostrongyle coming in second at 77.1%, Trichuris spp. at 35%, andMoniezia expansa at 14%.

A single infection was 15.2% (57/374), but the mixed infection incidence was 84.8% (317/374). There was a range of 50 to 600 eggs per gram (EPG) and 50 to 29,800 oocysts pergramme (OPG) in the faeces. Of the four nucleotide sequences that were isolated, three of them displayed 96.64-99.46% identification with Haemonchus contortus from Laos, China,India, and Mongolia, while one sequence had 94.10-94.47% similarity with Trichostrongylus colubriformis, which was reported from Laos. Since the study area had a rather high rate of gastrointestinal parasite infection in small ruminants, it was important to establish suitable treatment and control techniques in order to minimize production losses.

Souza et al. (2012) This study examined the intestinal parasitism caused by protozoaand helminths in sheep (Ovis aries) of the Santa Inês breed in the Rio Grande do Norte municipality of Lajes. From April 2005 to August 2007, stool samples were taken once a month from two tracer lambs on the first day of the experiment, and on the forty-fourth day, the animals were necropsied. Of the 64 lambs that were sampled, only 62 were put todeath. The deposition of the faecal samples in water was used for analysis. Helminth recovery was investigated in the contents of the abomasum, small intestine, and large intestine. The helminth groups' eggs were identified by the parasitological analysis as Strongyloidea, Strongyloides sp., Trichuris sp., and Moniezia sp..Additionally discovered were Giardia duodenalis cysts, Entamoeba ovis cysts, and oocysts of Eimeria spp. Haemonchus contortus, Cooperia pectinata, Cooperia punctata, Trichostrongylus colubriformis, Moniezia expansa, Oesophagostomum sp., Skrjabinema ovis, and Trichuris sp. were the helminths that were found after a thorough examination of the contents.

Pedreira et al. (2006) In Galicia (Northwest Spain), an Atlantic region where sheep agriculture is replacing cattle due to Agricultural Community Politics of the European Union, he conducted a

coprological survey to determine the existence of gastro-intestinal nematode parasites infecting sheep. Using the flotation technique, 1710 faeces samples were randomly selected from 49 sheep farms between September 2001 and November 2002in order to assess the presence of gastro-intestinal nematode parasites. Chabertia, Cooperia, Haemonchus, Nematodirus, Oesophagostomum, Teladorsagia, Trichostrongylus, and Trichouris spp. were the genera identified; the prevalence at the sheep level was 100%.

Concurrent with the sample process, a questionnaire regarding parasite control techniques in the previous year (2000) was given to the farmers. ninety percent (95% CI 81%, 98%) of the farmers reported using antiparasitic medications on occasion, but none of them requested a coprological investigation before starting therapy, and the medications' effectiveness was never assessed. The treated sheep (163) had a higher median EPG than the untreated animals (26).

Raza et al. (2014) proved through his experiment that small ruminants are a significant source of income for many rural inhabitants, particularly in developing countries' semi-arid and dry regions. Gastrointestinal parasites are frequently found in extensively managed livestock, and even persistent infestations can cause financial losses.We assessed the gastrointestinal helminth prevalence in sheep and goats in Pakistan's Cholistan desert, where cattle are the main source of income for the area. Five different places were used to gather 500 sheep and 500 goats' fresh faeces (10–15 g). Parasite eggs were identified using standard parasitological techniques, and individual helminths could be identified from larval determinations by copro-culture.

Among the 1000 animals, the overall helminth prevalence was 78.1%; pure nematode infestations accounted for the majority at 37.5%, followed by pure trematode infestations (7.9%), pure cestode infestations (2.6%), and pure protozoa infestations (0.8%). 6.4% of all animals had combined helminth and trematode infections, 3.8% had mixed nematode-cestode infestations, and 19.1% of sheep and goats had infestations of allthree. While sheep had the opposite infestation rate (73.6% males, 79.5% females), goats had a higher percentage of infested males (81.1%) than females (77.0%). In particular, suckling goats (85.2%) and sheep (88.5%) had higher parasite prevalence than did young (goats 80.6%, sheep 79.3%) and adult animals (72.8 % vs. 73.8%) animals.

The management of nursing animals needs special attention because of the high ratesof infestation. The thorough testing of conventional plant-based treatments against helminths for affordable and routine deworming of the cattle could be a general strategy tolower infection rates.

Alowanou et al. (2021) claimed that because to its ease of use, the McMaster (McM) method is one of the most used methods for assessing the shedding of faecal parasites in veterinary clinics. However, the Mini-FLOTAC (MF) has recently been presented as a potential substitute for faecal worm egg counts due to its light sensitivity. The purpose of this study is to evaluate how well the MF and McM techniques diagnose patients. 40 randomly chosen animals from farms raising sheep, goats, and bunnies provided faeces samples, which were gathered and individually analyzed using MF and McM procedures. Using both methods, there was a statistically significant difference ($p<0.001$) in the countsof strangled eggs in small ruminants and oocysts of Eumyces spp. in rabbits. Nonetheless, there was no discernible difference ($p>0.05$) between the strongylida eggs per gram of faeces in sheep (MF: 202.01 vs. McM: 174.75) goat (MF: 147.36 vs.

McM: 143.75) and rabbit oocysts of Emeria spp. per gram of faeces (MF: 130.75 vs. McM:130.5). In terms of prevalence (MF: 32.5–100% vs. McM: 7.5–70%) and precision values(MF: 85.52–90.44% vs. McM: 49.52–63.07%), MF demonstrated superior diagnostic performance. This study showed that, for veterinary clinics, MF seems to be the more effective alternative strategy.

Tsilipounidaki et al. (2022) The goals of this study were to: (a) use the FilmArray® GI Panel to identify gastrointestinal infections in sheep and goat faecal samples; and (b) assess the parameters that were linked to the presence of these pathogens. Using the BioFire® FilmArray® Gastrointestinal (GI) Panel, faecal samples from ewes or does in 70sheep flocks and 24 goat herds in Greece were examined for the presence of 22 gastrointestinal infections. The infections that were found most commonly were Campylobacter spp. (50.0% of farms), Giardia lamblia (59.6%), and Escherichia coli stx1/stx2, which produce Shiga-like toxin. Salmonella species, Enterotoxigenic E. Coli. lt/st, Yersinia enterocolitica, E. Coli. O157, Rotavirus A, Shigella/enteroinvasive E. Coli.,and

Pseudomonas strigelloides were among the other pathogens found.
For Salmonella spp. alone, there was a difference in the prevalence of pathogen detection across sheep and goat farms: 18.3% versus 0.0%, respectively. An average of 2.5
± 0.1 pathogens per farm were found in 76 (80.9% of farms) with mixed infections seen in57 sheep flocks and 19 goat herds. Compared to ewes in farms where at least two pathogenswere detected, the body condition score of ewes in farms where only one pathogen was found in faecal samples was substantially higher (2.55 ± 0.11 versus 2.31 ± 0.04). Faecal sample counts for sheep flocks showed that farms with semi-extensive management had amuch greater number of infections.
Within goat herds, there was an inverse relationship between the temperature range and the frequency of infections in faecal samples, and a positive correlation with average precipitation.

MATERIALS AND METHODS

Sample preparation

The obtained faecal samples were then put into Petri dishes and smashed while adding sometape water until a uniform mixture was obtained. The crushed faecal samples were passedthrough a sieve while adding tape water into appropriately labelled falcon tubes. The samples were then centrifuged at 3300rpm for 5mins at room temperature. The supernatant was discarded. 350 g of sugar was dissolved in 1L of water in a beaker to make sheather sugar flotation solution for parasitic egg flotation because specific gravity of parasitic eggs are1.1 and the specific gravity of sugar is 2.3. Therefore, the eggs will float on the surfaceof the solution. The solution was added to the pellet in each tube till it is full up to 40ml.

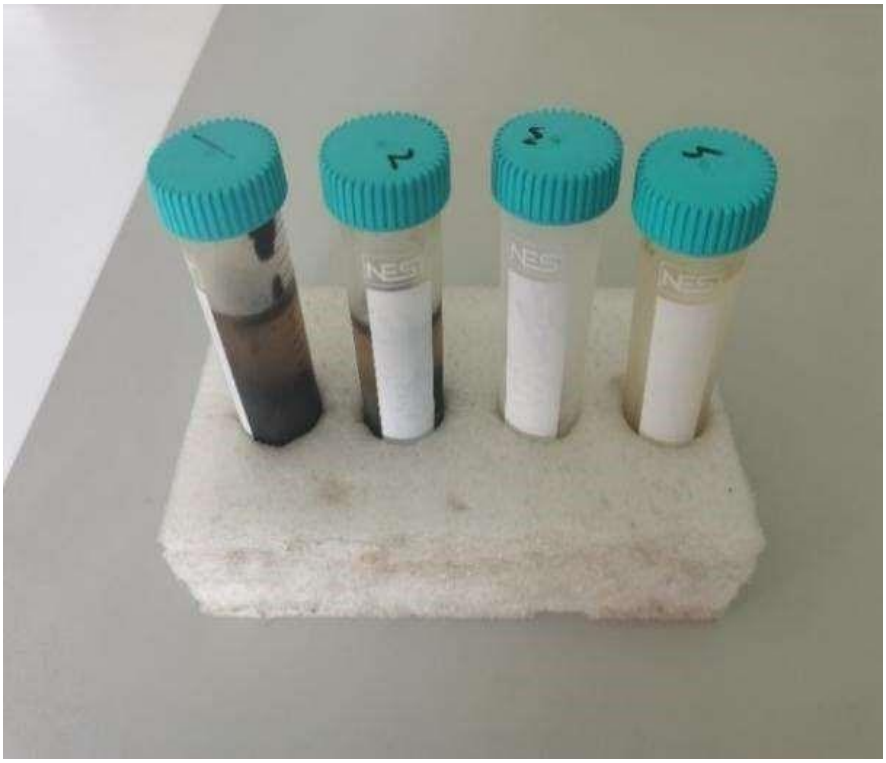

Figure 1: Samples in Falcon Tubes

Egg conformation

Egg confirmation was achieved through microscopic detection from samples. After adding sugar to form sheather sugar flotation solution, the samples were centrifuged at 3300 rpm for 10mins. After centrifugation drops were put on slides. The presence of parasitic egg in all the samples was confirmed through microscope analysis.

DNA Extraction

After confirming that parasitic egg was present in all the samples, 500µl from each samplewas added into eight Eppendorf tubes. The tubes were divided into three groups, each consisting of two tubes and labelled as 1A, 1B, 2A, 2B, 3A and 3B (Extraction Kit). ThreeDNA extraction methods were employed in each group described below in detail.

Figure 2: Samples added to labelled Eppendorf tubes.

1. TRIZOL DNA Isolation Method:

TRIZOL (Manual DNA extraction method) using an already optimized phenol-chloroformisoamyl alcohol (PCI) extraction was used for tubes 1A and 1B. After adding 500µl sample,
from sample 1 in both tubes 500µl lysis buffer and 10µl proteinase K were added. The tubes were kept overnight at 37 C in the incubator. On the second day, the tubes were takenout and 500µl PCI was added. The solution was mixed by inverting the tubes several timesand was centrifuged at 13000rpm for 10mins. After centrifugation, two phases were formed; the aqueous and organic phases. Take the aqueous phase (as it contains DNA) in new properly labelled Eppendorf tubes. 500µl solution D (Chloroform + Indole Acetic Acid) was added and the tubes were again centrifuged at 13000rpm for 10mins. Aqueous form was collected and 55µl Sodium Acetate and 1000 µl of 100% Ethanol were added.
The solution was mixed by inverting the tubes and centrifuged for 10mins at 13000rpm. Supernatant was discarded as the pellet contained the extracted DNA. 200 µl of 70% Ethanol was added and centrifuged at 13000rpm for 7mins to wash the DNA. Thesupernatant was again discarded and the pellet was dried. 30µl TE buffer was added to dissolve the extracted DNA.

2. TRIZOL with Sonication Technique:

For tubes 2A and 2B egg disruption by sonication was done prior to DNA extraction. Sonication of both tubes was performed at a frequency of 20-50 Hz at 10-30 pulses. The frequency was done for 10sec with 30secs of cooling down. The procedure was carried out 5 times for each tube. After sonication DNA extraction was carried out by TRIZOL method.

3. KIT Method (GeneJET Genomic DNA Purification kit):

DNA extraction from tubes 4A and 4B was achieved by the use of a GeneJET Genomic DNA Purification kit. 200 µl of samples were thoroughly mixed by vertexing with 400µl of Lysis solution and 20 µl of Proteinase K. After achieving a uniform suspension, the sample was incubated at 56 C while vertexing occasionally for 10mins. 200µl of 100% ethanol was added to the suspension and mixed by pipetting or vertexing. The prepared lysate mixture was then transferred to GenJET Genomic DNA Purification Columninserted in a collection tube and centrifuged at 6000g for 1min. The collection tube was discarded and GenJET Genomic DNA Purification Column was placed inside a new 2ml collection tube. 500µl of Wash buffer with added ethanol was added in the Column and again Centrifuged at 8000g for 1min. the flow-through solution was discarded from the purification column. The above step was repeated with the solution being centrifuged at a speed of 12000g for 3min. The collection tube containing the flow-through solution was discarded and the Purification Column was transferred to a sterile 1.5ml microcentrifuge tube. 200µl of Elution buffer was added to the centre of the Purification Column membranefor the elution of genomic DNA and was incubated at room temperature for 2min. after incubation, it was centrifuged at 8000g for 1min. the Purification Column was discarded and the purified DNA was visualized using Gel Electrophoresis.

Gel Electrophoresis

Gel electrophoresis was performed for DNA visualization and detection in the samples. 1%agarose gel was made for this purpose. For 1% gel preparation, 0.3g of agarose was addedto 30 ml of 1X TBE buffer and was heated in the microwave for 30sec to dissolve. For thevisualization of DNA 1.5µl of Ethidium bromide was added to the solution and mixed by gently shaking. The gel was poured into the gel casting tray and put until it solidified. Aftersolidification, the tray was immersed into a gel chamber filled with 1X TBE buffer. 3.5µl samples mixed with loading dye were loaded in the wells. The gel was run at 110V for 30mins. After running the gel the bands were observed under UV radiation and photographed with the help of the Gel Documentation System.

Data collection:

DNA bands were observed under UV radiation and captured using a Gel Documentation System after running the gel.

Data analysis:

The data obtained from gel electrophoresis was analyzed and took to determine the formation and presence or absence of DNA bands.

RESULTS AND DISCUSSION

The microscopic examination confirmed the presence of eggs in all the samples. The microscopic examination at different resolution confirmed the presence of different parasitic eggs as shown in (Fig 3 and 4). The result describes a microscopic analysis conducted on several samples, aiming to detect the presence of nematode eggs and isolateDNA from GI parasites.

After confirming that parasitic egg was present in all the samples, 500µl from each sample was added into eight Eppendorf tubes. The tubes were divided into three groups, each consisting of two tubes and labelled as 1A, 1B, 2A, 2B, 3A and 3B (Extraction Kit). Three DNA extraction methods were employed in each group described below in detail.

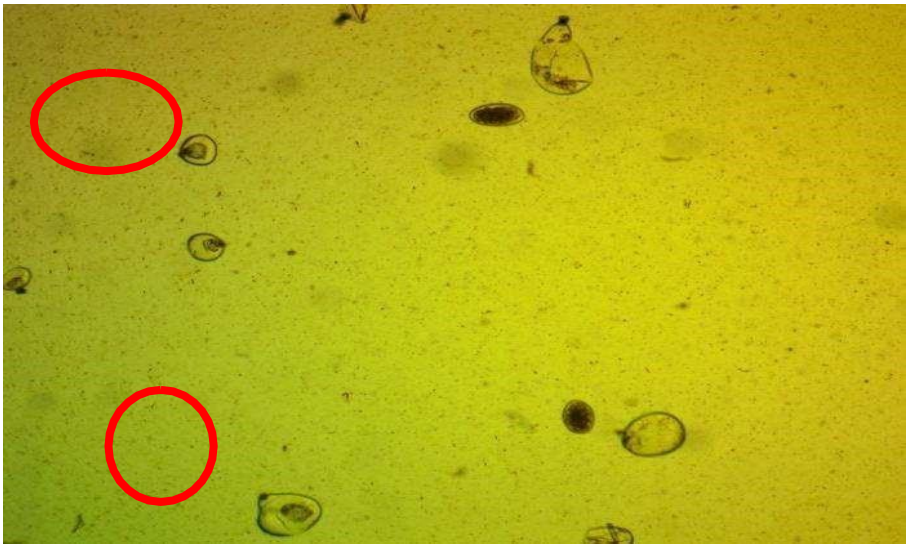

Figure 3: Gastrointestinal Parasitic Eggs using 4X lens.

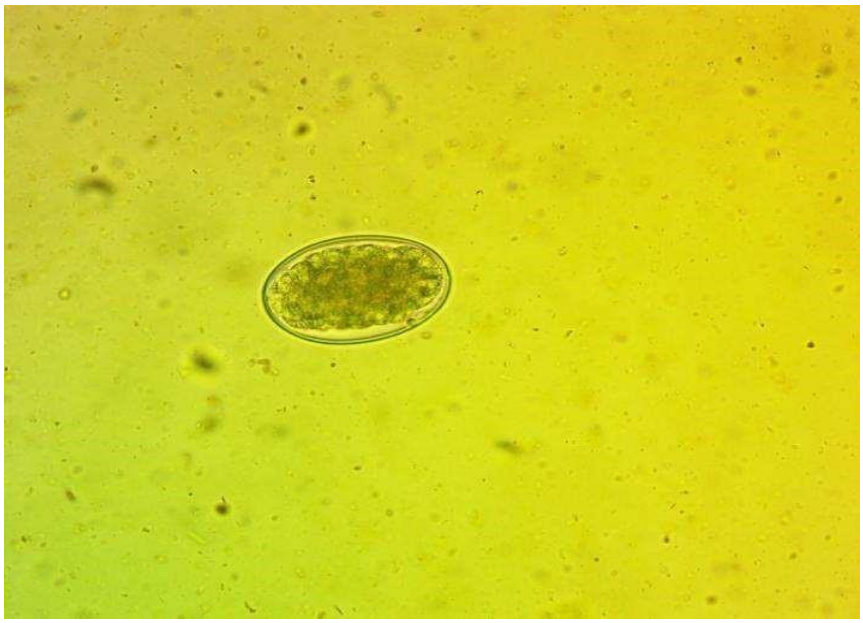

Figure 4: Gastrointestinal Parasitic Egg (40X image)

1. TRIZOL Method:

The samples that were processed on only TRIZOL method, after visualisations on geldoc, no clear DNA bands for samples 1A and 1B were observed as shown in (Fig 5). We repeated the protocol several times to omit any human errors but get only negative results each time. Which lead us to the conclusion that the DNA extraction process was unsuccessful for these samples, resulting in negative results.

The fact that no clear DNA bands were observed multiple times despite repeated attempts on the TRIZOL protocol suggests that there may be some technical or methodological issues during the DNA isolation process specifically for samples 1A and 1B. It is possible that there were inhibitory substances or contaminants in these samples that interfered with the DNA extraction, leading to the negative results.

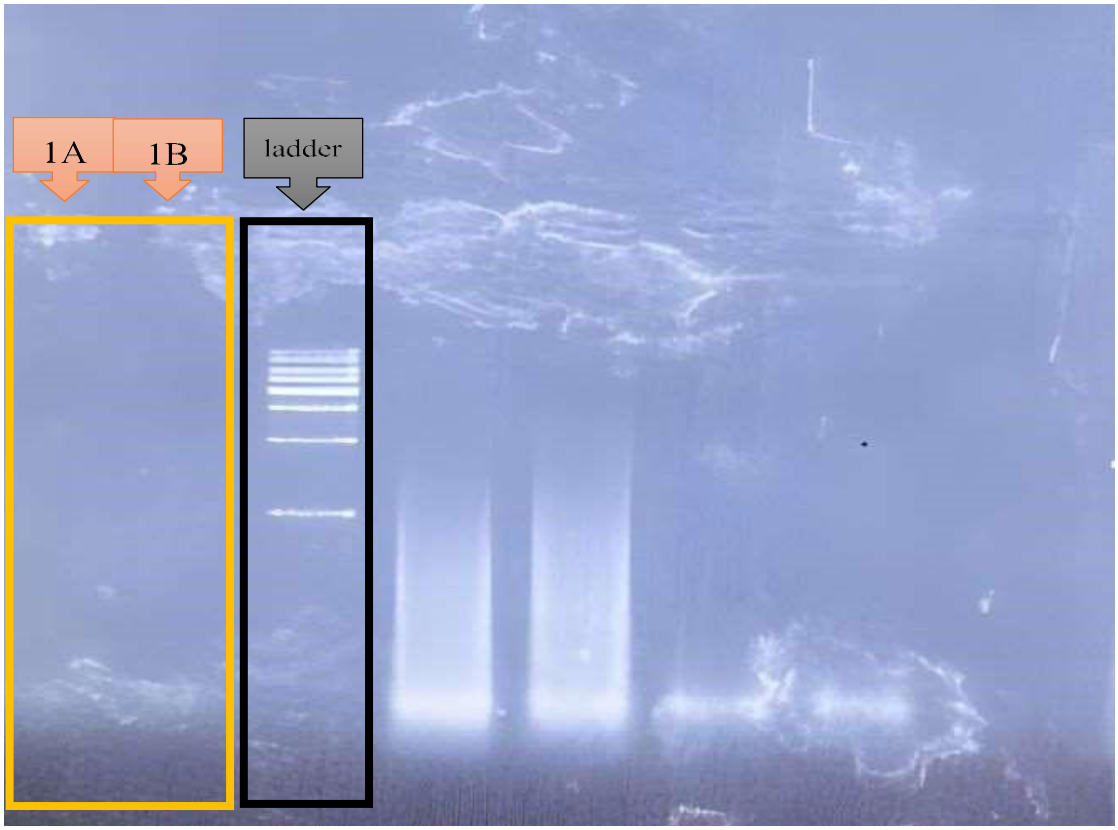

Figure 5: No DNA Bands in 1A & 1B

2. TRIZOL method with Sonication:

After repeated failure with TRIZOL method we modified the protocol and add sonication in the process. In this protocol we done sonication for samples 2A and 2b (10hz and 50hz, for 10 and 30 seconds as descried in material and method), we repeat the sonication with different frequency and time but each time get only smears on gel as shown in (Fig 6 2A and 2B). These results may be due to the shea ring of DNA due to sonication or because of overloading of DNA. We believe that in these samples DNA was extracted but damaged due to mishandling or time duration and frequency for sonication.

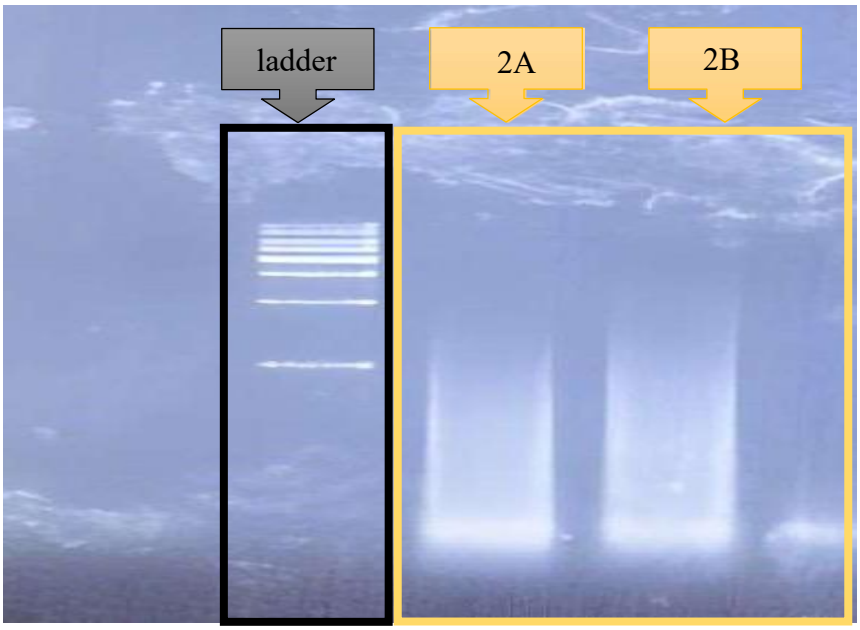

Figure 6: Smears formed in the samples, sonication (2A & 2B) smears

3. GeneJET Genomic DNA Purification kit:

After failing in above all protocols, we employed Gene Jet Genomic DNA extraction kit commercially available for this purpose. The results of kit extraction showed, clear DNA bands for samples 3A and 3B as shown in fig 7. The results suggest that the commercial kit method is more effective in isolating DNA from gastrointestinal parasites in sheep compared to the TRIZOL method. This highlights the importance of using appropriate DNA extraction protocols for accurate detection and characterization of parasites.

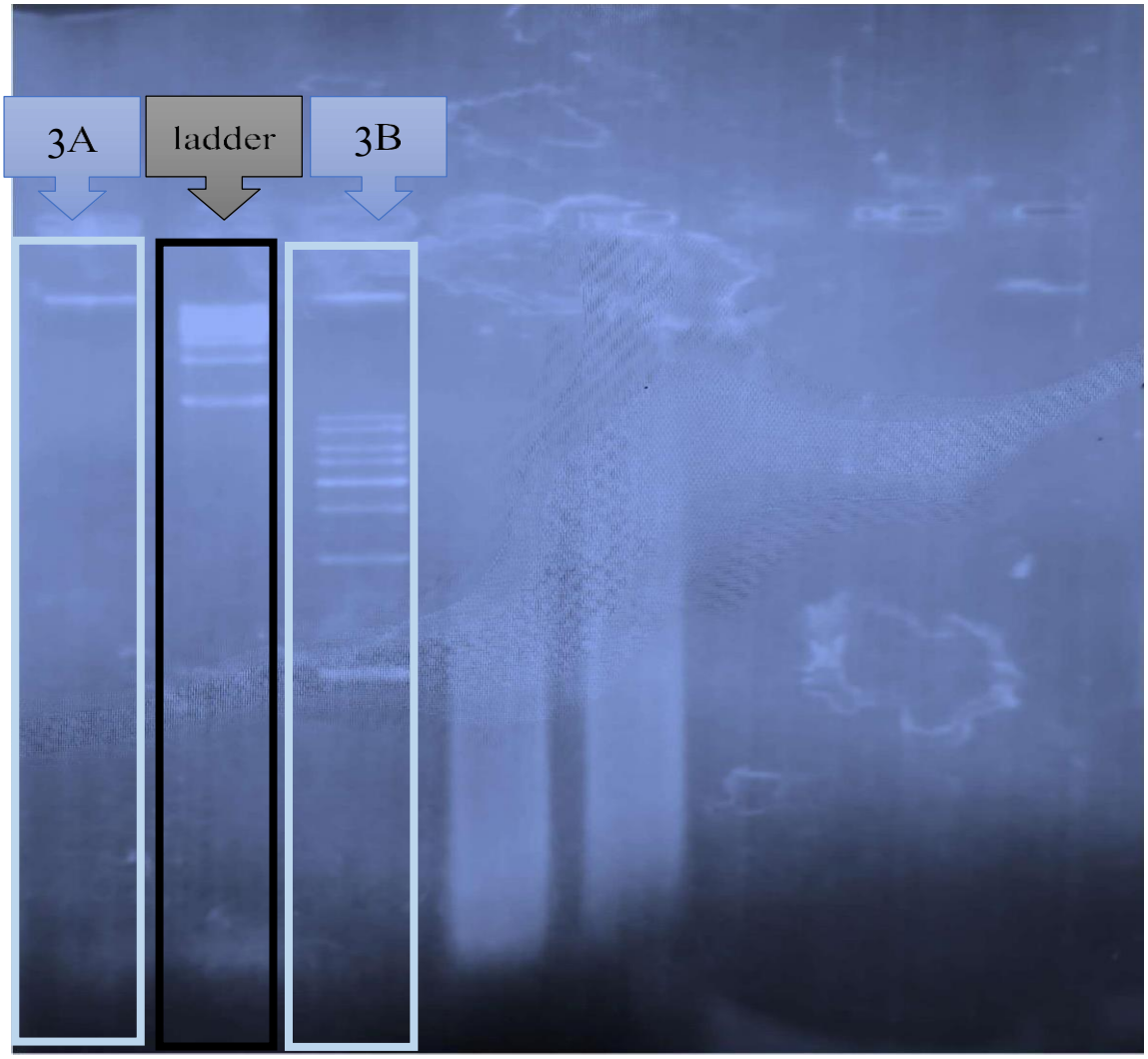

Figure 7: DNA Bands in 3A & 3B

CONCLUSION

Gastrointestinal parasites pose a significant threat to the health and productivity of sheep worldwide. These parasites can cause various diseases, leading to economic losses in the livestock industry. Accurate identification and understanding of these parasites are crucial for implementing effective control measures. Different methods of DNA extraction, including TRIZOL method, TRIZOL with sonication technique and commercial kits, have been used for DNA isolation from gastrointestinal parasites in sheep.

In the study conducted, fecal samples were collected from sheep, and parasitic eggs were confirmed through microscopic analysis. Three different DNA extraction methods were employed as mentioned above, however the TRIZOL method did not yield clear DNA bands for the samples, indicating unsuccessful DNA isolation. On the other hand, the commercial kit method resulted in clear DNA bands in the gel electrophoresis for the samples. The results suggest that the commercial kit method is more effective in isolating DNA from gastrointestinal parasites in sheep compared to

the TRIZOL method. This highlights the importance of using appropriate DNA extraction protocols for accurate detection and characterization of parasites.

It is worth mentioning that further research and optimization of DNA extraction protocols may be necessary to improve the success rate of DNA isolation from gastrointestinal parasites in sheep.

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