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Transfected *Eimeria tenella* Could Complete Its Endogenous Development In Vitro

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ABSTRACT: Live attenuated coccidiosis vaccines could be used as powerful carriers, expressing exogenous viral and bacterial antigens, to induce protective immunity against pathogenic organisms. We investigated the ability of *Eimeria tenella* to express an exogenous gene in vitro. *Eimeria tenella* sporozoites were transfected with the plasmid pH4-2EYFP-Actin3 containing the yellow fluorescent protein gene (yfp) and inoculated into primary chicken kidney cells (PCKCs), followed by incubation at 41 C in a 5% CO_2 chamber. Fluorescent sporozoites were observed as early as 15–20 hr post-inoculation (PI). Fluorescence displayed by the expressed YFP protein was visible throughout the schizogony and gametogony stages of the tranfected *E. tenella*. Fluorescent oocysts were found between 200–327 hr PI. Higher fluorescence intensity was observed in the nucleus than in other compartments of the transfectants, while little or no fluorescence was seen in the refractile globule. The diversity of schizonts, particularly of the first generation, was presented by fluorescent nuclei arranged in different patterns. Our results demonstrated the ability of *E. tenella* to express an exogenous gene throughout the endogenous development in vitro. Completion of the endogenous development of transfected *E. tenella* in cell cultures will facilitate the study of foreign antigen expression in *Eimeria* spp., paving the way for the development of an *Eimeria* spp. vector vaccine that also carries and delivers other vaccines by oral administration.

Eimeria spp. are apicomplexan parasites and are the most common causative agents of coccidiosis, which is prevalent in many domesticated animal species, particularly poultry. The universal loss from coccidiosis in chickens is estimated to be more than $2 billion annually (Shirley et al., 2004). Preventative and therapeutic control of coccidiosis using anti-coccidial drugs is being challenged by the problem of increasing drug resistance and residues. Fortunately, live attenuated *Eimeria* vaccines have offered a practical alternative to anti-coccidial drugs for the sustainable control of coccidiosis in chickens, and several such vaccines have been commercially available for a number of years (Suo et al., 2006).

Recently, because of their convenience for oral immunization (Kotton and Hohmann, 2004; Souza et al., 2005), many enteric microorganisms, including viruses and bacteria, were investigated for their ability to express heterogeneous proteins and induce specific immunological responses. Live attenuated coccidiosis vaccines are better candidates for expressing exogenous proteins because of their large capacity to carry foreign genes and their high immunization efficiency to chickens. However, gene transfection of *Eimeria* spp. has not been extensively studied due to the lack of sufficient genetic information and to the difficulty of culturing the parasite in vitro. Kelleher and Tomley (1998) reported, for the first time, a transient transfection system using β-galactosidase as the reporter enzyme for *Eimeria tenella*, which was cultured in the MDBK cell line. Later, another system using the yellow fluorescent protein (YFP) or red fluorescent protein (RFP) as a marker was described for *E. tenella* cultured in the same cell line by Hao et al. (2007). However, the above 2 investigations reported transfectants that differentiated only to first-generation schizonts. This limits the investigation of the transfectants to expression of exogenous genes over the entire development stages and to the development of multivalent coccidian vaccines carrying bacterial and/or viral antigens.

Here, we describe a transfection system in which transfected *E. tenella* complete the development stages and differentiate to oocysts in vitro. The plasmid pH4-2EYFP-Actin3, containing *yfp* as the reporter gene flanked by a 2.0 kb histone 4 5′ region (including a 90 bp coding sequence ligated in-frame with the *yfp* start codon) and a 1.5 kb actin 3′ region derived from the BJ *E. tenella* strain, was introduced into *E. tenella* as described below. The BJ strain *E. tenella* was isolated, sporulated, and maintained according to the method of Long (1982). Sporocytes were released from oocysts by grinding, followed by excystation with trypsin-bile solution (0.75% [w/v] trypsin and 10% [v/v] bile in PBS). Sporozoites were purified by DE-52 anion-exchange chromatography (Schmatz et al., 1984). The purified sporozoites were washed once in an incomplete cytomix buffer (10 mM K_2HPO_4:KH_2PO_4, pH 7.6; 120 mM KCl; 0.15 mM $CaCl_2$; 25 mM HEPES; 2 mM EGTA; and 5 mM $MgCl_2$) (Van den Hoff et al., 1992) and then suspended in the same buffer, supplemented with 2 mM ATP and 5 mM glutathione. The pH4-2EYFP-Actin3 circular plasmid DNA (200 μg in 50 μl cytomix) was then added to the sporozoite suspension (1×10^7 ml^{-1}). Transfection was completed by electroporation using a Gene Pulser Xcell™ Electroporation System (Bio-Rad, Hercules, California) with 0.4 mm-gap cuvettes at 2.0 kV and 25 μF. The electroporated cells were placed in a Laminar flow cabinet and left undisturbed for 20 min at room temperature. Primary chicken kidney cells (PCKCs) prepared from a 10-day-old chicken were used to cultivate the transfectants according to the modified method of Taylor and Baker (1978). The PCKCs were grown in 5 ml of RPMI1640 culture solution (Gibco, Grand Island, New York) containing fetal bovine serum (5% [v/v]), penicillin (200 U ml^{-1}), and streptomycin (20 mg ml^{-1}) at 41 C in an atmosphere containing 5% CO_2 using 25-cm^2 flasks. The development of transfected *E. tenella* was investigated using fluorescence microscopy (Olympus, Tokyo, Japan) with 488 nm excitation and 508 nm emission filters, under which the expressed fluorescence appeared green. The optimal spectrum for yellow fluorescent protein detection was 514 nm for excitation and 527 nm for emission (Patterson et al., 2001).

Fluorescent sporozoites were observed as early as 15–20 hr PI. The nucleus showed the highest intensity of green fluorescence emission, while the refractile globule showed little or no fluorescence at all, indicating the 90 bp coding sequence downstream from the histone 4 promoter worked as a nucleus-targeting signal sequence (Fig. 1A). One or 2 refractile globules were always visible at the posterior or anterior end of the fluorescent sporozoites and schizonts. The presence of refractile globules was an indication of the first-generation schizogony. After active entry into the PCKCs by gliding motility, fluorescent sporozoites often looked swollen and moved irregularly in the host cells, even after 200 hr PI. Transformation of the sporozoites to spherical mononucleate fluorescent trophozoites occurred by increasing the width of sporozoites or by formation of a lateral outpocketing at the anterior end (Figs. 1B, C). The first-generation schizonts divided exponentially without reduction in fluorescence intensity (Figs. 1D–F). Fluorescent schizonts showed a variety of features because of daughter merozoites replicating in different patterns. There were some schizonts with nuclei arranged in the shape of maize-cobs, some in the shape of flowers, and others in irregular patterns (Figs. 1G–K). Mature schizonts often lacked visible refractile globules (Figs. 1I–K).

Many fluorescent first-generation merozoites were observed scattered in the cell culture at 60 hr PI. Most free crescent-shaped merozoites contained a single nucleus with some invading cells becoming trophozoites in the vicinity of the parent schizonts (Figs. 2A, B). Mature second-generation schizonts were differentiated from first-generation schizonts by their larger size and an absence of refractile globules, according to Strout and Ouellette (1970) (Figs. 2C, D). Consistent with observations by the same authors, fluorecent second-generation merozoites are also longer than the first-generation merozoites (Fig. 2E). In addition, fluorescent merozoites smaller than first-generation merozoites were observed in the culture; these smaller merozoites were probably third-generation (Fig. 2F). Tyzzer (1929) and Strout and Ouellette (1970) shared the opinion that third-generation schizogony does occur, but believed the third-generation merozoites are longer than those of the first-generation but shorter than those of the second-generation.

With further development, fluorescent immature microgamonts, mature microgamonts, and free microgametes were observed between 133 and 262 hr PI (Figs. 3A–D). Fluorescent nuclei of microgametes in

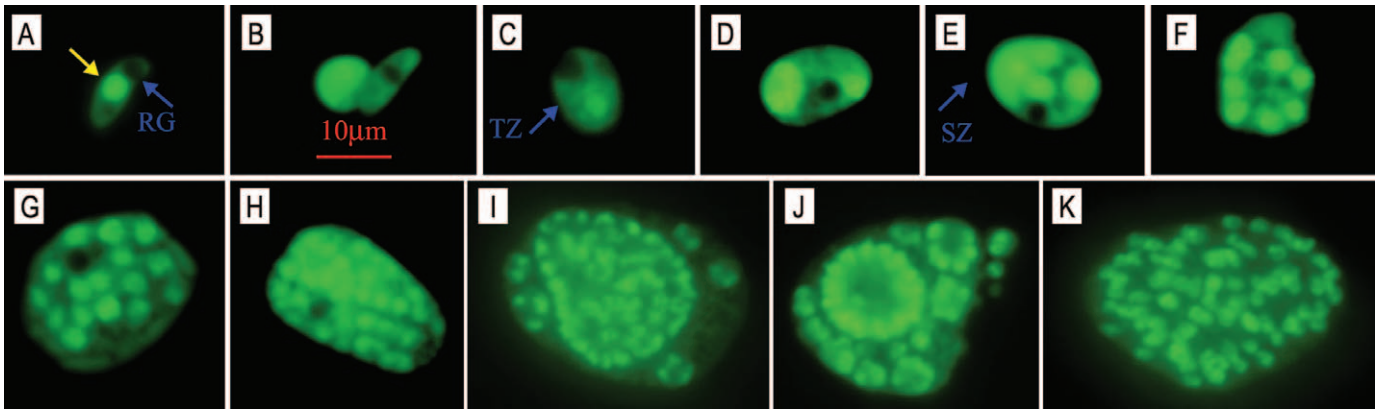

FIGURE 1. Development of transfected *E. tenella* from sporozoite to mature first-generation schizont in primary chicken cells (PCKCs). (**A**) A sporozoite in a PCKC at 24 hr PI. (**B**) A sporozoite with a lateral outpocketing at the anterior end at 42 hr PI. (**C**) A trophozoite at 42 hr PI. (**D–F**) Exponential development of schizonts with 2, 4, and 8 nuclei between 42 and 44 hr PI. (**G–K**) Schizonts with nuclei arranged in different patterns between 42–63 hr PI. (The same magnification was used for all figures shown in Fig. 1A) RG, refractile globule; N, nucleus; OP, outpocketing; TZ, trophozoite; SZ, schizont.

immature microgamonts were not clear. Most mature micogamonts detached from the PCKCs, with comma-shaped micogametes actively moving inside the microgamonts. Fluorescent macrogametes were observed between 173 and 360 hr PI (Figs. 3E–G). Higher fluorescence intensity in the nuclei of macrogametes was visible. At the same time, fluorescent immature oocysts were also found (Fig. 3H). To propagate fluorescent oocysts, we collected them from the culture and orally inoculated them into chickens after sporulation. Only a few non-fluorescent oocysts were sporulated and recovered from inoculated chickens (Fig. 3I). No fluorescence was detected in control parasites, which were not transfected with the plasmid DNA, or only mixed with the plasmid but not electroporated, and cultured in PCKC cells.

The YFP was highly expressed in the nuclei of sporozoites, schizonts, merozoites, microgamonts, and mature icrogamonts, and brilliant fluorescence from expressed YFP was visible throughout the developmental stages of *E. tenella*. This study demonstrated that transfected *E. tenella*

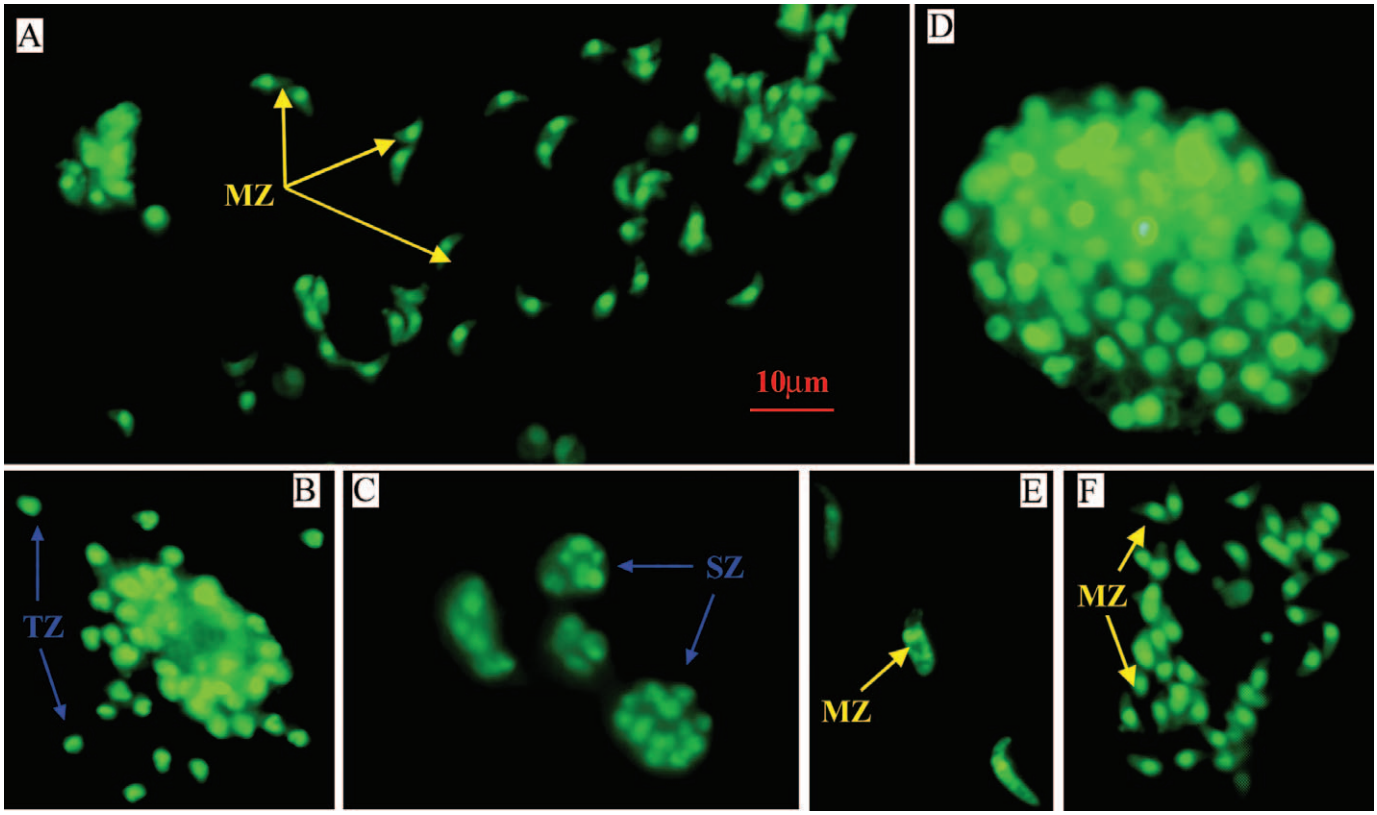

FIGURE 2. Merozoites and schizonts in non-synchronized schizogony. (**A**) First-generation merozoites at 63 hr PI. (**B**) Second-generation trophozoites at 87 hr PI. (**C, D**) Immature and mature fluorescent schizonts in second-generation schizogony at 100 and 188 hr PI. (**E**) Second-generation merozoites at 110 hr PI. (**F**) Possibly third-generation merozoites at 142 hr PI. (The same magnification was used for all figures shown in Fig. 2A). TZ, trophozoite; SZ, schizont; MZ, merozoite.

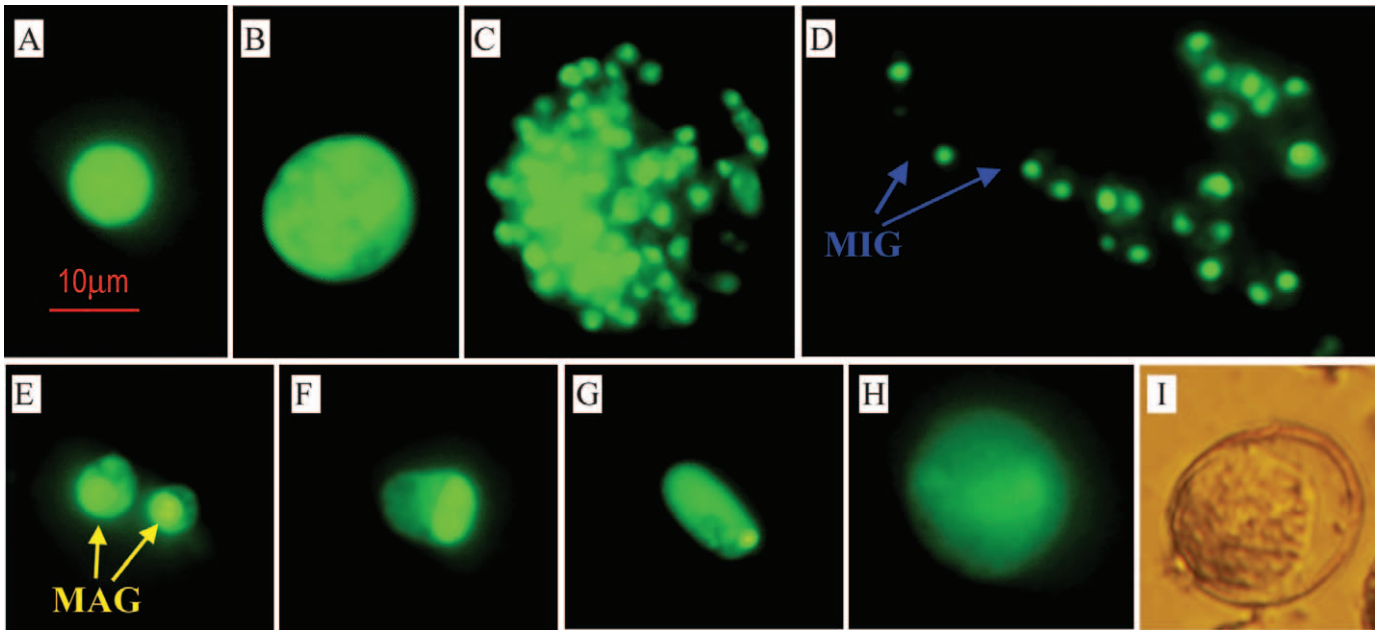

FIGURE 3. Gamogony and oocysts. (**A–D**) Microgametogenesis between 133 and 262 hr PI. (**A, B**) Different developmental stages of immature fluorescent microgamonts. (**C**) Fluorescent mature microgamonts. (**D**) Fluorescent microgametes released from microgamont. (**E–I**) Macrogametogenesis and oocyst formation between 173 and 360 hr PI. (**E–G**) Different forms of fluorescent macrogametes at different developmental stages. (**H, I**) Fluorescent from the PCKC culture and non-fluorescent oocysts obtained from a chicken inoculated with cultured oocysts (The same magnification was used for all figures shown in Fig. 3A). MIG, microgamete; MAG, macrogamete.

could complete its endogenous development in vitro and express exogenous genes. The above-described method could be used to investigate the basic biology of *E. tenella* in vitro and to develop a transfected *E. tenella* strain. More significantly, it could facilitate the study of foreign antigen expression in *E. tenella*, and of antigen presentation and CTL responses of the host, thereby paving the way for the successful development of oral multivalent vaccines carrying coccidian, viral, and bacterial antigens for the prevention of multiple diseases.

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