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Parasporal crystal proteins of *Bacillus thuringiensis* strain BT6, originating from the faeces of Sika Deer, demonstrate anticoccidial activity against *Eimeria tenella* in chickens

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The parasporal crystal proteins (Cry) of *Bacillus thuringiensis* (Bt) strain BT6, which was isolated from Sika Deer faeces, exhibited a similar effect with diclazuril for controlling the infection with *Eimeria tenella* in chickens. A far more considerable body weight gain was found in the chickens treated with BT6 Cry proteins, as compared to chemical drug-treated groups, infected-untreated groups and even the uninfected group. This work is a preliminary contribution aiming to develop Bt Cry proteins for fighting coccidiosis in chickens.

Key words: *Bacillus thuringiensis*, faeces, parasporal crystal proteins, coccidiostatic, *Eimeria tenella*, chicken.

INTRODUCTION

Coccidiosis, an ubiquitous avian disease that is recognized as a serious challenge for the poultry industry, is caused by protozoan parasites of the genus *Eimeria*, which is responsible for the destruction of the enteric epithelium, resulting in malabsorption, reduction of feed conversion, impaired growth rate and body weight gain in broilers, and egg production in layers as well as increased morbidity and mortality (Dalloul and Lillehoj 2006; Lillehoj et al., 2004, 2007). Since chickens are usually reared in direct contact with their faeces, the resilience of oocysts ensures the continued presence of coccidia wherever chickens are raised. The Achilles' heel of chemotherapy has always been the acquisition of drug resistance by parasites (Chapman et al., 2010). Moreover, prophylactic drug usage creates deep anxiety over concerns with chemical residues in food products (Min et al., 2004; Zhao et al., 2011).

The first 100 years of *Bacillus thuringiensis* (Bt) topical sprays and transgenic crops have been extraordinarily successful and advantageous, with a strong record in terms of safety, efficacy and environmental beneficence (Sanahuja et al., 2011). The parasporal crystal proteins (Cry proteins) produced in *B. thuringiensis* have high specificities against insects that attack crops and trees. Cry proteins are also able to kill nematodes, bacteria and human cancer cells (Yudina et al., 1997; Mizuki et al., 1999; Cappello et al., 2006). The identification of *B. thuringiensis* with novel or improved activities continues to be a focus of attention. Herein, we present the first report of *B. thuringiensis* Cry proteins demonstrating

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Table 1. Inhibition of oocyst development in chicken embryo by BT6 Cry proteins.

	0ocyst number/ embryo (1×10^4)	Inhibition rate (%)
150 µg BT6 Cry per embryo	$8.3+15.48^c$	87.9
300 µg BT6 Cry per embryo	$0.0+0.15^d$	100
150 µg BT1 Cry per embryo	$67.8+29.83^b$	1.4
300 µg BT1 Cry per embryo	$67.7+37.23^b$	1.6
150 µg BT14 Cry per embryo	$68.0+21.85^a$	1.2
300 µg BT14 Cry per embryo	$67.8+30.26^b$	1.5
0.1 mL 0.01 mol/L PBS (pH7.4) per embryo	$68.2+12.91^a$	0.8
Infected-untreated	$68.8+18.36^a$	0

Column data marked with same superscripts mean no significant difference (P>0.05), with different superscripts mean significant difference (P<0.05). Ten days old chicken embryos, 20 embryos in each treatment, were injected with BT6 Cry proteins or 0.1 mL PBS. The sporulated oocysts (1×10^4) of *E. tenella* PT0705 were injected 24 h later. Infected-untreated was used as control.

activity against coccidiosis in chickens.

MATERIALS AND METHODS

Isolation of *B. thuringiensis* from faecal samples

The center region of fresh faeces from herbivorous animals including sika deer, Francois's Leaf Monkey and Red-crowned Crane were collected from Fuzhou Zoo, Fujian Province, China before visitors came in. Three grams of faecal material were suspended in 10 ml of LB broth supplemented with 325 µl of 2 mol/L NaOAc and 40 µl of 100 mg/ml penicillin, and then incubated at 30°C and 250 rpm for 4 h, after which 200 µl suspension were thermally shocked at 80°C for 3 min, and immediately cooled down, then centrifuged at 5 000 rpm for 15 min. The pellets were washed with sterile water once and resuspended in 200 µl of sterile water, after which 200 µl aliquots were plated on nutrient agar plates and incubated for 72 h at 30°C. The colonies exhibiting protein crystals considered to be *B. thuringiensis* were identified using Phase Contrast Microscopy, which were then stored in the Bt culture collection of the Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, Fujian Agriculture and Forestry University, China.

Preparation of parasporal crystal proteins

The parasporal crystal proteins were prepared according to the method of Zhou et al. (2005). Protein concentration was measured with bovine serum albumin as the standard by the method of Bradford (1976).

Preliminary test for anticoccidial activity based on chicken embryo

Eimeria tenella isolate PT0705, which was isolated from local chickens, exhibited the highest pathogenicity when compared with other isolates (Wu et al., 2010). A rapid test aiming to determine whether BT6 Cry has anticoccidial activity was conducted according to the method of Zhang et al. (1997).

Evaluation of the anticoccidial efficacy of preventive treatment with BT6 Cry in comparison with two chemicals

The evaluation of the anticoccidial efficacy of preventive treatment with BT6 Cry in comparison with two chemicals was performed according to the method described by Chapman (1998), Li et al. (2005) and De Pablos et al. (2010).

RESULTS

The development of oocyst in chicken embryo was strongly inhibited by BT6 Cry protein

Three *B. thuringiensis* strains, BT1, BT6 and BT14, were isolated from the faecal samples of Francois's Leaf Monkey, sika deer and Red-crowned Crane, respectively. Results of a preliminary test (Table 1) showed that the development of oocysts in chicken embryo was strongly inhibited (87.9 %) when BT6 Cry reached 150 µg per embryo, and was completely inhibited at 300 µg. Cry proteins prepared from the other two *B. thuringiensis* strains, BT1 and BT14, however, hardly inhibited the development of oocysts, similarly to 0.01 mol/L PBS (pH7.4) and infected-untreated. The test indicated that BT6 Cry had potential for controlling the infection with *E. tenella* in chickens.

Efficacy of preventive treatment with BT6 Cry in comparison with two chemicals against the infection with *E. tenella* in chickens

The efficacy of preventive treatment with BT6 Cry against the infection with *E. tenella* in chickens was investigated in comparison with two chemical drugs and control (Table 2). The anticoccidial index (ACI) of groups III, IV and V are 202.3, 184.1 and 128.2, respectively, which indicated that BT6 Cry exhibited a significantly higher anticoccidial efficacy in comparison with chemical drugs diclazuril and amprolium.

Oocyst excretion is the most sensitive parameter for evaluating coccidial infection. The goal of a treatment is thus the suppression of oocyst excretion as much as possible (Mundt et al., 2007). The mean values of oocyst

Table 2. Efficacy of preventive treatment with BT6 Cry in comparison with two chemicals against the infection with *E. tenella* in chickens.

	Group I	Group II	Group III	Group IV	Group V
%RGW	100	37.5	112.3	95.1	51.2
%S	100	70	100	100	100
IL	0	26	5	6	13
OI	0	/	5	5	10
ACI	/	/	202.3	184.1	128.2

150 two week old apparently healthy chickens were divided into 5 groups, 30 chickens each. Each of these groups was subdivided into three cages of ten individuals. Group I chickens were left as the uninfected-untreated (control). Chickens from group II (infected-untreated) were inoculated with 1×10^4 sporulated oocysts of *E. tenella* PT0705. Chickens from groups III, IV and V were supplied with drinking water containing 2.5 g/L BT6 Cry proteins, 0.5 g/L diclazuril and 0.5 g/L amprolium, respectively, and 6 h later they were infected. For the lesion scores, chickens were sacrificed 10 days postinfection. For counting of the number of oocyst excretion, the faeces of each group were collected from 5^{th} to 10^{th} day and the mean values of oocyst count per gram faecal dropping (OPG) was calculated. Body weight (BW) was determined at the beginning and termination of the observation period, and weight gain was calculated. Feed consumption was recorded every day. Feed conversion (feed: gain ratio) was calculated as the ratio between feed intake and BWG. Treatment effectiveness was determined according to Chapman (1998), who describes histopathological observations and the different indices as oocyst index (OI), body weight gain (BWG), relative weight gain (RWG), lesion scores (LS), and anticoccidial index (ACI). To analyze the effectiveness of the treatment at the end of the experiment, we calculated the anticoccidial index using the formula: (ACI) = (%S + %RGW)-(IL + OI), where ACI is the anticoccidial index, %S the percentage of survival, %RGW the percentage of relative weight gain, IL the lesion index and OI the oocyst index (De Pablos et al., 2010). We considered a product to lack anticoccidian activity when values were lower than 120, low effective at values of 120 to 160, and moderate effective at values of 160 to 180, high effective at values higher than 180 (De Pablos et al., 2010).

Table 3. Oocyst-excretion values per gram of faeces of different groups corresponding to days 5 to 10 post-infection.

	Days 5 (1×10^4)	Days 6 (1×10^4)	Days 7 (1×10^4)	Days 8 (1×10^4)	Days 9 (1×10^4)	Days 10 (1×10^4)
Group I	0	0.00^c	0.00^d	0.00^d	0.00^d	0.00^d
Group II	0	$10.65+18.56^a$	$28.15+23.15^a$	$16.33+20.18^a$	$9.26+28.72^a$	$5.66+31.93^a$
Group III	0	0.00^c	$2.89+15.39^c$	$1.98+14.68^c$	$0.88+16.52^c$	$0.68+12.31^c$
Group IV	0	0.00^c	$2.58+18.24^c$	$1.08+15.53^c$	$0.69+36.38^c$	$0.48+21.96^c$
Group V	0	$0.65+12.36^b$	$11.68+13.21^b$	$6.63+19.31^b$	$4.35+38.75^b$	$2.38+25.53^b$

Column data marked with same superscripts mean no significant difference (P>0.05), with different superscripts mean significant difference (P<0.05). For counting of the number of oocyst excretion, the faeces of each group were collected from 5^{th} to 10^{th} day and the mean values of oocyst count per gram faecal dropping (OPG) was calculated.

count per gram faecal dropping (OPG) were reduced significantly in BT6 Cry-treated group (0.68) and two chemical-treated groups (0.48 and 2.38, respectively) when compared with infected-untreated group (5.66) (Table 3). It was demonstrated clearly that treatment with BT6 Cry was almost as effective in controlling oocyst excretion as treatment with diclazuril (0.68 against 0.48) (Table 3).

There were distinct differences between the groups in terms of body weight development (Table 4). The group receiving BT6 Cry exhibited a significantly higher weight gain (458.9 g) in comparison with two drug-treated groups and infected-untreated group (388.5, 209.1 and 153.2 g, respectively). It was very surprising that BT6 Cry-treated group had a higher weight gain than uninfected group (458.9 against 408.5 g). The feed conversion ratio reached 49.9% in BT6 Cry-treated group against 44.3, 25.4, 19.2 and 45.5 % in diclazuril-treated group, amprolium-treated group, infected-untreated group and uninfected group, respectively (Table 4). It would seem reasonable to suppose that the weight gain was due to BT6 Cry that exhibited activities against both *E. tenella* PT0705 and some other intestine parasites infection in chickens.

DISCUSSION

The present results showed that the parasporal crystal proteins of *B. thuringiensis* BT6 may aid in combating infection by some unknown means. Generally, Cry proteins are solubilized under alkaline pH in the insect midgut and activated by endogenous gut proteases, then interact with specific receptors present in the epithelial cells of the insect midgut, causing pore formation and consequent osmotic cell lysis (Bravo et al 2007). The prepared solubilized Cry proteins were toxic to larvae, adult, and ova of *Ascaris suum in vitro*, and they were effective for pig to fight against Ascaridasis after orally

Table 4. Comparison of body weight gain and feed conversion ratio among different groups 10 days postinfection.

	Group I	Group II	Group III	Group IV	Group V
Initial bogy weight (g)	106.1+21.36[a]	107.5+23.45[a]	106.8+34.18[a]	107.2+22.68[a]	106.8+15.47[a]
Body weight gain (g)	408.5+39.42[a]	153.2+31.59[d]	458.9+27.98[a]	388.5+33.85[b]	209.1+32.42[c]
Food consumption (g)	898.7+31.77[b]	796.9+41.32[d]	918.8+37.48[a]	876.6+40.18[b]	823.3+37.35[c]
Feed conversion ratio (%)	45.5	19.2	49.9	44.3	25.4

Data in row marked with same superscripts mean no significant difference (P>0.05), with different superscripts mean significant difference (P<0.05). Weight was determined at the beginning and termination of the observation period, and weight gain was calculated. Feed consumption was recorded every day. Feed conversion (feed: gain ratio) was calculated as the ratio between feed intake and body weight gain.

administered (Deng et al., 2004). After drinked, the prepared solubilized BT6 Cry proteins entered the gut of chickens, however, did Cry proteins enter the gut of *E. tenella* and then activated? Or did they directly act against the larvae, adult and oocyst of *E. tenella in vitro*? Further studies should be done to uncover the mode of action.

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