



E-ISSN: 2320-7078

P-ISSN: 2349-6800

www.entomoljournal.com

JEZS 2021; 9(2): 1010-1014

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Received: 07-01-2021

Accepted: 09-02-2021

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Comparison of different DNA extraction procedures for *Eimeria* oocysts

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DOI: <https://doi.org/10.22271/j.ento.2021.v9.i2l.8568>

Abstract

A study was carried out to compare the efficacy of different DNA extraction procedures for *Eimerian* oocysts. A total of 753 litter and fecal samples and intestines were collected from desi poultry farms and slaughter houses in Bengaluru, Karnataka. Out of five different methods of DNA extraction procedures the glass bead method was found to be the best method. Since the above method involves breaking of the oocysts and sporocysts walls and dissolving the sporozoites membrane. This greatly simplifies the currently used DNA extraction procedures for *Eimeria* species. The liquid nitrogen method was also found to be good but was more critical and time consuming. Other methods such as sodium hypochlorite, direct and sonication has failed to yield DNA from *Eimeria* oocysts. The present study indicates that glass beads method yielded better quality of DNA for molecular analysis and it is suitable for both large and small number ($<1 \times 10^3$) of oocysts in a sample. Hence, can be used as a routine procedure for DNA extraction for *Eimerian* oocysts.

Keywords: chickens, DNA, *Eimeria*, extraction, methods, oocysts

Introduction

Poultry production in India has emerged as one of the key segments of the livestock economy. The total value of the poultry sector is 162 billion rupees in 2005-06, which accounted for 10.5 per cent of the total value of livestock output and 2.6 per cent of the agricultural sector as a whole. More than three million people directly or indirectly depend on this sector for income and employment [1]. Among poultry diseases, coccidiosis has been reported as a major constraint to successful in both commercial and backyard poultry farming and ranks high among factors that threaten native chicken production [2]. Coccidiosis, caused by intracellular protozoan parasites belonging to the genus *Eimeria*, is one of the commonest and most economically important enteric diseases of chickens' worldwide [3]. Seven species of *Eimeria* are known to infect the chicken (*Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox* and *Eimeria tenella*) causing huge economic loss to the poultry industry, in terms of decreased conversion ratios, decreased egg production and increased mortality [4, 5]. Infection with one or several species can cause growth deficiencies, malnutrition, blood loss, dehydration and increasing the susceptibility to secondary infection [6, 7].

Currently, many commercially available DNA extraction kits have been used for extraction of DNA. Although the majority of these kits was originally designed for nucleic acid extraction from pathogens other than enteric protozoa, these kits were tried for protozoan DNA extraction from faeces [8, 9].

Recently, DNA based molecular biology techniques, which are more sensitive and less subjective, have been utilized with *Eimeria* spp, making them ideal identification methods. However, the gene amplification with field samples remains complicated by the need of lysing the thick and rigid oocyst wall to release its DNA and by PCR inhibition of faecal material [10]. Numerous methods for lysing the cell wall, such as sonication, repeated freezing and thawing hot phenol incubation ammonia, ethanol or lysozyme have all failed to sufficiently rupture the coccidian oocyst wall [11, 12]. Good disruption is achieved only when using strong mechanical forces, viz, glass bead vortex or mini pestle grinding [13, 14]. Hence, there is inadequate information on extraction of DNA from oocysts of desi chickens.

Therefore, the present study has been carried out to evaluate five different methods of DNA extraction from oocysts of *Eimeria* spp, affecting desi chickens.

Material and Methods

Collection and Processing of the Samples

The study was conducted during the period from September 2019 to December 2020 in around Bengaluru, India, to evaluate the convenient procedure, for DNA extraction from oocysts of *Eimeria* spp in desi chickens. A total of 753 fecal and litter samples and 178 intestinal samples were collected from desi farms and local poultry slaughter houses. The collected samples were preserved at 4 °C until further processing. The litter and droppings were processed as per procedure described by Conway and McKenzie [15]. The positive samples consisting of *Eimeria* oocysts were subjected either directly for DNA extraction or kept in 2.5% potassium dichromate for sporulation.

Sporulation of oocysts

The extracted oocysts from the samples were suspended in 2.5% potassium dichromate ($K_2Cr_2O_7$) solution in petri dishes (6mm thickness). The petri dish was covered partially for oxygenation and was incubated between 23 to 29 °C at humidity of 70 to 80 percent for 48 hours [16]. The contents in petri dishes were frequently stirred, to ensure the oxygenation of the oocysts. The sporulation of the oocysts was confirmed by taking a drop of the mixture and examined under the microscope. During the study, an electric aquarium pump was evaluated for the sporulation of *Eimeria* oocysts for continuous supply of air (O_2) to the oocysts culture.

Purification of sporulated oocysts

The purification of oocysts was carried out with slight modification [16]. The sporulated oocysts suspension was mixed thoroughly with equal quantity of 2.5 per cent potassium dichromate ($K_2Cr_2O_7$) solution. The suspension was then filtered through a sieve followed by muslin cloth. The filtrate was centrifuged at 3000 rpm for 5 minutes and washed by water 2 to 3 times. Almost 90 percent of the supernatant was discarded and the remaining portion of the supernatant in the centrifuge tubes was poured in a fresh tube and mixed with saturated chloride (NaCl) for flotation. The mixture was centrifuged at 2000 rpm for 2 minutes. The supernatant having sufficient number of sporulated oocysts was aspirated by pipetting system and collected separately in a tube. The sediment was processed in the same way until no sporulated oocysts remain in the supernatant. The supernatant thus collected was mixed with water (1:5) in a falcon tube and kept undisturbed for overnight at 4°C. The sporulated oocysts settled in the bottom were collected by removing all water (one inch above the bottom of the tube) through suction by pipetting system. The supernatant (3/4th portion) was removed and the remaining mixture at the bottom, having the sporulated oocysts, was centrifuged at 3000 rpm for 5 minutes and to the sediment 2.5% potassium dichromate ($K_2Cr_2O_7$) was added to prevent the growth of fungal and long-time viability and stored at 4 °C until further use.

Mechanical disruption of oocysts by different methods

1. Direct method

The 1ml of purified oocysts, stored in 2.5% potassium dichromate solution, was washed 3 times by PBS solution and

centrifuged at 10,000 rpm for 3min. The sample pellet (200mg) approximately containing 500,000 oocysts were used. DNA was then purified as per protocol of DNA Fast Stool Kit (Qiagen, Germany). The DNA visibility was analysed by 1.5% agarose gel electrophoresis as per standard procedure [17, 18].

2. Sodium hypochlorite method

In this method, the purified oocysts, stored in 2.5% potassium dichromate solution, were washed 3 times by PBS solution and centrifuged at 10000 rpm for 3min. The sample pellet of 200mg was resuspended in 200µl of 4% sodium hypochlorite and incubated for 1h at 4 °C. Freeze thaw for 2min at -20 °C and kept in water bath at 100 °C for 5min for three cycles [19]. DNA was then purified as per protocol and procedure of DNA Fast Stool Kit (Qiagen).

3. Sonication method

1ml of purified *Eimeria* oocysts suspension containing approximately 500,000 oocysts were transferred into 2ml micro centrifuge tube, and was subjected for sonication (Model, CML-4) at 10 volts power for 5 cycles each cycle for about 30 sec interval. DNA was extracted by using DNA Fast Stool Kit (Qiagen) as per manufacture instruction.

4. Liquid nitrogen method

DNA extraction was carried out with slight modification [19]. Ten cycles of freezing and thawing, in water bath at 100 °C, was carried out for complete rupturing of oocysts walls without adding sodium hypochlorite or use of glass beads. During this process, 10ul was taken and was examined under the microscope (40X) to ensure complete oocyst wall destruction. Fast DNA Stool extraction kit was used for genomic DNA extraction and in the final step DNA was eluted in 60µl of EL buffer.

5. Glass bead method

The DNA extraction by glass-bead grinding is currently the one of the most used method for DNA extraction for *Eimeria* oocysts. To compare this method with other methods, DNA was extracted using a glass bead grinding method with minor modification [20, 21]. Briefly, to 1ml of purified suspended oocysts sample containing approximately 500,000 sporulated or unsporulated oocysts. 500µl inhibit EX buffer was added into a sterile, 2ml round bottom microfuge tube, and 200mg of sterile, 425-600µm glass beads acid wash (Sigma) was added, and the tube vortexed by vortexer (GeNei Vortexer, Bangalore, India) with high speed for around 15 to 20 min to complete rupture the oocysts wall and sporocysts. Breakage was monitored using a compound microscope, 40x at 5min intervals until all the oocysts and their sporocysts appeared to be ruptured approximately. After complete rupture of the oocysts, 100µl AL buffer and 10µl of proteinase K were added and vortex it for 1min and incubated at 56 °C for 2h. After incubation in water bath vortex the sample for 1min and kept the sample in ice for 5min. Then added the 500µl remaining inhibit EX buffer and again incubated at water bath for 15min at 95 °C, centrifuged the sample for 1min at 3000rpm (775rcf) speed (Fig 1). Finally DNA was purified as per protocol by DNA Fast Stool Kit (Qiagen) with slight modification. The DNA was eluted at volume of 60µl and the quantified using absorbance at 260 and 280nm.

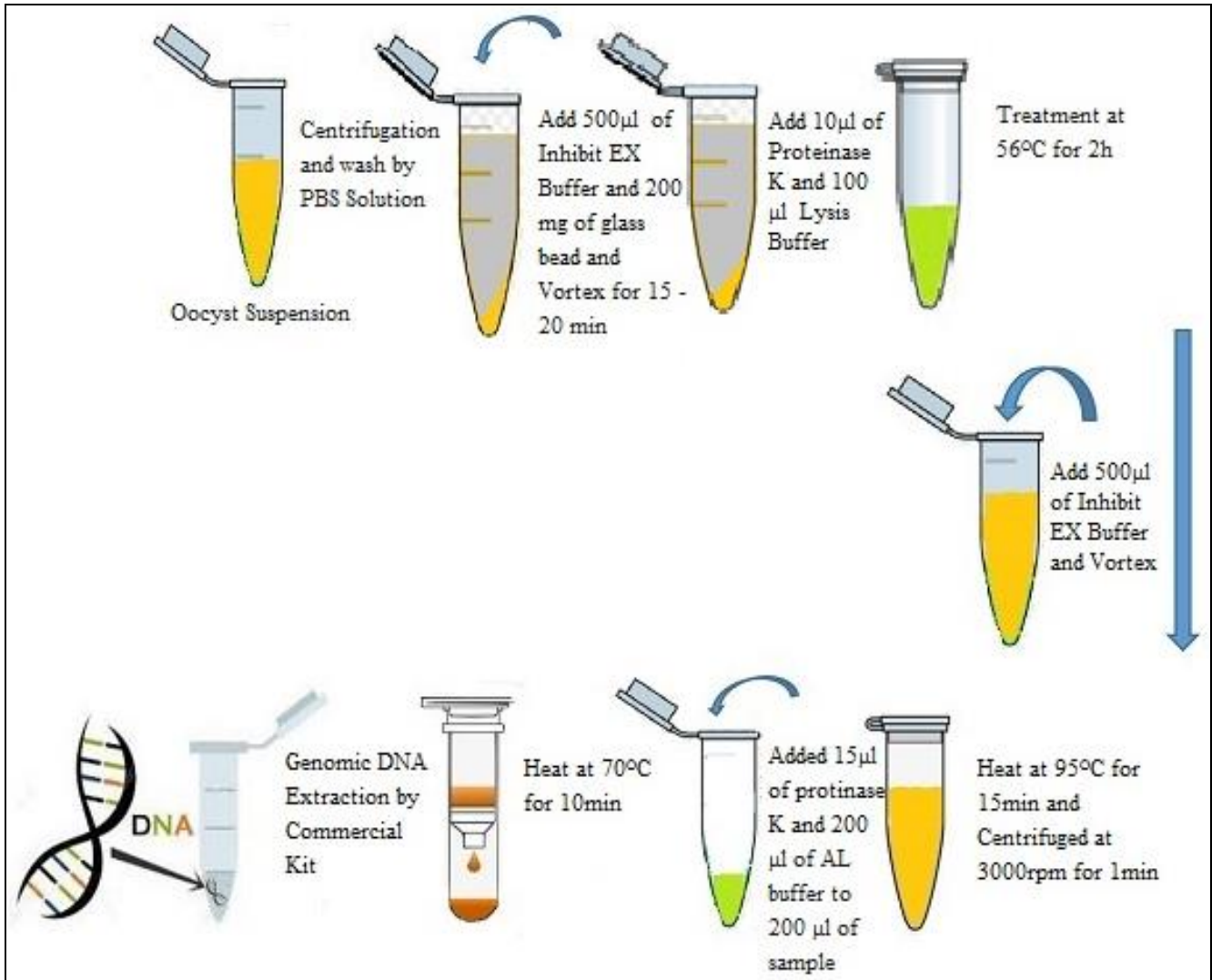


Fig 1: The flowchart of the DNA extraction by glass bead method

Result

A total of 753 samples (litter, faecal and intestines) were examined for eimerian oocysts from different desi chicken farms and poultry slaughter houses in Bengaluru, Karnataka state. The positive samples after processing and purification were subjected for DNA extraction by five different methods *viz.*, direct, sodium hypochlorite, sonication, liquid nitrogen and glass beads. Among these methods, glass bead method resulted in good DNA yield and the DNA visibility was observed by 1.5% agarose gel electrophoresis (Fig 2). The liquid nitrogen method also resulted in good DNA yield similar to glass bead method. However, the above method was time consuming and critical analysis was required at the time of DNA extraction procedure. The DNA yield was estimated by Nano drop (Eppendorf AG, Germany). The estimated DNA yield by glass bead method and liquid nitrogen method was found to be 118.24 and 113.82 ng/ μ l, respectively (Fig 3). During this study, other three methods such as direct, sonication and sodium hypochlorite did not yield DNA.

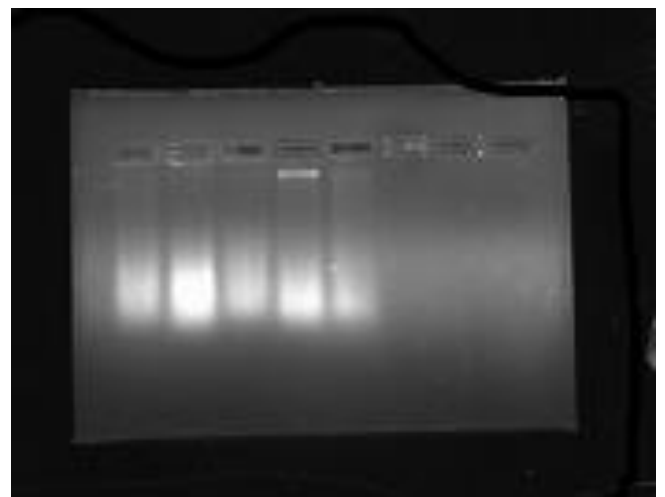


Fig 2: Visibility of DNA 1.5% gel electrophoresis

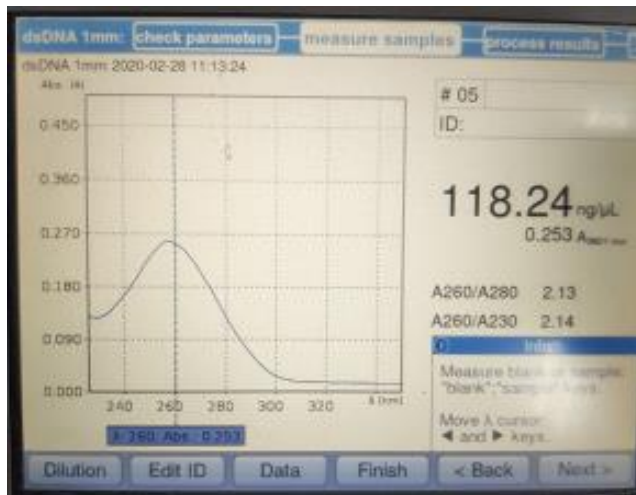


Fig 3: Concentration of DNA by glass bead method analysed by Nano drop

Discussion

DNA extraction from prokaryotes and eukaryotes usually involves two steps; rupture of the cell to release DNA contents, followed by extraction of DNA from a lysate using a phenol chloroform extraction [17]. During this study, glass bead method resulted in good DNA yield because the mechanical disruption by this method resulted in rupture of both oocysts and sporocysts wall which enabled the release of sporozoites before solubilised. Further the oocyst wall of *Eimeria* species usually consists of two or more layers [22, 23]. The outer skeletal layer is a thick, elastic substance composed of a chitin-like material and the inner layers is composed of approximately 70% protein and 30% lipid, with the protein localized in the outermost portion and the lipid mainly distributed in innermost portion of the inner layer [22, 24]. Because the inner layer of the oocyst wall mainly is composed of protein and lipid, it can be digested and dissolved by adding proteinase K and a lysis buffer to the sample (AL Lysis buffer included Kit) once the outer layer of the oocyst wall is removed. During this study, slight modification was carried out in the procedure of commercial kit (Qiagen- Fast DNA Stool Mini Kit) for extraction of DNA from oocysts, Proteinase K and lysis buffer (AL buffer) was used to strip off the outer layer of the oocyst wall and dissolve the inner oocyst wall and sporocyst walls and sporozoites membranes to release the genomic DNA.

The glass bead protocol requires only one step to solubilize and rupture the cells and then is followed by standard DNA extraction techniques as per kit with slight modification in the procedure. It is quite simple and rapid compared to other existing methods. The Glass-bead grinding required slightly longer grinding times was also reported with equal rupture efficiency of the oocysts. When heavy burdens of fecal remains were present, the final fraction of oocysts required longer time to grind regardless of method and also described that 0.5 mm glass beads to be optimal glass bead size. During this study, same size of glass beads was used and 15 to 20 min time was required to rupture all the oocysts wall and sporozoites by vortexing [14].

Several means of disruption of the oocysts wall have been described by many authors including sonication [25], hot phenol incubation [12, 26], repeated freezing and thawing [11], enzyme digestion after sodium hypochlorite incubation [26], passage through a high pressure cell [27], grinding in liquid nitrogen [11, 28] and grinding by glass beads [13, 29]. However the

resistance of oocysts of *Eimeria* species, due to the outer oocysts wall, an important protective barrier, can also be a limiting factor for obtaining good yield DNA. The use of glass beads has been the most commonly used procedure for disruption of oocyst walls and previously reported by different authors [30, 31]. It was also reported, that fifty cycles of freezing and thawing, using liquid nitrogen and boiling in water bath at 50 °C could completely rupture the eimerian oocysts walls [16].

The incubation of culture of oocysts in sodium hypochlorite for 1.5h at 4 °C followed by treatment with saturated salt solution for 1h at 55 °C broke the wall of *Eimeria tenella* oocysts and other coccidian species of chickens and rabbit and the DNA was also extracted from approximately 50 oocysts using Kit (Tiagen, Beijing, China) [32]. Disruption of oocysts by sonication method was also which successfully ruptured the eimerian oocysts wall and sporocysts and released the sporozoites and DNA was extracted by Stool Mini Kit (Qiagen, Germany) [33]. Further methods to rupture oocysts may also be employed, although the results are variable and large numbers of oocysts are often not affected.

Conclusion

Eimeria have particularly thick oocyst walls that are highly resistant to mechanical and chemical forces. Additional steps to break down the oocysts and sporocyst walls to enable release of sporozoites are needed before the sporozoites can be solubilized. Because the inner layer of the oocyst wall mainly is composed of protein and lipid, it can be digested and dissolved by adding proteinase K and a lysis buffer (AL buffer) to the sample. The present study recorded that glass bead method is suitable for extraction of DNA from small (<1x10³) and large numbers of oocysts. And can be used as routine procedure for DNA extraction from chickens *Eimeria* oocysts.

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