ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Differential Sporulating Oocyst Count and Cross Protection Assessment of Two Immunologically Distinct Strains of *Eimeria maxima*; Guelph and M6 Strains

Saeed El-Ashram[1,2,*], Shujian Huang[1*], Ibrahim Al Nasr[3,4], Mahmoud Goda[5] and John R Barta[6]

[1]*College of life science and Engineering, Foshan university, 18 Jiangwan street, Foshan 528231, Guangdong province, China*

[2]*Faculty of Science, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt*

[3]*College of Science and Arts in Unaizah, Qassim University, Unaizah, Saudi Arabia*

[4]*College of Applied Health Sciences in Ar Rass, Qassim University, Ar Rass, Saudi Arabia*

[5]*Faculty of Science, Tanta University, Egypt*

[6]*Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada*

***Corresponding Author**: Saeed El-Ashram (saeed_elashram@yahoo.com); Shujian Huang (sjhuang.foshan@163.com).

Received: June 21, 2017; **Published:** July 25, 2017

Abstract

The members of the genus *Eimeria* infect a wide range of vertebrate hosts, such as swine, cattle, goats, sheep, rabbits, turkeys and chickens. It is now generally accepted that seven *Eimeria* species are recognised to parasitize the intestinal tract of the domestic fowl (*Gallus gallus domesticus*). Of all the *Eimeria* species that infect domestic fowl, *Eimeria maxima* is the most immunogenic species. Recent live oocyst vaccine failures to protect against infection with field isolates of *E. maxima* have demonstrated that there are immunologically distinct strains of this species. The degree of cross protection between the Guelph and M6 strains was evaluated by measuring oocyst shedding following homologous and heterologous infections. Furthermore, differential sporulating oocyst count for each strain was characterized in detail.

Oocyst outputs were determined following primary, homologous and heterologous challenge inoculations. Oocysts were not observed until the day 5-6 sample period in the primary and heterologous challenge groups. No oocysts were produced during homologous challenges with GS strains. Birds infected with oocysts of GS strains are fully protected against challenge by the same strain (GS) but not by the other strain. Six stages can be differentiated in the course of sporulation between the two strains of the same species (i.e. *E. maxima* GS and M6 strains) as follows: unsporulated or dead oocysts, less concentrated sporoblasts, more concentrated sporoblasts, pyramidal stage, four rounded-sporoblats, cigar-shaped sporoblasts. Differential sporulating oocyst count of all stages showed insignificant differences at 50 and 70 h of sporulation process. In the present study, the oocyst shedding following heterologous challenge further support the notion of the incorporation of multiple strains in the inoculum to provide protection against each individual strain of the same species. Furthermore, differential sporulating oocyst count of *E. maxima* M6 and GS were found to be virtually identical despite the protein expression profile differences of these two parasite strains.

Keywords: *Eimeria maxima Guelph and M6 strains; Domestic fowl; Differential sporulating oocyst count; cross protection.*

Introduction

Eimeria species are obligate intracellular apicomplexan protistan parasites. They are the major cause of chicken coccidiosis, a disease that leads to economic losses in livestock industries, particularly poultry, due to intensive rearing conditions. In domestic fowl (*Gallus gallus domesticus*), seven species of *Eimeria* are known to localize two predilection sites, ceca and intestine. The forms of the disease in poultry depending upon the localization can be divided into cecal coccidiosis that is caused by *E. tenella* and intestinal coccidiosis that is caused by *E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis and E. praecox. Eimeria acervulina, E. maxima, E. tenella* and *E. necatrix* are known to cause significant problems in the poultry industry due to their ubiquity, pathogenicity and immunological features. In 1929, Tyzzer was the first to describe *Eimeria maxima* and it was so named because it is the largest oocyst (30 x 20 μm2) of all avian coccidial parasites [1]. Midgut or mid-intestine coccidiosis; *Eimeria maxima* has a worldwide distribution, a sanitation related disease and most frequently diagnosed species of avian coccidiosis. On either side of Meckel's diverticulum (the remnant of the yolk sac); an important anatomical landmark locates at the junction between the jejunum and the ileum is the preferred site for *Eimeria maxima* infection. The highly resistant sporulated oocyst is the infective form of *E. maxima*, which is shed in the feces as an undifferentiated stage (i.e. unsporulated) of infected chickens. Oocysts contain four sporocysts, each with two sporozoites.

Seven morphologically distinct stages have been characterized during the sporulation of *Eimeria stiedai* [2]. For *Eimeria maxima*, six morphologically distinct stages were illustrated by Tyzzer (1929) as follows: (a) unsporulated oocyst as discharged from intestine; (b) oocyst with band stretching through sporoplasm; (c) oocyst in stage just prior to division (anlagen); (d) oocyst with four sporoblasts; (e) oocyst with immature sporocysts (sporocyst wall present but Stieda body not fully formed); and (f) fully mature oocyst with four mature sporocysts, each with a fully formed Stieda body and containing two fully formed sporozoites possessing refractile bodies. It has been demonstrated that sporulation was divided into five morphologically distinguishable stages whose abundance peaked at the following times during sporulation: unsporulated oocysts at 0 h; sporoblast anlagen at 18 h; sporoblasts without sporocyst walls at 22 h; and sporocysts without mature sporozoites at 38 h [3]. Early reports of strain variation in *E. maxima* concerned the United Kingdom Houghton and Weybridge strains. Challenge with the heterologous strain imparted only partial protection against the other [4]. The implications of strain variation in *E. maxima* to vaccination are considerable. The control of *E. maxima* through vaccination against several strains was considered, including the Houghton, Weybridge, Watton AL, Thrapston AL and 7 field isolate strains. The strains were only partially cross protective (infection with one strain only conferred partial protection to infection with the heterologous strain) meaning that incorporation of multiple strains in the inoculum was necessary to provide protection against each individual strain [5]. The aim of the present study was to assess the cross protection to infection with different strains of a single species and compare the oocyst differential count differences between the two strains of *Eimeria maxima*; M6 and GS at the sporulation level at a constant access to oxygen, temperature and humidity.

Materials and Methods

Chickens

Inbred male Shaver strain White Leghorn chickens were used (S haver Poultry Inc., Cambridge. Ontario). Animals were provided a 12 h/12 h light-dark cycle and provided feed and water *ad libitum*. All experiments were approved by the Animal Care Cornmittee, University of Guelph, and were conducted according to Canadian Council of Animal Care (CCAC) guidelines.

Eimeria maxima strains

Two strains of *Eimeria maxima*; the Guelph strain (GS), which is a single oocyst- derived. This strain was isolated from litter samples obtained from a commercial broiler house in Ontario in 1973 and has been maintained under laboratory condition from that time in the Ontario Veterinary Medicine, Guelph, Canada. The other strain is M6, which is a clonal isolate (i.e. a single sporocyst-derived strain) of the Florida strain (FL). The latter was isolated from litter samples during the period between 1994 and 1995 from a commercial broiler house in Florida, USA[6]. Both strains were maintained by cryopreservation in liquid nitrogen.

Sporulation experiment for oocyst differential count

Experimental infections were initiated in 14-day-old White Leghorn chicks. The sporulation process was commenced after scraping the unsporulated oocysts from the middle part of the small intestine and the unsporulated oocysts were isolated from the middle gut contents by simple fecal floatation technique using saturated sodium chloride. Potassium dichromate (2.5%) and perforated aluminum foil as a coverlid for Erylenmyer flask on shaker at ambient temperature were a prerequisite for sporulation process [7-9]. More than 100 microscopic fields from different prepared slides of coccidian oocysts were investigated for each time interval.

Assessment of cross-protection between GS and M6 strains

A cross protection experiment was conducted to evaluate cross protection on the basis of differences in oocyst output during primary, homologous and heterologous challenge infections with the GS and M6 strains of *E. maxima*. For the two groups (GS/GS, GS/M6), the initial infections were with 2 x 10^3 oocysts and the challenges with 5 x 10^5 oocysts. At 7 days of age the chicks were infected with 2 x 10^3 oocysts of GS strain per os using a syringe. At 21 days of age, the birds were challenged with 5 x 10^5 oocysts of each strain. Prirnary infection control group (0/GS) was only inoculated at 21 days with 5 x 10^4 oocysts of GS strains. Samples were obtained daily from day 0 post-challenge (day of challenge) through day 9 post-challenge. The birds were al1 housed in cardboard boxes prepared for fecal collection. Briefly, an approximate 6 cm slot was cut into the bottom edge of each box and the boxes were fitted with wire mesh upon which the chickens were placed. In order to collect daily fecal samples, a clean piece of aluminum foi1 was placed into the box through the slot. Feces were collected from days 4-5, 5-6, 6-7, 7-8 and 8-9 following challenge by removing and replacing foi1 pieces daily. Each time period represented a 24-hour collection period. Feces were manually removed from the foi1 pieces, mixed and weighed. Contaminants, such as food and feathers were removed from the collected fecal samples that were then placed into individual sterile beakers. The oocysts were counted using a McMaster Counting Chamber on a phase-contrast microscope (Carl Zeiss, Germany). The following equation was used to calculate the total number of oocysts in a fecal sample:

$$\text{Total oocysts} = \frac{[\text{total sample volume(ml)} \times \text{dilution factor}]}{[0.15(\text{ml})(\text{grid volume}) \times \text{no of grids counted}]}$$

Statistical analysis

Differences between the morphologic forms were determined by unpaired Student's *t*-test using Instat software (Graphpad Software, San Diego, Calif). The threshold of significance was $p < 0.05$.

Results

The mid-intestinal area on either side of the yolk sac diverticulum is the preferred site for *Eimeria maxima*. In severe infections, the lesions extended from the duodenum to the ileo-cecal junction (Figure 1). The epithelial surface showed somewhat more numerous petechiae with bloody intestinal contents (Figure 2).

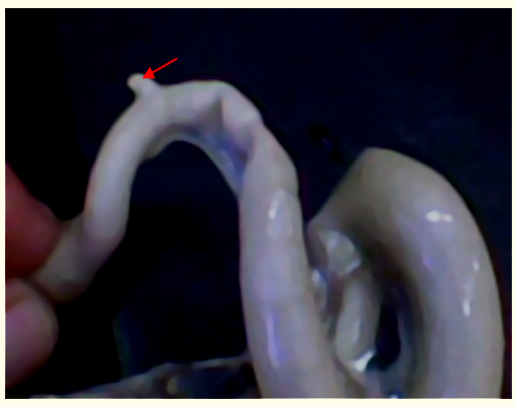


Figure 1: *Section from the formalinized chicken small intestine to illustrate the yolk sac diverticulum (normal intestine)(arrow head)*

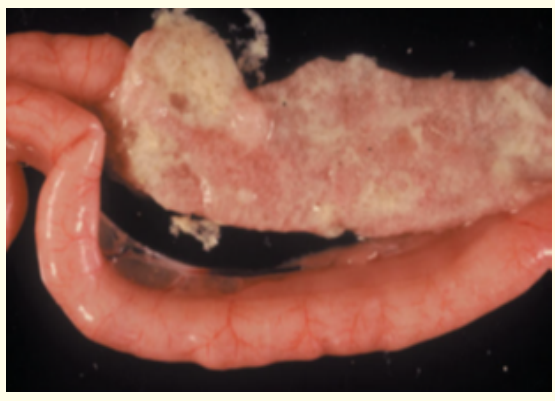


Figure 2: *Section from the chicken small intestine to show the epithelial surface with numerous petechiae along with bloody intestinal contents (infected intestine)(After Conway and McKenzie, 2007).*

Oocyst outputs were determined following primary, homologous and heterologous challenge inoculations. Oocysts were not observed until the day 5-6 sample period in the primary and heterologous challenge groups. No oocysts were produced during homologous challenges with GS. Birds infected with oocysts of GS strains were fully protected against challenge by the same strain (GS) but not by the other strain (Figure. 3).

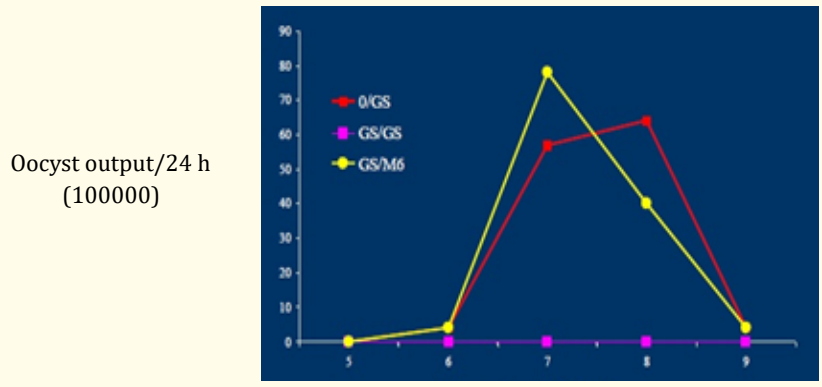


Figure 3: *Oocyst output after challenge infection with two immunologically distinct strains of Eimeria maxima; Guelph and M6 strains.*

The unsporulated coccidian oocysts of the two strains of *Eimeria maxima* M6 and GS were allowed to undergo sporulation in a conical flask that contained 2.5% (w/v) potassium dichromate ($K_2Cr_2O_7$) at 20-22˚ C on a shaker. Stages of sporulaton were checked and photographed using Nomarski interference contrast (NIC) microscopy at regular intervals in order to determine the morphological differences between the two strains of *Eimeria maxima*; M6 and GS at sporulation level. The sporulation process of *Eimeria maxima* parasite includes rounded sporont stage, pyramid-stage (pyramidal stage), four-globe stage (four-sporoblast stage), sporozoite differentiation stage and complete sporulation stage (Figures 4-8). Furthermore, in-depth differential morphological studies of the different stages of two strains of *Eimeria maxima* (strain GS and M6) revealed no siginificant differences between the two strains of *Eimeria maxima* (strain GS and M6 at 50 and 70 h of sporulation process). By 35 h after scraping and purifying the unsporulated oocysts from the middle part of the small intestine, 15.7% and 12.5% of total *Eimeria maxima* GS and M6 oocysts respectively were fully sporulated and the percentage increased to 62.9% and 60.6% of total *Eimeria maxima* GS and M6 sporulated oocysts, respectively at 50 h (Table 1).

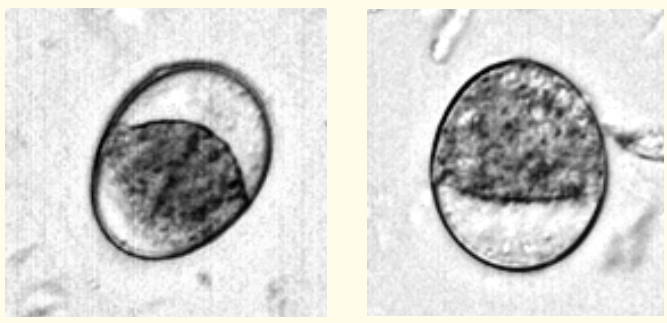


Figure 4: *Photomicrographs the dead oocysts of Eimeria (?).*

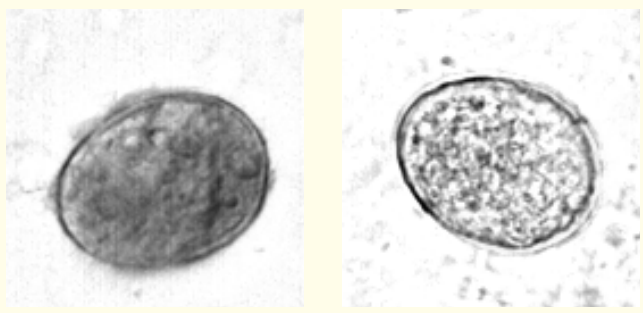


Figure 5: *Photomicrographs of the newly formed oocyst (unsporulated oocysts) of Eimeria maxima.*

Figure 6: *Photomicrographs of the oocysts with concentrated sporont of Eimeria maxima.*

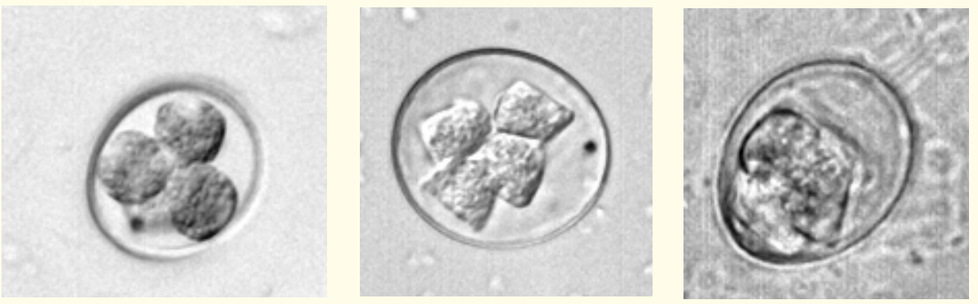


Figure 7: *Photomicrographs of the sporulating oocyst showing:*

A: Oocyst with protrusions

B: The four-celled early sporoblasts (pyramidal-shaped)

C: Spherical sporoblasts

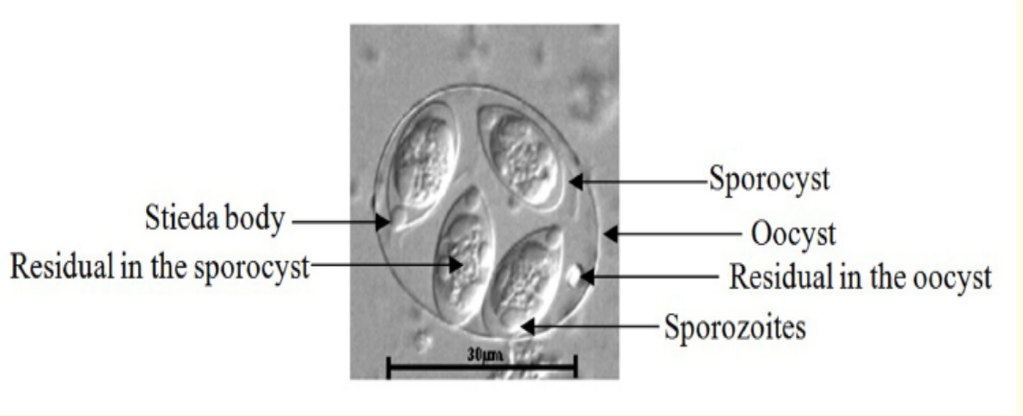


Figure 8: *Photomicrographs of the fully formed oocyst of Eimeria maxima. Scale bar 30µm.*

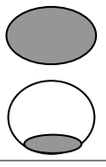
Morphologic forms						
	Unsporulated or dead oocysts (?)	Less concentrated sporoblasts	More concentrated sporoblasts	Pyramidal stage	Four rounded -sporoblats	Cigar-shaped sporoblasts
GS strains		12.8%	87.2%			
M6 strains		17.5%	82.5%			
Sporulation time	5 h					
GS strains	8.5%	2.6%	88.9%			
M6 strains	17.6%	3.2%	79.2%			
Sporulation time	24 h					
GS strains	21.6%	4.9%	25.7%	4.9%	21.6%	15.7%
M6 strains	7.1%	8.9%	48.2%	8.9%	23.2%	12.5%
Sporulation time	35 h					
GS strains	15.3%		16.7%±0.98%	0.8%	3.2%	62.9%
M6 strains	23.8%		12.3%	0.0%	3.3%	60.6%
Sporulation time	50 h					
GS strains	25.3 %		4.6 %			70.1 %
M6 strains	25.3 %		8.4 %			66.3 %
Sporulation time	72 h					

Table 1: *Morphological differences between the two strains of Eimeria maxima; Guelph and M6 strains.*

Discussion

To date, there are seven species of *Eimeria* described and named from domestic fowl (*Gallus gallus domesticus*); *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis, E. praecox*. The Guelph and Florida strains of *E. maxima* have been found previously to provide minimal immunological cross-protection against each other in chickens [6]. This is not surprising as [10] observed differences in protein expression profiles between the two aforementioned strains. Measuring oocyst shedding following homologous and heterologous infections assessed the degree of cross-protection between the Guelph and Florida strains. No oocysts were observed in the feces of birds following homologous challenge with GS strains. Thus, the initial doses administered to these birds elicited a fully protective immune response in terms of oocyst production. The absence of oocysts following homologous challenge of GS strain-inoculated chickens is not in agreement with results obtained in other studies. Oocyst shedding after homologous challenge of birds with the Florida strain has been reported by [6, 11]. Unsporulated oocysts of the two strains of *E. maxima* were collected from an area on either side of Meckel's diverticulum of the barred-chicken to eliminate the possibility of sporulated oocysts. The sporulation process of coccidian parasites can be divided into endogenous, such as *Eimeria* caepelli in fish and exogenous sporulation process, for example in the case of chicken *Eimeria*, the sex stages can be differentiated in the course of sporulation as follows: stage of rounded sporont, stage with protrusions, pyramidal stage, four-sporoblast stage, stage of sporozoite differentiation, and stage of complete sporulation. The same scenario was repoted for *Eimeria tenella* [12], *Eimeria magna* of the rabbit [13] and *Eimeria burdai* [14]. The same stages with the exception of pyramid-stage (pyramidal stage) were observed [15] for snowy owl; *Eimeria nycteae*.

Six stages can be differentiated in the course of sporulation between the two strains of the same species (i.e. *E. maxima* GS and M6 strains) has been described as follows: unsporulated or dead oocysts, less concentrated sporoblasts, more concentrated sporoblasts, pyramidal stage, four rounded-sporoblats, cigar-shaped sporoblasts. There were no differences between the two strains of the same species (i.e. *E. maxima* GS and M6 strains) at the sporulation level employing differential sporulating oocyst count at 50 and 72 h of sporulation process. In the present study, the oocyst shedding following heterologous challenge further support the idea of the incorporation of multiple strains in the inoculum to provide protection against each individual strain of the same species. Furthermore, differential sporulating oocyst count of *E. maxima* M6 and GS were found to be virtually identical despite the protein expression profile differences of these two parasite strains.

Acknowledgments

This research was supported by the Start-up Research Grant Program provided by Foshan University, Foshan city, Guangdong province for distinguished researchers.

Bibliography

1. DP Conway, *et al*. "Poultry coccidiosis: diagnostic and testing procedures" (2007).
2. U Durr., *et al*. "Sporogony of Eimeria stiedai (Protozoa, Sporozoa)". *Acta Veterinaria Academiae Scientiarum Hungaricae* 21.4 (1971): 421-432.
3. R Al-Badri and JR Barta. "The kinetics of oocyst shedding and sporulation in two immunologically distinct strains of Eimeria maxima, GS and M6". *Parasitology Research* 111.5 (2012): 1947-1952.
4. TK Jeffers. "Genetic transfer of anticoccidial drug resistance in Eimeria tenella". *The Journal of Parasitology* 60.6 (1974): 900-904.
5. PL Long and BJ Millard. "Immunological differences in Eimeria maxima: effect of a mixed immunizing inoculum on heterologous challenge". *Parasitology* 79.3 (1979): 451-457.
6. AG Martin., *et al*. "Analysis of immunological cross-protection and sensitivities to anticoccidial drugs among five geographical and temporal strains of Eimeria maxima". *International Journal for Parasitology* 27.5 (1997): 527-533.
7. MH Kogut and PL Long. "Extraintestinal sporozoites of chicken Eimeria in chickens and turkeys". *Zeitschrift fur Parasitenkunde* (Berlin, Germany) 70.3 (1984): 287-295.
8. S El-Ashram and X Suo. "Corrigendum: Electrical cream separator coupled with vacuum filtration for the purification of eimerian oocysts and trichostrongylid eggs". *Scientific Reports* 7 (2017): 45799.
9. S El-Ashram., *et al*. "Electrical cream separator coupled with vacuum filtration for the purification of eimerian oocysts and trichostrongylid eggs". *Protocol Exchange* (2017).
10. S El-Ashram., *et al*. "From the Macro to the Micro: Gel Mapping to Differentiate between Sporozoites of Two Immunologically Distinct Strains of Eimeria maxima (Strains M6 and Guelph)". *PloS One* 10.12 (2015): e0143232.
11. SE Beattie., *et al*. "A comparison of sporozoite transport after homologous and heterologous challenge in chickens immunized with the Guelph strain or the Florida strain of Eimeria maxima". *Parasitology Research* 87.2 (2001): 116-121.
12. D Mielke and G Alabdul Rahman. "The sporogony of Eimeria tenella". *Angewandte Parasitologie* 32.1 (1991): 39-41.
13. EM Kheisin., *et al*. "Life cycles of Coccidia of domestic animals". Heinemann Medical, London (1972).
14. B Koudela., *et al*. "Eimeria burdai sp. n. (Apicomplexa: Eimeriidae), a new parasite species from subterranean African silvery mole-rat, Heliophobius argenteocinereus". *Folia Parasitologica* 47.4 (2000): 97-99.
15. J Volf., *et al*. "Eimeria nycteae sp n (Apicomplexa: Eimeriidae), a new parasite species from the snowy owl, Nyctea scandiaca". *Folia Parasitologica* 46.4 (1999): 168-170.

Volume 10 Issue 1 July 2017