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Transmission Electron Microscopy (TEM) Study of the Oocyst of *Eimeria tenella*

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Abstract: The present work describes isolation, cell wall preparation, morphological as well as molecular detection of oocyst of *Eimeria tenella*. Based on transmission electron microscopy, the oocyst of this species showed the outer and inner wall without veil forming bodies; cross section of sporozoites with different organelles and macrogamete structure at ultrastructural level. Polymerase Chain Reaction (PCR) based on Internal Transcribed Spacer 1 (ITSI) regions of ribosomal DNA (rDNA) demonstrated 270 bp specific for *E. tenella*. Results of this study provide the basis for further ultrastructural studies of the organism. The present study also suggested that morphological characteristics along with molecular identification are prerequisite for validation and identification of *Eimeria* species.

Key words: Oocyst, *Eimeria tenella*, organism

INTRODUCTION

The coccidia are a large ubiquitous group of protozoa classified under the phylum Apicomplexa on the basis of certain anatomical features that were revealed by electron microscopic studies, collectively known as the apical complex (Levine *et al*., 1980). The most important coccidian parasites in poultry are species of the genus *Eimeria*. These parasites are characterized by oocysts that contain four sporocysts, each containing two infective sporozoites. The discharge of the protein contents of micronemes, rhoptries and dense granules during invasion of host cell that has been studied in various apicomplexan parasites has led to the suggestion that these organelles are involved in the process of recognition, host cell invasion and parasite adaptation (Aikawa *et al*., 1990; Leriche and Dubremetz 1991; Dubremetz, 1993; Rick *et al*., 1998). The precisely controlled chronology of the exocytosis of apical organelles is quite intriguing and forms the basis of the ongoing search aimed at describing the events in functional and structural terms (Caruthers and Sibley, 1997).

The present study was undertaken to study Transmission Electron Microscopy (TEM) and characterize the Internal Transcribed Spacer 1 (ITS1) regions of ribosomal DNA (rDNA)) species specific for *Eimeria tenella*.

MATERIALS AND METHODS

Oocyst floatation: The sporulated oocysts in 2.5% potassium dichromate were centrifuged at 1000 g for 10 min and the sediment saved. The pellet was resuspended in PBS and centrifuged a second time to replace the potassium dichromate with PBS. For the separation of oocysts from fecal/tissue debris saturated sterile sodium chloride floatation was used according to the procedure described by Rose *et al*. (1984). The washed pellet of the oocysts was mixed with saturated sodium chloride solution. The mixture was blended briefly and then transferred into 50 ml centrifuge tubes. Two ml of distilled water were layered carefully on the top of each tube. The tubes were centrifuged at 1000 g for 15 min. The oocysts formed a whitish layer on the top of the salt solution and were collected by aspiration. The oocysts were washed 4 times with distilled water using centrifugation to collect the oocysts between two washings (1000 g for 10 min). The pelleted oocysts were stored in PBS at 4°C until experimentation. The sporozoites were excysted through mechanical and enzymatic digestion of the oocysts and sporocysts

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respectively. The oocysts in PBS were centrifuged at 1000 g for 10 min in a 15 ml centrifuge tube. The supernatant was discarded and 0.9 ml of the thick white slurry of oocysts was added to 3.3 gm of glass beads contained in a glas vial. The stoppered vial was held in a shaker for 10-12 sec. The released sporocysts were separated from the glass beads by washing with PBS and the resulting washings containing sporocysts, oocyst walls and a few intact oocysts were filtered through a cellulose nitrate membrane filter (Whatman GmbH, Germany). The filtrate was collected in a beaker on ice and then centrifuged at 1000 g for 10 min to collect the sporocysts. The pelleted sporocysts were suspended in excystation fluid (0.25% trypsin, 5% chicken bile) (v/v) in PBS, adjusted to pH 7.4 with 1.0 M NaOH) and placed in shaking water bath at 40°C in flasks, each containing 40-50 ml of sporocyst suspension, for 90-120 min. When the majority of the sporozoites had existed, the excystation was halted by 3-fold dilution with PBS. Four centrifugations at 1000x g for 10 min were used to wash the excystation fluid with PBS. The pellet containing sporozoites, sporocyst walls and the unexcysted sporocysts was then suspended in PBS.

Collection and isolation of macrogamete: Collection of macrogamete was made by taking the scraping of the ceacal mucosa and subsequently macrogametes were floated and stored until experimentation in similar way to that of oocysts.

Purification of sporozoites: The mixture of sporozoites, sporocyst walls and unexcysted sporocysts was fracactionated using percoll gradient. Ninety- percent isotonic Percoll was prepared by adding PBS to Percoll. The gradient was created by mixing 3 parts of 90% isotonic Percoll with 2 parts of sporozoite suspension in PBS. The resulting mixture was centrifuged at 18000 g for 20 min. The sporozoites were recovered from near the bottom of the resulting continuous gradient and washed 4 times with PBS to remove the Percoll. The centrifugations between the washings were done at 1000 g for 10 min each time. The purified sporozoites were stored over night in PBS at 4°C.

Isolation of purified oocysts wall fragments: Purified oocysts wall fragments were prepared as described previously (Eschenbacher *et al*., 1996) with little modification and stored as pellets at -80°C until required. Briefly, sporulated oocysts (approx. 10^7) were washed in distilled water (1750 g for 10 min at 4°C) to remove 2.5% potassium dichromate solution within which they were stored. Oocysts were then re-suspended in 40 mM Tris-cl (pH 9), mechanically disrupted by being vortexed in the presence of glass beads and examined microscopically to ensure that at least 90% of the wall had been broken. The walls were then pelleted (1750 g, 10 min, 4°C) and washed in wash buffer (1mMEDTA [pH 8.0], 0.1% Triton x-100) by centrifugation (2500 g, 10 min, 4°C). The walls were then resuspended in 5 ml of wash buffer and sonicated (OMNI Ruptor 400, Made in USA) for 30 sec intervals over 1 min. The sonicate was then centrifuged (2500 g, 10 min, 4°C) and the pellet was collected and washed in 1 M sucrose in wash buffer three times (2500 g, 10 min, 4°C) in a swing-out rotor. When the supernatant was clear, it was discarded and the oocysts pellet wall was stored at -80°C until required. To obtain inner wall, washed oocysts were kept and incubated in 5.75% HCl_3 solution at 4°C to remove the outer wall. The rest of the procedure was same as described above.

Transmission electron microscopy: Purified oocysts of *Eimeria tenella* were washed with distilled water to remove the 2.5% potassium dichromate solution within which they were stored. Each sample of oocysts wall fragments, unsporulated oocysts, gamatocytes and the sporozotes was centrifuged and the pellets were fixed in 4% glutaral dehyde in 0.1 M phosphate buffer. The pellets were post-fixed in 1% osmium tetraxide in phosphate buffer, dehydrated in absolute ethanol, treated with propylene oxide and embedded in spurr's epoxy resin. Thin sections of oocysts walls, sporozoites, macrogamate and unsporulated oocysts were then mounted on copper grids and stained with uranyl acetate and lead citrate for routine electro microscopy.

DNA extraction: The pure oocysts, stored in 2.5% potassium dichromate solution, were washed 4 times by centrifugation (1.4 x 10^3 rpm for 5 min, each wash) in autoclaved, high-salinity, phosphate buffered saline (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4) (Reece *et al*., 1997). The pellet was resuspended in 200 µl of 5.75% sodium hypochlorite and incubated on ice for 30 min. The oocyst suspension was then diluted with 1 ml of sterile double distilled water and pelleted by centrifugation. The pellet was again washed 3 times in the above PBS. The pellet was resuspended in 1 ml deionized water and sonicated at high speed sonication using ultrasonicator to disrupt the oocysts (OMNI Ruptor 400, Made in USA). The DNA was extracted from sonicated oocysts and analyzed by agarose gel electrophoresis as per standard procedures (Sambrook *et al*., 1989). The genus specific forward primer coded 'BSEF' 5'-CTGTGAATCCATCGGA-3' and the reverse primer coded 'BSER'-5'-ATCGCATTTCGCTGCGTCCT-3'(Bangalore Genei, India). The species specific forward primer for *Eimeria*

tenella coded 'ETF' 5'-AATTTAGTCCATCGCAACCCT-3' and the reverse primer coded 'ETR' 5'-CGAGCGCTCTGCATACGACA-3' as per Devereux *et al.* (1984).

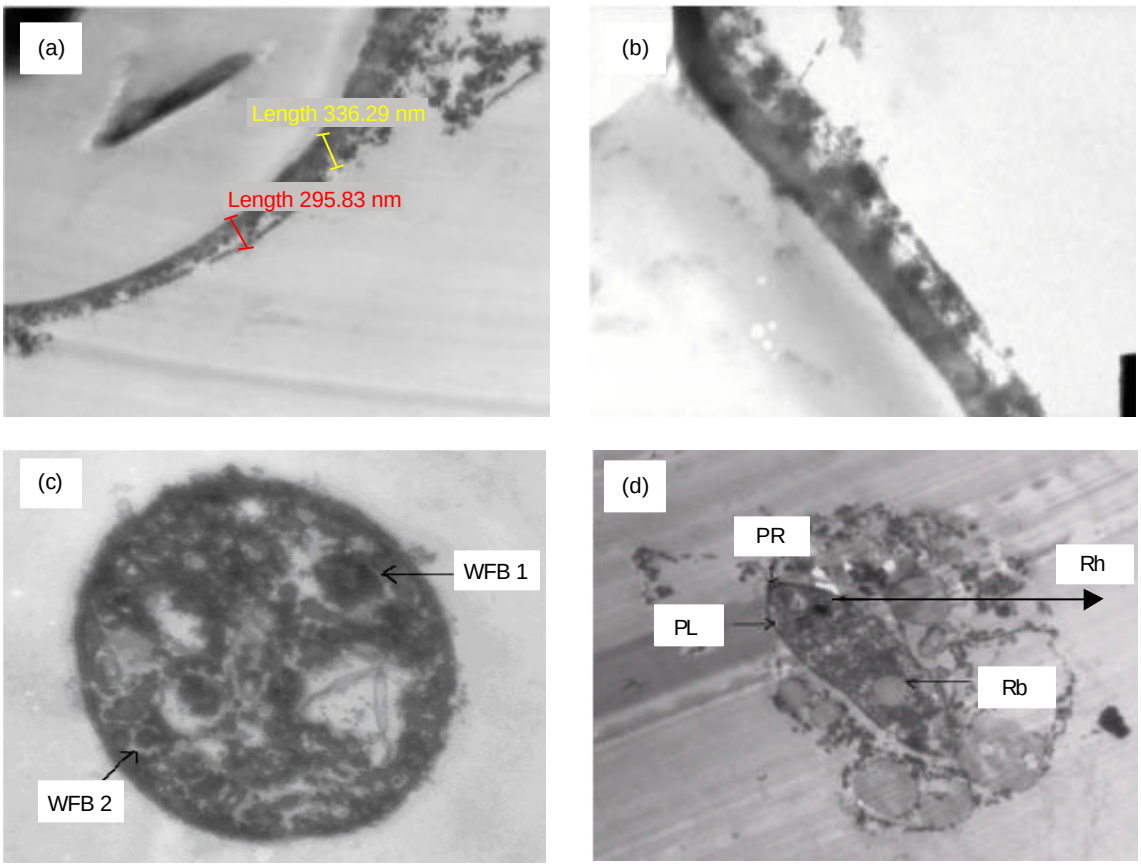
Amplification of the ITS1: BSEF and BSER primers were used to amplify the ITS 1 region of rDNA from the field samples by PCR. The PCR product was analyzed by gel electrophoresis.

PCR: The PCR was done with 50 µl of reaction mixtures for each sample that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each deoxyribonucleotide, 100 pmol oligonucleotide (25 pmol of each genus and species specific for *E. tenella*) primer and target DNA. The samples were kept at 94°C for 4.5 min before adding IU of Taq DNA polymerase (Bangalore Genei). The cycling programme was performed with system Mastercycler Gradient machine (Eppendrof, Germany), each of the 30 cycles consisted of 50 sec denaturation at 94°C, 50 sec of annealing at 62°C and 1 min extension step at 72°C with the final extension for 2 min. Ten-microlitre aliquots of the reaction mixtures were electrophoresed through 1.5% agarose gels and stained with ethidium bromide.

RESULTS

TEM: TEM of the outer and inner walls of oocysts of *Eimeria tenella* are shown in (Fig. 1a, b). The outer layer of the oocysts wall was electron dense with a roughened outer surface and an increased density on the inner side. The thickness of the layer was 295.93 nm. The inner layer was having a uniform electron lucent appearance. Two cytoplasmic structures were identified in the present study (Fig. 1c): The wall forming bodies 1(WFB1) and wall forming bodies 2(WFB2). Figure 1d represents an early stage of oocyst wall formation, showing secretion of the WFBS to initiate formation of the outer layer of the oocysts wall. Transverse section of the sporozoites of *Eimeria tenella* under TEM showed outer plasmalemma and the various subcellular organelles like rhoptries, microtubules, refractile bodies, plant like storage amylopectin granules, polar ring, nucleus with nucleolus etc. (Fig. 1e, f and g).

Gene amplification by PCR: The specific annealing temperature of both the genus and species specific primer pairs was found to be 62°C. The amplification of the ITS1 region using the genus and species specific primer pairs at optimum temperature yielded a product of approximately 570 bp and 270 bp, respectively (Fig. 2) (Patra *et al.*, 2010).



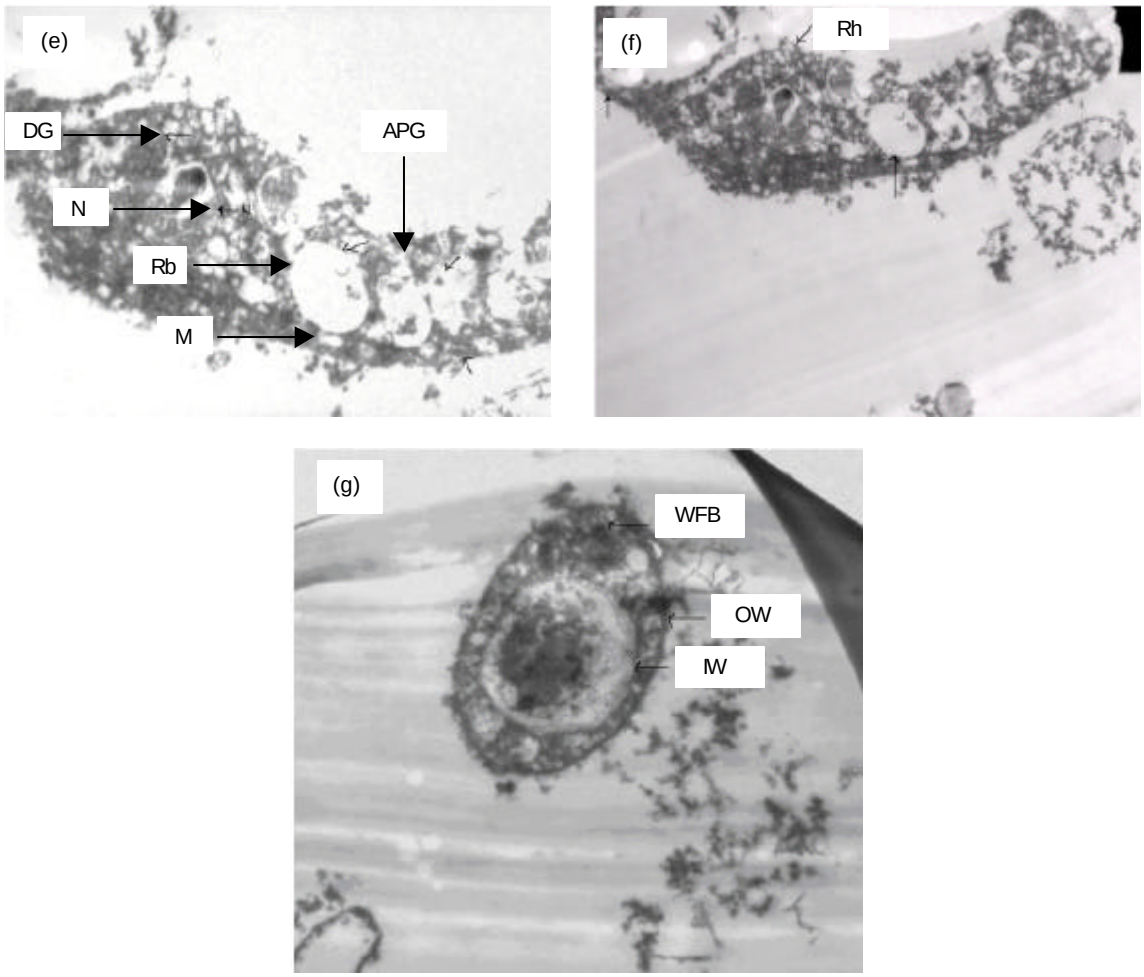


Fig. 1: (a) Outer wall of oocysts of *Eimeria tenella* (5000x). (b) Inner wall of oocysts of *Eimeria tenella* (1300x). (c) Macro gamete of *Eimeria tenella* (6300x). (d-f) Sporozoite of *Eimeria tenella* (1600x, 4000x, 2500x) (g) An early stage of oocyst wall formation of *Eimeria tenella* (2500x). DG: Dense Granule, N: Nucleus, APG: Amino Pectin Granule, WFB1: Wall Forming Bodies 1, WFB2: Wall Forming Bodies 2, Rh: Rhoptries, M: Microtubule, OW: Outer Wall, IW: Inner Wall, Rb: Refractile body, PL: Plasma Lemma, PR: Polar Ring

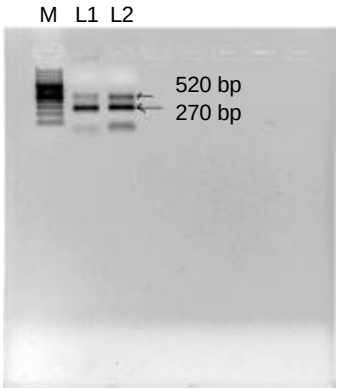


Fig. 2: Agarose gel electrophoresis of the PCR products. Lane 1 and Lane 2: Amplified PCR products obtained using both genus and *E. tenella* specific primers. Lane M: 100 bp marker

DISCUSSION

The oocyst wall is impervious to many common disinfectants, allowing oocysts to survive and remain infective in a moist environment for long period of times (Ryley, 1973). The early oocysts are enclosed by loose outer membrane, termed the outer veil (Ferguson *et al.*, 2000; Ferguson *et al.*, 2003) and underlying this is the oocysts wall comprising two distinct layers. This delicate veil is normally absent in mature oocysts isolated from faeces. The thickness of the outer layer of *Eimeria tenella* (295.93 nm) observed in the present study differed not only from other genera but also from the species of the same genera, being-200 nm for *Eimeria maxima* (Ferguson *et al.*, 2003) and 20-40 nm for *Toxoplasma gondii* (Ferguson *et al.*, 2000). Oocysts develop from macrogametes and this appears to occur before their release from the host cell into the lumen.

Two cytoplasmic structures i.e. WFB1s and WFB2s were observed in the macrogamete by TEM in the present investigation. The WFBs1 have been the features of storage granules and unlike the VFGs, which are secreted during macrogamete maturation, they are retained until their simultaneous secretion from the mature macrogamete to form the outer layer of the oocysts wall. The WFB2s are unusual structures in that they are not cytoplasmic granules but consist of irregular shaped electron dense deposits located within the rough endoplasmic reticulum (REM). Thus, the question of how material locate in the endoplasmic reticulum could be secreted to form the inner layer of the wall is intriguing (Sabina *et al*., 2006). However, by immune electron microscopy, it was observed that, only after the release of the contents of WFBs was the WFB2s material retained in the RER transferred to the surface via the golgi body (Ferguson *et al*., 2003). In the fully formed oocysts wall, the WFBs were no longer present in the present study had been also observed previously (Lindsay, 1999; Elwasila, 1984; Ferguson *et al*., 2003).

Transmission electron microscopy study of the ultra-section of sporozoite of *Eimeria tenella* showed plasmalemma, polar ring, rhoptris, refractile bodies, nucleus and other subcellular organelles. Apicomplexan sporozoites are motile, infective stages that invade cells of various tissues. In addition to their motility, zoites are characterized by possessing ultrastructurally a pellicle, conoid, polar ring, rhoptris and dense granules concentrated in the apical parts of the parasites. Dubremetz (1993) has suggested that three distinct extrusomes (rhoptries, micronemes and dense bodies) present in all apicomplexan sporozoites may be involved in sporozoite host cell reactions.

Although the organelles are conserved among different apicomplexan parasites and despite seemingly conserved functions, very few homologies have been found beyond the genus level. However, our knowledge is too incomplete to detect homologies which would very likely point to conserved properties and would help identify conserved functions. Coccidian sporozoites are characterized by their ability to penetrate into intact host cells without damaging the host cell membrane. Within the cytoplasm of the infected cells, the parasite resides within a vacuole comprised of a modified host cell membrane. The invasion process of coccidian sporozoites has been divided into a) recognition, b) internalization of the sporozoite and c) maturation of the vacuole into a metabolic compartment suitable for parasite growth. Based largely on ultrastructural observations organellar involvement in cell invasion has been suggested as: micronemes at recognition, rhoptries during internalization and dense granules during parasitophorous vacuole formation (Hemphill *et al*., 1998).

In this study, the specificity of PCR assay was assessed by positive amplification of DNA from *E. tenella* with species specific primer. No cross amplification by the other species specific primer was observed. Thus, the result of this study clearly demonstrates that the PCR assay based on amplification of ITS1 regions of *Eimeria* species in poultry can be used for detection and identification of the parasite. An attractive genomic DNA target for PCR analysis is the internal transcribed spacer-1 gene of ribosomal DNA (Cai *et al*., 1992; Holmdahl and Mattsson, 1996). This spacer separates the 3' end of the 16S like ribosomal RNA (rRNA) gene within individual rDNA transcription unit, it is also of a multiple copy gene family and thus provide large numbers of potential PCR targets.

Results of this study suggest that the ultrastructural studies of *E. tenella* at molecular level may help validation and identification of *Eimeria*.

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