Global Veterinaria 3 (6): 489-494, 2009
ISSN 1992-6197

A Preliminary *In Vitro* Trial on the Efficacy of Products of *Xenorhabdus* and *Photorhabdus* Spp. on *Eimeria* Oocyst

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Abstract: The increased resistance of avian coccidia to anticoccidial drugs currently used by in poultry industry has stimulated the search for new methods of its control. In the last decades plant extracts were widely used for the controlling of avian coccidiosis and improving poultry performance worldwide. Anticoccidial efficacy of symbiotic bacterial proteins of *Xenorhabdus* and *Photorhabdus spp.* has been investigated against sporulated oocysts of mixed *Eimeria* species under laboratory conditions. *Xenorhabdus spp*. revealed higher effecacy than *photorhabdus sp*. on *Eimeria* oocysts as monitored by mortality percentage. In the treated group, most oocysts exhibited some deformities such as shrinking and fracture of oocystic cell wall as well as disappear of the embryo if compared with non treated group. It could be concluded that both *Xenprhabdus* and *Photorhabdus* bacteria produced protein toxin or Lecithinases secretion from phase I of *Xenorhabdus spp*. may have an important role in the induced of pathogenicity of coccedial oocyst, however further investigations are still recommended.

Key words: Entomopathogenic bacteria · *Xenorhabdus* · *Photorhabdus* · *Eimeria* · Control

INTRODUCTION

Avian coccidiosis is an intestinal disease which is a common health problem in poultry production in deep litter system around the world. It is considered to be one of the most economically devastating parasitic diseases that currently plague the industry as it is responsible for high mortality and morbidity rates as well as poor feed conversion in infected birds [1]. Poultry industry all over the world is under threat of the disease and its prevalence results in the loss of millions of birds, accounting for more than 800 million US dollars in lost revenue annually all over the World [2]. A central feature of avian coccidiosisis that it is caused by protozoan parasites, most notably *Eimeria* species. Chemoprophylactic use of anticoccidial such as Amprolium® or chemotherapy has been the primary means of controlling the disease in most poultry farms all over the world. Predictably though expensive and cumbersome, drug regimens have played a significant role in the growth of the poultry industry [1].

Unfortunately, the emergence of drug-resistant strains of coccidia has made the currently available anticoccidials less effective and this has threatened the economic stability of the industry, especially in developing countries where the problem has become a major concern to resource-poor farmers. Consequently, many different types of substances have been investigated in the search as alternative methods for controlling coccidiosis.

A number of natural products have been tested as anticoccidal dietary additives [3]. Sources of fats continuing of n-3 fatty acids (n-3 FA) such as fish oils, flaxseed oil and whole flaxseed, when added to starter rations from one day of age, effectively reduced lesions resulting from challenge with *E. tenella* but not *E. maxima*. Curcumin (0.05%), appear to be effective in reducing upper-and mid small intestinal infections caused by *E. acervulvna* and *E. maxima* [3], but not beneficial for *E. tenella* infections. Other traditional medical plants were applied by Allen *et al*.[4,5], Massoud *et al*. [6] and Abu El-Ezz, [7] for controlling Coceidiosis in chickens.

Xenorhabdus and *Photorhabdus* are motile Gram-negative bacteria that are highly pathogenic to insects [8]. The bacteria live in symbiosis with rhabditoid nematodes belonging to the genera *Steinernema* and

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Heterorhabditis.[9,10]. *Xenorhabdus* occurs naturally in a special intestinal vesicle of *Steinernama* infective juveniles [11]. while *Photorhabdus* are mainly located in the anterior part of *Heterorhabditis* infective juveniles [12]. Upon invasion of the insect, the IJ enters the hemocoel and releases *Xenorhabdus* or *Photorhabdus* into the hemolymph. Together the bacteria and nematode contribute to the pathogenic process involved in killing the insect host. *Xenorhabdus* spp. secrete proteinaceous toxin complexes that are similar to the insecticidal complexes produced by the sister taxa, *Photorhabdus* spp. [13]. In the insect cadaver, *Xenorhabdus* spp. grow to high cell density and secrete a diverse array of antimicrobial and nematicidal products which are believed to protect the insect cadaver from invasion by other soil organisms. Also *Xenorhabdus* spp. produce exoenzymes that degrade insect tissues and macromolecules which contribute to the nutrient base that supports bacterial and nematode reproduction. After several rounds of nematode reproduction, the dauer juvenile form of the nematode develops which possesses the gut vesicle that is colonized by the bacterium [14]. The dauer stage enters the soil environment as the infective juvenile which initiates a new life cycle upon invasion of an insect host. These nematode-bacteria symbiotic pairs have been used as biological control agents several agricultural pests [15]. In this study *Xenorhabdus* and *Photorhabdus* proteins were used for controlling *Eimeria* ooceyst as an in-vitro application under laboratory conditions.

MATERIAL AND METHODS

Source of *Eimeria spp.*: Mixed species of *Eimeria*" *E.necatrix; E.maxima; E.praecox* and *E.acervulina; E.mitis and E.tennela* were collected form chicken flocks suffered from coccidioses.These species were previously subjected to molecular characterization by Kutkat *et al.* [16].

Bacterial Isolation: About 20 infective juveniles (IJs) of three species of *Steinernema* (*Steinernema carpocapsae* DD136, *S.carpocapsae* all strain and *S.riobrave*) and one species of *Heterorhabditis* (*Heterorhabdetis bacteriophora* HP88) were used for infection of a greater wax moth larvae (*Galleria mellonella*). After 24 h, a leg of *G.mellonella* larvae was removed and the released haemolymph was streaked on Nutrient-Agar (NBTA) plates containing 8% nutrient broth (LAB) and 12% agar. Plates were incubated at 25°C. After 3days characteristic of bacterial colonies developed these were repeatedly subculture until a pure culture was obtained. Pure cultures were transferred to nutrient broth slants. Incubated at 25°C for 3days stored at 6°C and subculture at least once a month to maintain viability. Bromothymol Blue (BTB) 0.025g and 0.04g Triphenyltetrazolium Chloride (TTC) were added as an indicator where the growing bacteria will deny the stain giving dark blue-green colonies [17].

Bacterial Colonies: *Xenorhabdus nematophila* (DD), *Xenoprhabdus sp* (all), *xenorhabdus sp*.(Sr) and *Photorhabdus luminescens laumondii* (HP88) are derived from *Steinernema carpocapsae* DD136, *S.carpocapsae* all strain, *S.riobrave* and *Heterorhabdetis bacteriophora* HP88 nematodes respectively. Nematodes are propagated on 6th instar larvae on *Galleria mellonella* according to Dutky *et al*. [18].

Bacterial Protein Preparation: Purified colonies were harvested and inoculated in 250ml conical flasks containing 50ml includes Lb broth medium and incubated in a shaking incubator at 180 rpm at 25°C for 72h. or till the pH of the culture reaches 8.5. The colonies free supernatant (CFS) was collected by centrifugation at 13000 rpm for 30min at 4°C. The proteins in the CFS were precipitated by ammonium sulfate 85% saturated solution. The precipitated proteins were dialyzed over night at 4°C against distilled water. The dissolved CFS proteins were concentrated 10X by sucrose using dialysis bag Whattman cut off 12-14 kDa. [19]. The protein concentration was monitored during the process of purification according to Bradford [20].

Anticoccidial Agent Bioassay: A Serial two fold dilutions of symbiotic bacterial products started by 4.864 to-0.038µg/ml of four symbiotic bacterial spp., *Xenorhabdus nematophila* (DD) *Xenorhabdus sp*. (all), *Xenorhabdus sp*. (Sr) and *Photorhabdus luminescens laumondii* (HP88) with equal volume of 7.2pH saline buffer were occurred in micro titer plates. Five hundred of purified and molecular characterized local field Sporulated oocysts of mixed *Eimeria* species were added per well. The treatment were replicated three times in every bacterial product. Control oocyest treated by 7.2pH saline buffer. Micro titer plates were covered with parafilm to prevent the evaporation and incubated at 25C°. The experiment was examined daily for the oocysts activity. Results obtained after 3 days by using inverted microscope for accounting the deed/or deformities in oocysts of *Eimeria* in compare with the control.

Statistical Analysis: The data were subjected to statistical analysis using F test (one way classification least significant differences "L.S.D.") according to Snedecor and Cochron [21]. The significance of the main effects was determined by analysis of variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range test ($P<0.05$, 0.01). All analysis was made using a software package "Costat", a product of Cohort Software Inc. Berkeley, California.

RESULTS

The efficiency of *Xenorhabdus* and *Photorhabdus* protein complex on *Eimeria* oocyst had been detected as comparing with control. Deformities in embryo were observed, as well as shrinking and fracture in cell wall of most oocysts. Some *Eimeria* oocysts were empty from embryo and some of them were disappeared completely from the examined field (Fig. 1). Mortality percentages of *Eimeria* oocysts affected by symbiotic bacterial proteins of *Xenorhabdus spp* and *Photorhabdus sp*, were significantly varied (Table 1). The LC_{50} indecated that *Xenorhabdus sp.* (all) protein was the most effective one whereas it revealed 0.008µg/ml with F value 146.15. It was followed by *X.nematophila* (DD) with LC_{50} 0.166µg/ml and *Xenorhabdus sp*. (Sr) with LC_{50} 1.704µg/ml *P. luminescens laumondii* (HP88) protein was the lowest effect one which gave LC_{50} 3.692µg/ml with F value 10.040 if compared with control.

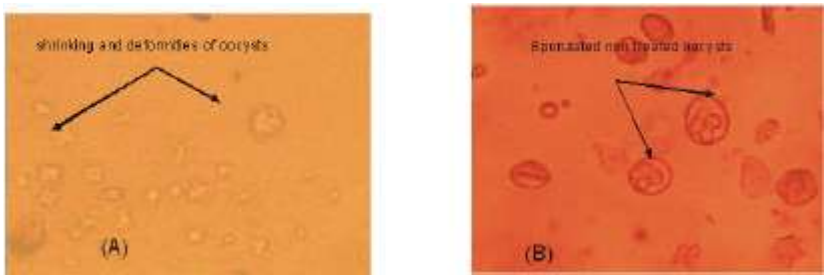

Fig. 1: Effect of symbiotic bacterial proteins of both *Xenorhabdus* and *photorhabdus spp.*on coccedial oocyst. (A) Most oocyst exhibited some deformities such as fracture, shrinking of oocystic cell wall and disappear of the embryo (X40). (B) sporulated non treated oocysts of Eimeria(X20).

Table 1: Efficiency of Symbiotic bacteria proteins on Mortality percentage of *Eimeria* oocysts

Conc.	DD *X.nematophila*	all *Xenorhabdus sp*	Sr *Xenorhabdus sp*	HP88 *P.l.laumondii*
Control	2.78±0.56^{a}	2.78±0.56^{a}	2.78±0.56^{a}	2.78±0.56^{a}
1- 0.038	12.22±2.00^{b}	71.67±2.55^{b}	8.33±2.89^{a}	4.44±0.56^{a}
2- 0.076	40.56±1.47^{c}	73.89±2.42^{b}	12.78±7.09^{a}	10.56±3.64^{a}
3- 0.152	52.22±4.94^{d}	76.67±2.89^{b}	29.44±2.00^{b}	11.67±3.33^{a}
4- 0.304	58.89±3.64^{d}	77.78±2.42^{b}	37.78±2.42^{bc}	14.44±0.56^{a}
5- 0.608	75.56±1.47^{e}	87.22±4.44^{c}	42.78±0.56^{cd}	32.78±4.94^{b}
6- 1.216	100±0.00^{f}	100±0.00^{d}	47.78±1.47^{cde}	37.78±7.72^{b}
7- 2.432	----------	----------	52.78±4.55^{de}	39.44±8.41^{b}
8- 4.864	----------	----------	60.00±7.26^{e}	55.56±8.73^{c}
F	175.752	146.15	31.837	10.040
P	0.000	0.000	0.000	0.000
LC50	0.166	0.008	1.704	3.692

a, b, c, …. indicates different concentrations. Means with different letters within the same column are significantly different according to Duncan test $P <$ 0.01 .

Steinernema carpocapsae DD136 (DD). Xenorhabdus (X).

S.carpocapsae all strain (all). S.riobrave (Sr).

Heterorhabditis bacteriophora HP88 (HP88). Photorhabdus (P).

The results were adjusted according to About formula [22]).

DISCUSSION

High resistance of avian coccidioses to the classical anticoccidial drugs currently used by the poultry industry has stimulated the search for an alternative solution for controlling these disease. In the last decades plant extracts were widely used for the controlling of avian coccidiosis and improving poultry performance worldwide [6,7,23]. *Xenorhabdus* and *Photorhabdus* bacteria live in symbiosis with rhabditoid nematodes belonging to the genera *Steinernema* and *Heterorhabditidis*. In particular, both the bacteria and the nematodes produce a range of toxins that are responsible for killing the insect host [24]. The efficacy of *Xenorhabdus* and *Photorhabdus* Protein complex was tested against *Eimeria* oocyst under laboratory conditions. No literature could be available on the mode of action of symbiotic bacterial protein and *Eimeria* oocyst. So, these experiments were applied for the first time as a preliminary studies for field application to control coccediosis in chicken deep litter system house. Mortality percentages of *Eimeria* oocysts was found to be affected by symbiotic bacterial protein complex of *Xenorhabdus* and *Photorhabdus* and were significantly varied. Moreover, embryo deformities, as well as shrinking and fracture in cell wall of most oocysts were clearly observed in comparing with control. Some oocysts exhibit empty from the embryo and some of them disappeared completely from the examined field in high concentrations. Analysis of the genome of *Photorhabdus luminescens* resulted in identification of more putative toxin genes than have been found in any other bacterium sequenced to date [25]. The only toxins that have been studied in detail from *Xenorhabdus* and *Photorhabdus* bacteria are the Tc toxins from *P.luminescens* Strain W14 [13, 26, 27]. The Tc toxins form a large protein complex consisting of about 10 polypeptides ranging in size from 30 to 200 KDa that is toxic to insects after either ingestion or injection [13, 26]. In this study *Xenorhabdus spp.* in general, showed significant higher toxicity than *Photorhabdus sp*. The details of mode of action of the various toxins of *Xenorhabdus* and *Photorhabdus* are still not known. Injection of the Tca complex of *P.luminescens* strain W14 or ingestion by *Manduca sexta* larvae damaged the midgut cells, resulting in shedding of the midgut epithelium into the gut lumen, followed by lysis of epithelium [28]. Production of phosphatidylcholine-hydrolyzing phospholipases (or lecithinase) is detected on soild media as opalescent zoon surrounding colonies grown on agar supplemented with egg yolk (the egg yolk test) [29]. Many of these lecithinases have been purified and charachterized as single secreted-polypeptide proteins. Lecithinases are toxic determinants, as well as a means of securing bacterial supplies of phosphates [29] Oocyst is the most resistant stage of the *Eimeria* parasites life cycle [30]. The wall of oocysts provides an effective protective barrier against the extremes of pH, the action of the detergents an enzymes (proteolytic, glycolytic and lipolytic), mechanical disruption and reagents such as sodium hypochlorite and dichromate [31]. Our results revealed that *Xenorhabdus spp*. has harmful affect on oocysts than *photorhabdus spp*. It may be due to exoenzymes which are elaborated by many strains of Xenorhabdus and Photorhabdus although their production can be variable in individual strains [32].

It was concluded that *Xenorhabdus* and *Photorhabdus spp.* proteins gave promising results for controlling *Eimeria* oocysts in a deep-litter system, but, further investigations are needed to purified and characterize the entomopathogenic bacterial protein toxins.

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