FULL PAPER *Parasitology*

Characterization of Stage-Specific and Cross-Reactive Antigens from *Eimeria acervulina* by Chicken Monoclonal Antibodies

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ABSTRACT. The characterization of five chicken monoclonal antibodies (mAbs) that were developed against apical complex antigens of *Eimeria acervulina* sporozoites is realized and the mAbs reactivity to merozoites belonging to this species is tested. Using immunofluorescence assay (IFA), one mAb (HE-4) that recognized apical antigens common to sporozoites of *E. acervulina* and *E. brunetti* bound antigens localized on the apical tip of merozoites from all stages of development examined. The mAb 8E-1, reactive with antigens found on the apical tip of all chicken *Eimeria* sporozoites, also showed binding to antigens common to merozoites from all generations. Another mAb, 8C-3, which identified an antigen shared by sporozoites apical tip and sporocysts wall of *E. acervulina* reacted very weak and inconstantly with the merozoites from all generations whereas the mAbs 5D-11 and 8D-2 that recognized antigens shared by the sporozoites of *E. acervulina* and *E. maxima* (mAb 5D-11) and *E. acervulina* and *E. brunetti* (mAb 8D-2) did not react with the merozoites from any generation. Collectively, these results showed that the invasive stages of chicken *Eimeria* share cross reactive apical complex antigens which are inter-species and inter-generation-specific that might be components of a potential recombinant vaccine.
KEY WORDS: apical complex, *Eimeria*, immunofluorescence, merozoite, monoclonal antibody.

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Coccidiosis is the most important protozoan disease of chickens due to its worldwide prevalence and significant economic impact on poultry industry [20, 22]. For example, in 1995, coccidiosis cost UK chicken industry approximately 40 million pounds with annual poultry production of 625 million broilers [34]. At least seven species of *Eimeria* have been recognized to infect chickens [11], they parasitize specific areas within the digestive tract and they have different degrees of pathogenicity and immunogenicity. *Eimeria* species are obligate intracellular parasites with a complicated life cycle which consists of asexual stages and sexual stages.

Although the poultry industry has relied upon chemoprophylaxis method to control coccidiosis [7], alternative control strategies are needed due to high costs associated with the development of new drugs [20], rapid emergence of drug resistant field strains of *Eimeria* [6, 12], the public's attitude toward the drug treated-meat products [8, 20], the increasing demand for organic products [34] and the disadvantages that accompany the use of live vaccines [20, 33]. Therefore, new strategies such as vaccination with recombinant proteins and genetic improvement of poultry immunity, are now actively pursued [22].

Major drawbacks in developing recombinant vaccines are the *Eimeria* specie-specific host immunity [29] and the identification of the protective molecules and mucosal delivery methods [20, 22]. An optimal vaccine should include antigens common to many *Eimeria* species and developmental stages which induce protective immunity [20] and preferably, antigens associated with host cell recognition and invasion [5, 20, 33].

Intense research has focused on the identification of the immunogenic proteins of *Eimeria* using mouse monoclonal antibodies (mAbs) [1, 20]. However, the recombinant vaccines based on the molecules identified by mouse mAbs, generally, showed weak immunogenicity [4, 14, 17, 18, 26, 33] and further studies are needed to identify more effective vaccine candidates.

The mechanisms that govern the antibody diversity in chickens are different from those in mammals [28], they provide avian species with a robust system for the production of a primary antibody repertoire. Furthermore, the chicken has approximately one hundredfold more B-cells than a mouse and therefore has a larger initial cell repertoire from which to select reactive B-cells after *in vivo* priming [25]. In chicken eimeriosis it has been shown that the immune sera from chickens, mice and rabbits react with different intensities with antigens derived from *E. acervulina* invasive stages [13].

As a new approach to identify coccidia antigens relevant to the natural host immune system, chicken B-cell hybridomas were produced [19, 30]. Out of several chicken hybridomas developed, one designated as 6D-12-G10, secreted a mAb which identified an apical complex protein shared

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among all invasive stages of *Eimeria* spp. and related coccidian parasites including *Toxoplasma*, *Neospora* [31] and *Cryptosporidium* [24]. This mAb significantly inhibited the invasion of *E. acervulina* sporozoites into host cells in an *in vitro* model.

Recently we have developed additional chicken B-cell hybridomas secreting mAbs that reacted with antigens of the initial invasive stages (sporozoites) of all chicken *Eimeria* species [9]. In this paper, we further analyzed these chicken mAbs using *E. acervulina* merozoites from all generations fixed by different methods.

MATERIALS AND METHODS

Monoclonal antibodies: Five chicken monoclonal antibodies named 8C-3, 5D-11, 8D-2, 8E-1 and HE-4 were produced as culture supernatants as described [30] and used in indirect immunofluorescence assay with *E. acervulina* merozoites. The antibody isotypes were of the immunoglobulin G class and the antibody concentrations were adjusted to 0.93 μg/m*l* on the basis of protein assay (Bethyl, Montgomery, U.S.A.) for the studies described in this paper.

Merozoites: Merozoites were obtained by orally inoculating 27-day-old male chickens (Hyline) with 3 different doses (1×10^8, 2×10^7 and 1×10^6) of sporulated *E. acervulina* oocysts, USDA #40 strain (Beltsville, MD, U.S.A.), in Phosphate Buffered Saline (PBS). Chickens were euthanized by cervical dislocation at 48 hr, for the first and second generation merozoites, 72 hr, for the second and third generation merozoites and 85 hr, for the third and fourth generation merozoites, post infection (PI). Since the intracellular development of *Eimeria* is not synchronized and the shizogonic stages overlap [23, 35, 36], parasites from three different time points were pooled to identify the presence of the common merozoite antigens on all stages. When chickens are infected with large doses of parasites, after invasion some sporozoites may delay their development, and indeed we and other researchers [23, 36] observed in histological sections undeveloped or developing sporozoites at least up to 60 hr PI. In choosing the time points for getting the merozoites we took into consideration *E. acervulina* life cycle, the overlapping schizogonic stages and our preliminary data using the strain mentioned above (data not shown).

For merozoites preparation, the intestines were quickly opened and washed two times in cold PBS, cut in small pieces (4×4 mm) and incubated for 30 min at 41°C in a water bath with gentle shaking according to the method previously described [32]. The incubation medium consisted of 0.25% (w/v) trypsin (Merck, Darmstadt, Germany) and 1% (w/v) taurodeoxycholic acid (Sigma, St. Louis, U.S.A.) in Hanks' balanced salt solution at pH 7.4 (HBSS, Sigma, St. Louis, U.S.A.). After incubation the mixture was filtered through a cotton gauze and briefly centrifuged for 2 min at $700 \times$ g at 5°C to remove the large debris. The merozoites were purified on diethylaminoethyl (DEAE) cellulose (DE52, Whatman International Ltd, Maidstone, Kent, UK) columns using a modified method as described [32]. The purified merozoites were either air dried on siliconized glass slides (Dako, Kyoto, Japan) or fixed after drying using either cold methanol (2 min) or cold acetone (5 min) followed by 3 washes in PBS. Fresh merozoites were fixed for 15 min in 2% glutaraldehyde in PBS followed by two washes in PBS and allowed to dry at room temperature on siliconized glass slides. The siliconized glass slides with merozoites were preserved at –20°C until use.

Indirect Immunofluorescence Assay: The merozoites on the siliconized glass slides were incubated in a humid box with 100 μ*l* of each chicken monoclonal antibody for either 40 min at room temperature or overnight at 4°C, washed 3 times with PBS and incubated with 100 μ*l* of Rabbit anti-chicken IgG FITC conjugate (1:1000 in 1% BSA/PBS, Sigma, St. Louis, U.S.A.) for 40 min or 4 hr, respectively. The slides were then washed 3 times with PBS and mounted using Vectashield (Vector Laboratories, Burlingame, U.S.A.). The mAb 6D-12-G10 which reacts with all chicken *Eimeria* merozoites [31] was used as positive control whereas Iscove's Modified Dulbecco's Medium (IMDM, Sigma, St. Louis, U.S.A.) and chicken IgG (Cappel, ICN, Ohio, U.S.A.) were used as negative controls at the same concentration as the monoclonal antibodies. The slides were visualized and photographed using a Nikon Optiphot microscope (Nikon, Tokyo, Japan). Taking into account the small size of the merozoites, the different positions the merozoites can adopt on the glass slides and the weak staining for some merozoites and monoclonal antibodies the slides examination was conducted very carefully.

RESULTS

The summary of the reactivity of the chicken mAbs with *E. acervulina* merozoites fixed by different methods is presented in Table 1.

Monoclonal antibodies 8E-1 and HE-4 recognized an antigen present on the apical tip of air dried, acetone and methanol fixed merozoites from all generations (Figs. 1 and 2). MAb 8E-1 in general stained the merozoites more intensely than mAb HE-4. The mAb 8C-3 stained very weak and inconstantly the apical tip of merozoites from all stages regardless of the method of fixation (data not shown). For all these mAbs the merozoites collected 72 hr PI stained more intensely than the merozoites collected 48 or 85 hr PI.

Table 1. The reactivity of chicken monoclonal antibodies under study with *E. acervulina* merozoites fixed by different methods

Method of fixation	Dried	Methanol	Acetone	Glutaraldehyde
mAb 8C-3	–/+	–/+	–/+	–
mAb 5D-11	–	–	–	–
mAb 8D-2	–	–	–	–
mAb 8E-1	+	+	+	–
mAb HE-4	+	+	+	–

\+ = Intense and constant staining.
–/+ =Weak and inconstant staining.
– = No staining.

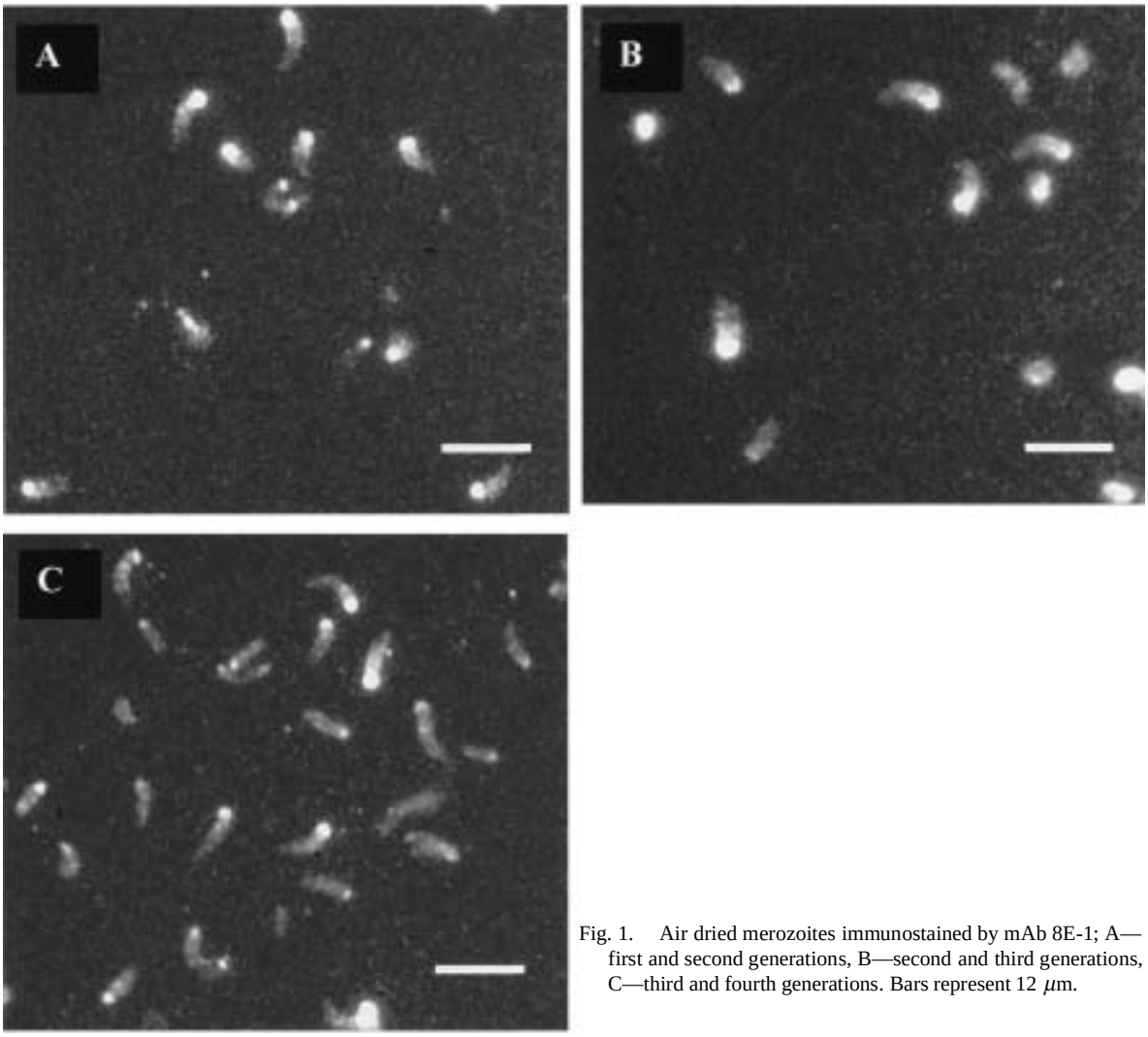

Fig. 1. Air dried merozoites immunostained by mAb 8E-1; A—first and second generations, B—second and third generations, C—third and fourth generations. Bars represent 12 μm.

The significance of this fact is unknown but Jenkins and Dame (1987) observed that while the *E. acervulina* merozoites do not differ essentially in their protein make-up some antigens might be expressed more intensely in some stages. The intensity of merozoites staining correlated well with that of sporozoites stained using these three mAbs (mAb 8E-1 stained the *E. acervulina* sporozoites most intensely followed by mAb HE-4 and 8C-3), implying that the antigens they recognize are represented the same in all invasive stages, most likely fulfilling the same task.

Irrespective of the method of fixation, mAbs 5D-11 and 8D-2 did not react with any antigen on the merozoites from any generations.

Glutaraldehyde (2% in PBS) seems to destroy epitopes which are recognized by chickens mAbs since none of the merozoites tested stained no matter what mAb was used.

DISCUSSION

The important role of the apical complex in host cells recognition, invasion and initial development of Apicomplexan parasites is generally established. Altering host cell recognition and/or blocking the sporozoites and merozoites invasion of host cells should prevent coccidian infection. In chicken eimeriosis the protective immunity is elicited by the sporozoite-infected intestinal cells [15] and further boosted by the merogonic stages [16]. Therefore, the identification and characterization of the antigens shared by *Eimeria* invasive stages have been actively pursued in the last years. The immunization with such molecules may induce an immune response that is capable of blocking the sporozoites invasion and further asexual development of merogonic stages [5].

Encouraged by the promising results we obtained using the chicken mAb 6D-12-G10 which recognized a conoid antigen shared among the invasive stages of *Eimeria*, *Toxo-*

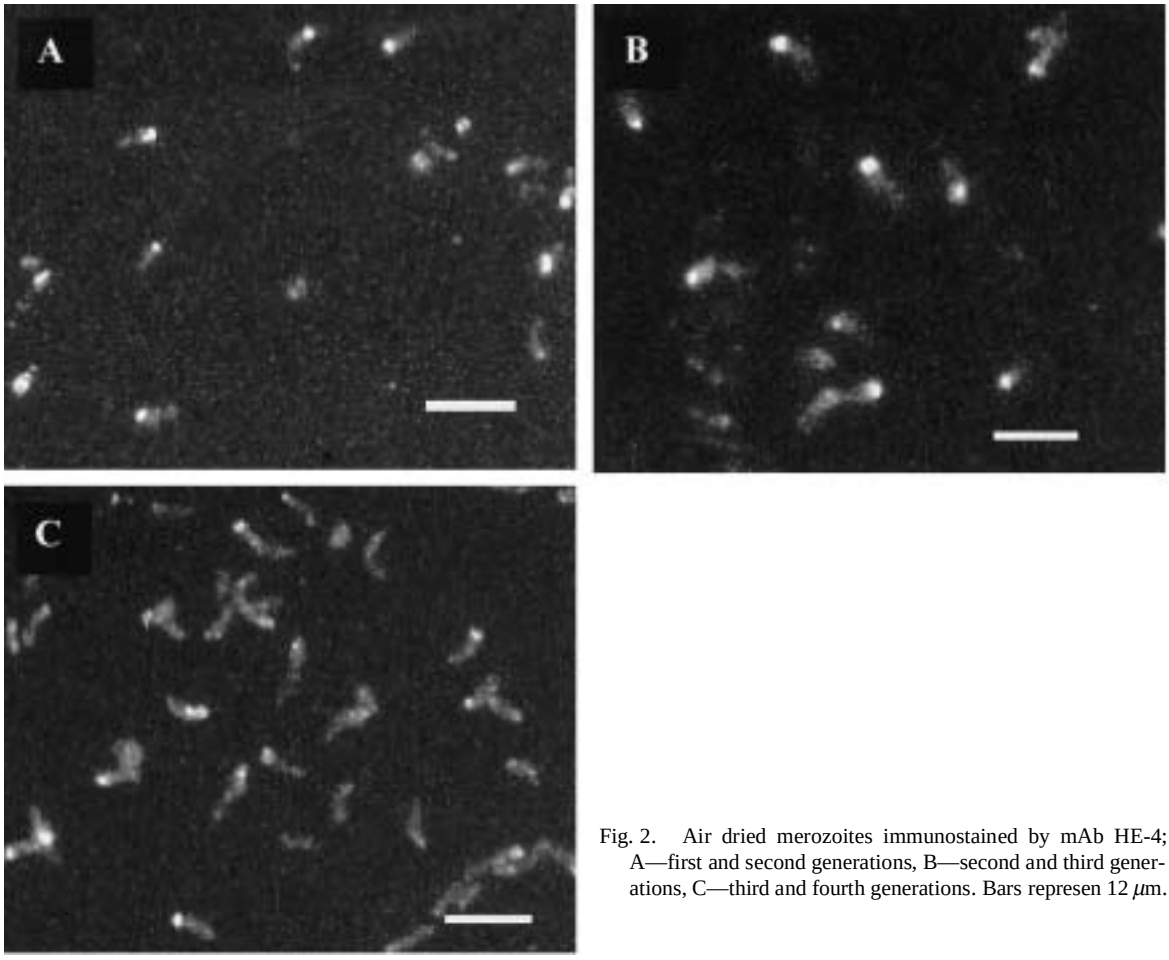

Fig. 2. Air dried merozoites immunostained by mAb HE-4; A—first and second generations, B—second and third generations, C—third and fourth generations. Bars represen 12 μm.

plasma, *Neospora* and *Cryptosporidium* and blocked the *E. acervulina* sporozoites invasion of $CD8^+$ T lymphocytes [30], we produced additional chicken mAbs that have been analyzed using sporozoites belonging to all chicken *Eimeria* species [9].

Out of the five chicken mAbs produced against *E. acervulina* sporozoites, one mAb (8C-3) recognized an antigen present on the sporozoites and sporocysts of *E. acervulina*, three mAbs recognized antigens presented on the sporozoites of two different *Eimeria* species, e.g. mAb 5D-11 detected an epitope on *E. acervulina* and *E. maxima* and mAbs 8D-2 and HE-4 identified *E. acervulina* and *E. brunetti* antigens, whereas the last mAb, 8E-1 recognized a sporozoite antigen shared by all chicken *Eimeria* spp.

In this paper, we showed that two mAbs (8E-1 and HE-4) recognized an antigen present on *E. acervulina* merozoites from all generations. Interestingly, while the antigen recognized by mAb HE-4 was shared by the sporozoites of only 2 species, *E. acervulina* and *E. brunetti*, the antigen recognized by mAb 8E-1 was present on the apical tip of sporozoites of all chicken *Eimeria*. Thus, mAb 8E-1, like mAb 6D-12-G10, binds an antigen conserved among the invasive stages of chicken *Eimeria*. The mAbs 5D-11 and 8D-2 which react with sporozoites antigens common to two *Eimeria* species did not recognize any merozoite epitopes whereas the mAb 8C-3 stained the merozoites very weak and inconstantly. Regarding the *E. acervulina* merozoites apical antigens we can say that our set of monoclonal antibodies (including 6D-12-G10) could not detect any antigenic differences among the merozoites from the different generations. These mAbs either recognized antigens on merozoites from all stages (6D-12-G10, 8E-1, HE-4 and 8C-3) or they did not (5D-11 and 8D-2).

The air dried merozoites stained most intensely followed by acetone and methanol fixed parasites. This showed the sensitivity of the antigens recognized by these mAbs for organic solvents, especially for methanol. This observation was previously reported. For example, Augustine *et al.* [3], also observed decreased reactivities of methanol-treated *Eimeria* sporozoites immunostained with mouse mAbs. The high sensitivity of the antigens recognized by our mAbs (except for 6D-12-G10) for organic solvents might be the reason we could not determine either their molecular weight by Western blotting or the exact location inside the sporozoites by immunoelectron microscopy [9]. The observation that the glutaraldehyde-fixed parasites did not stain with

mAbs may be explained by the mode of action of this fixative. Glutaraldehyde cross-links the antigens making intermolecular bridges that do not allow antibody access to the specimen. A similar phenomenon was observed by Augustine and Danforth [2] using mouse mAbs and *Eimeria* sporozoites. They reported that the staining patterns of air-dried and glutaraldehyde-fixed parasites were different, the latter method more likely destroy epitopes.

The results obtained using these chicken mAbs proved that there are conserved epitopes on the apical complex of the chicken *Eimeria* sporozoites and *Eimeria acervulina* merozoites from all generations. To our knowledge, this is the first report to show the presence of common antigens which are shared by *E. acervulina* merozoites from all generations. Danforth and McAndrew [10] and Lillehoj *et al.* [19], using mouse or chicken mAbs, also identified common antigens, most of them located on the apical tip of chicken *Eimeria* sporozoites and merozoites from one generation. This imply that *Eimeria* invasive stages (sporozoites and merozoites) are using common proteins for host-cell recognition and invasion. Although this study clearly demonstrates the existence of *Eimeria* shared antigens the possibilities that these common antigens be potential vaccines need to be explored.

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