Int.J.Curr.Microbiol.App.Sci (2018) *7*(8): 4364-4372

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 08 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.708.458

A Preliminary *in vitro* Study to Evaluate Poly-3-Hydroxy Butyrate as an Anticoccidial Agent against Oocysts of *E. tenella*

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Keywords

Coccidiosis, Sporulation inhibition, TEM and SEM analysis, *E. tenella* oocyst, PHB

Article Info

Accepted:
26 July 2018
Available Online:
10 August 2018

A B S T R A C T

The present study was conducted to evaluate the effect of Poly-3-Hydroxy butyrate against the sporulation of oocyst of *E. tenella*. In this study four different doses of PHB which was extracted from the *Bacillus subtilis* culture, 10, 20, 50and 100mg were used with 2.5% potassium dichromate as positive control and distilled water as negative control. The experiment was carried out in 24 well plates. The sporulation efficiency was evaluated by counting the sporulated and unsporulated oocysts using haemocytometer and percentage efficiency of sporulation inhibition was calculated. Data were analysed using chi square test. The PHB at 100mg concentration showed significant effect on sporulation inhibition when compared to other doses of PHB and positive control.

Introduction

Coccidiosis is the major parasitic disease which causes serious threat to the poultry industry. It is caused by the Apicomplexan protozoa called as *Eimeria* which consists of many species which affects the poultry either individually or in combination. Severe outbreaks resulted in tremendous economic loss due to increased morbidity and mortality. The genus Eimeria most commonly affects the intestinal tract, thereby affects the intestinal epithelium which inturn leads to reduced feed efficiency and body weight gain (Min *et al.*, 2004; Dalloul and Lillehoj, 2005). The most common species of *Eimeria* which affects poultry industry was *E. tenella, E. acervulina* and *E. maxima*. Coccidiosis is mainly caused by the ingestion of sporulated oocysts which will be able to survive in the environment for several months. This disease is mainly controlled by the use of some chemotherapeutic agents and some anticoccidial chemicals. Due to extensive and

indiscriminate use of anticoccidial drugs in poultry industry leads to the development of drug resistance against all the drugs. Despite the global acceptance and success of these drugs in controlling avian coccidiosis, the poultry industry is under constant pressure to reduce the dependence on anticoccidial drugs (Williams, 1999). These problem become public health concern about the presence of drug residues in poultry products which made the industry to look for the alternatives. Although various natural products were used for controlling coccidiosis, still none of the studies were repeated and not led to large scale applications of any of these compounds in practice. Inspite of all these drawbacks, associated with control strategies still there is need for alternatives. Poly-3-Hydroxy Butyrate which is a biopolymer produced by various groups of bacteria, a neutraceutical compound which was used as a feed additive as well as it suppress or inhibit the pathogenic bacteria in GI (Gastro intestinal) tract such as *E.coli, Vibrio, Salmonella* and it also has antimicrobial activity (Singh and Parmar, 2011). The attractive features such as Biocompatibility, biodegradability and non toxicity which renewed the interest of using this as an alternative source. Hence in the present study PHB (Poly-3-hydroxy butyrate) was evaluated for its effect against *E. tenella* oocyst in terms of sporulation inhibition and cell wall integrity (Fig. 1).

Materials and Method

Collection of faecal sample for recovery of oocyst

Fresh faecal droppings were collected from Translational Research Platform in Veterinary Biologicals animal shed, Madhavaram Milk Colony, Chennai-51. About 50-100g of fresh faecal sample was collected from the *E. tenella* oocyst challenged birds.

Processing of faecal sample

The faecal sample of about 25-30 grams were weighed and mixed with 75-100ml of distilled water. The suspension was mixed thoroughly and stained using double layer of nylon sieve with pore size approximately 1mm and filtrate was transferred into 50ml Falcon tubes (Tarson, India) and centrifuged at 3000 rpm for 10mins. The supernatant was discarded after centrifugation and saturated salt solution was added to the pellet and left for few minutes for the oocyst to reach the top and 5ml of the supernatant was taken in a new 50ml tube and 30ml of distilled water was added and centrifuged at 3000 rpm for 10min. The above step was repeated 3-5 times to remove all the salt solution. Final pellet was mixed with water and the oocyst count were eneumerated using McMaster counting chamber (Long *et al.,* 1986).

Extraction of PHB from *Bacillus spp*

Preparation of NDMM medium

For the production of PHB by *Bacillus spp* NDMM medium was used. The NDMM Medium was prepared by using the following constituents such as Dextrose (5g), Sodium chloride (0.05g), Magnesium sulphate (0.05g), Potassium dihydrogen phosphate (0.125g), Peptone (1.25g), Yeast extract (1.25g) and distilled water (500ml) (Panigrahi and Badveli, 2013).

The medium was prepared for 2500ml and was autoclaved at 121ºC for 20 mins at 15lbs pressure to avoid contamination. After autoclaving, *Bacillus spp* culture was added into the medium at the rate of 25ml per 500ml of medium.

Large scale production of PHB from *Bacillus spp* was carried out using Bioreactor.

Extraction of PHB from NDMM medium

PHB was extracted using the dispersion method of sodium hypochlorite and chloroform (Singh and Parmar, 2011) with minor modifications.

In vitro anticoccidial activity of PHB against oocyst of *E. tenella*

The oocyst of *E. tenella* collected from fresh faecal sample was used for *in vitro* study. *In vitro* evaluation was performed as per the protocol described by (Mikail *et al.*, 2016). *In vitro* evaluation was performed in 24 well plates to study the sporulation inhibition efficiency of the compound.

The study was conducted using PHB at different doses 10mg, 20mg, 50mg and 100mg and Amprolium 1mg (anticoccidiostat), 2.5% potassium dichromate was taken as positive control and oocyst suspension as negative control.

The sporulated and unsporulatedoocyst were counted at 0, 24 and 48 hours using haemocytometer. Each treatment contains ≤50,000 oocyst.

Evaluation of PHB against cell wall integrity of oocyst

Four doses of PHB were taken 10, 20, 50 and 100 mg with amprolium as positive control and 2.5% potassium dichromate as negative control. Amprolium was taken at the rate of 1g/ml. Approximately 10µl of sample was taken and diluted with water and added to 24 well plates with different doses of PHB, positive and negative control.

All the samples were maintained at room temperature and observed for lysis of cell wall after 48 hours. The cell wall integrity was assessed by subjecting the sample to SEM and TEM analysis.

Transmission Electron Microscope (TEM) analysis of PHB (100mg) treated *E. tenellaoocyst*

TEM analysis was carried out at Centralised Instrumentation Laboratory, Madras Veterinary College, Chennai-7. A drop of PHB treated oocyst suspension was pipetted onto the specimen plug for Transmission Electron Microscope. The mounted specimens were placed in an incubator and allowed to dry. The plug containing PHB (100mg) treated oocyst was examined by Transmission Electron Microscope. Photographs were made with a polaroid camera.

Scanning Electron Microscope (SEM) analysis of PHB (100mg) treated *E. tenellaoocyst*

SEM analysis of PHB (100mg) treated *E. tenella* oocyst was carried out at the Department of Mechanical Engineering, Anna University, Guindy. A drop of PHB (100mg) treated oocyst suspension was pipetted onto a specimen plug for the scanning electron microscope, and allowed to air dry. Mounted specimens were placed in. a vacuum evaporator and coated with a layer of gold, 100 Å thick. The plug containing coated oocysts was placed in a Japanese Scanning Microscope (JSM-2) and examined. Photographs were made with a polaroid camera.

Statistical analysis

Both sporulated and unsporulated oocyst was counted and sporulation inhibiting percentage at 0, 24 and 48 hours were, calculated, tabulated and statistical analysis was carried out. The data were analysed by one way ANOVA.

Results and Discussion

Different doses of PHB showed dose dependent inhibition for the sporulation of *E. tenella* oocysts as compared to the control group (2.5% Potassium dichromate). The statistical analysis showed that the doe of 100mg PHB inhibits the sporulation to certain extent followed by 50mg of PHB.

Different doses of PHB showed dose dependent inhibition of sporulation of *E. tenella* oocysts as compared to Positive control groups ($K_2Cr_2O_7$) and negative control group (Oocyst suspension). It can be seen from the Table 1a–1d 91.4% of oocysts of *E. tenella* managed to sporulate in the control incubations containing $K_2Cr_2O_7$ (Positive control) and oocyst suspension (Negative Control) whereas in incubation containing 10, 20, 50 and 100mg of PHB 86%, 81%, 74% and 68% of the oocyst were able to sporulate at 48 hrs. The maximum sporulation of *Eimeria spp* differs between the species. The different dose of PHB showed maximum inhibition at 100mg followed by 50mg, 20mg and 10mg respectively when compared to control groups. The sporulation inhibition of *E. tenella* oocyst treated with PHB at 0, 24 and 48 hrs is given in the Table 1d.

Transmission Electron Microscopic analysis of PHB (100mg) treated *E. tenella* oocyst

E. tenella oocyst treated with 100mg of PHB analysed by TEM (Fig. 2) revealed shrinkage in the proteinaceous layer of micropylar area.

Scanning Electron Microscope analysis of PHB treated *E. tenella* oocyst

SEM analysis of PHB treated *E. tenella* oocyst showed Breakage at the outer proteinaceous wall of oocyst is shown in the Figure 3.

Table.1a The effect of PHB on sporulation inhibition of *E. tenella* oocyst at 0 hours

Dose of the compound	Sporulated oocysts (n=6) (Mean ±SD)	Unsporulated oocysts (n=6) (Mean ±SD)	Percentage of unsporulated oocysts	Percentage of sporulated oocysts
PHB(10mg)	2666±1032	47000±2097	94.63	5.37
PHB(20mg)	2666±1032	47666.66±1505	94.70	5.30
PHB(50mg)	2333 ±816	48333±1505	95.39	4.61
PHB(100mg)	2333 ±816	46666±1632	95.20	4.8
Amprolium(1mg)	2333 ±816	47333±2065	95.30	4.70
Positive control	2333±816	48333±1505	95.39	4.61
Negative control	2333±816	48333±1505	95.39	4.61

Positive Control- 2.5% Potassium Dichromate. Negative control- Oocyst suspension. Each treatment contains ≤ 50,000 oocyst

Table.1b The effect of PHB on sporulation inhibition of *E. tenella* oocyst at 24 hours

Dose of the compound	Sporulated oocysts (n=6) (Mean ±SD)	Unsporulated oocysts (n=6) (Mean ±SD)	Percentage of unsporulated oocysts	Percentage of sporulated oocysts	F value between the groups	P value Significance between the groups
PHB(10mg)	26000±1786	22333.83±1505	46.20	53.80	53.998	.000**
PHB(20mg)	28666±1632	22000±1264	45.42	54.58		
PHB(50mg)	23333±1632	26666±1032	53.33	46.67		
PHB(100mg)	19000±1095	31666.66±1505	62.40	37.60		
Amprolium (1mg)	27333.33±1032	23000±2097	54.30	37.60		
Positive control	46000±1264	4333.33±1505	8.60	91.40		
Negative control	35000±2097	14333.33±1505	29.05	70.95		

Positive Control- 2.5% Potassium Dichromate. Negative control- Oocyst suspension. Each treatment contains ≤ 50,000 oocyst.

** Indicates highly significant. ** P ≤ 0.001 between the groups indicates highly significant.

Table.1c The effect of PHB on sporulation inhibition of *E. tenella* oocyst at 48 Hours

Dose of the compound	Sporulated oocysts (n=6) (Mean ±SD)	Unsporulated oocysts (n=6) (Mean ±SD)	Percentage of unsporulated oocysts	Percentage of sporulated oocysts
PHB(10mg)	43333±1032	7000±1095	13.90	86.10
PHB(20mg)	39666±1505	9333±1032	19.00	81.00
PHB(50mg)	37666±1505	13000±2756	25.65	74.35
PHB(100mg)	33000±2097	15333±1632	31.70	68.30
Amprolium(1mg)	36000±1786	13666±2943	27.51	72.49
Positive control	46000±1264	4333±1505	8.60	91.40
Negative control	42000±1264	8333±1505	16.56	83.44
F value between the treatments	134.813			
P value Significance between the groups	.000**			

Positive Control- 2.5% Potassium Dichromate. Negative control- Oocyst suspension. Each treatment contains ≤ 50,000 oocyst.

** Indicates highly significant.

** P ≤ 0.001 between the groups indicates highly significant.

Table.1d The effect of PHB on sporulation of *E .tenella* oocysts at 0, 24 and 48 hours

Dose of the compound	Percentage of sporulation inhibition at ''0'' hours	Percentage of sporulation inhibition at ''24'' hours	Percentage of sporulation inhibition at ''48'' hours
PHB(10mg)	94.63	46.20	13.90
PHB(20mg)	94.70	45.42	19.00
PHB(50mg)	95.39	53.33	25.65
PHB(100mg)	95.20	62.40	31.70
Amprolium(1mg)	95.30	54.30	27.51
Positive control	95.39	29.05	8.60
Negative control	95.39	29.05	8.60

Positive Control- 2.5% Potassium Dichromate. Negative control- Oocyst suspension. Each treatment contains ≤ 50,000 oocyst.

Figure.1 Photomicrograph of Sporulated and unsporulated oocyst of *E. tenella* (400X)

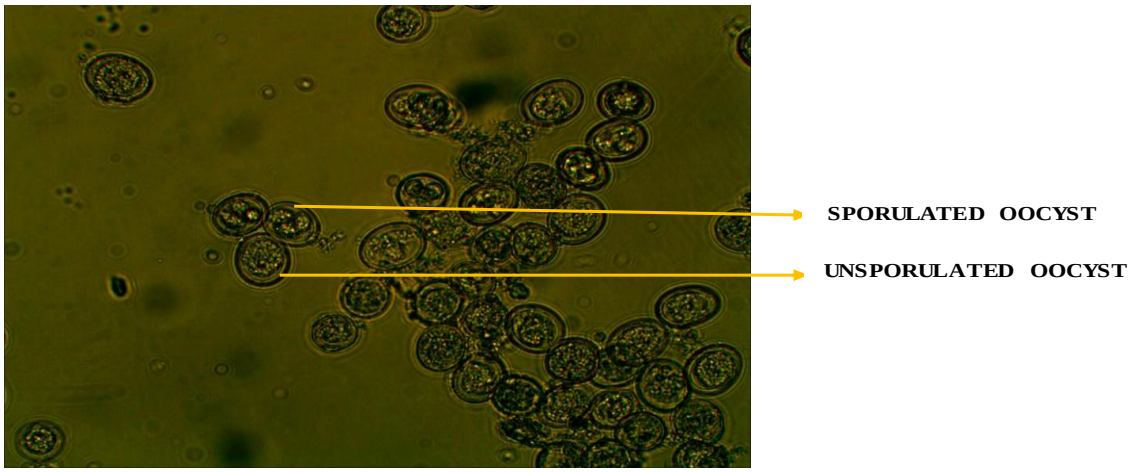


Figure.2 Transmission Electron Micrograph (TEM) of PHB (100mg) treated *E. tenella* oocyst at 48hrs at 3000X

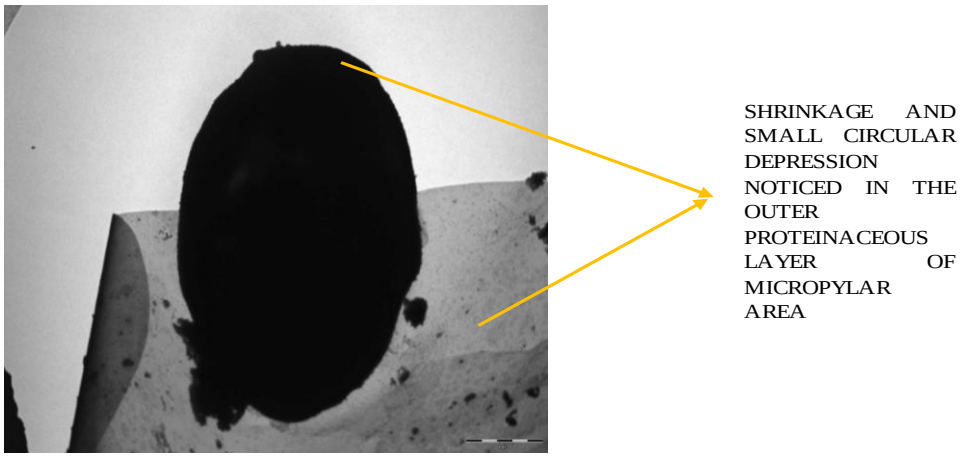
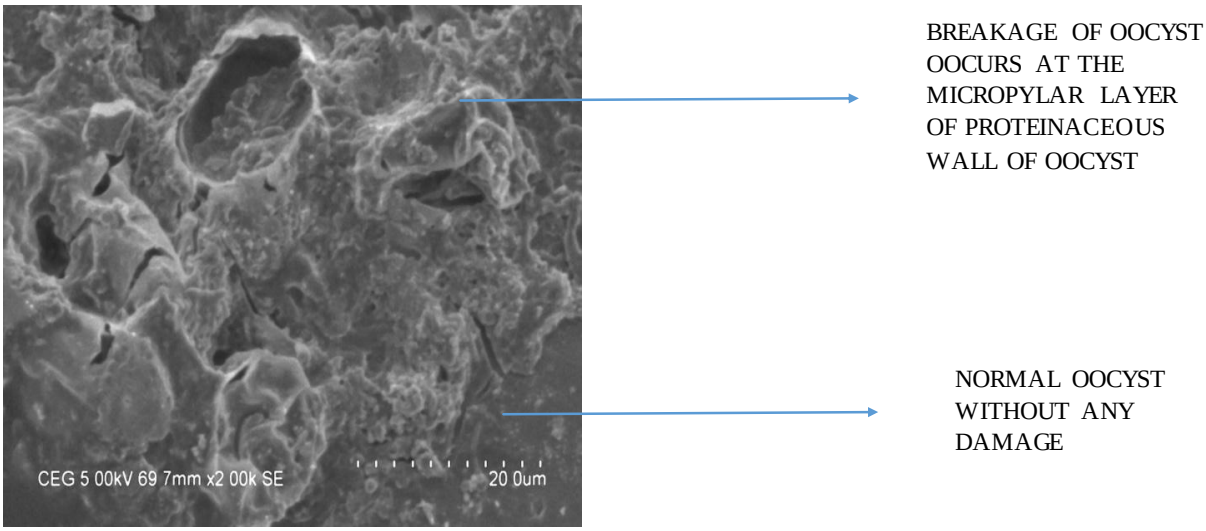


Figure.3 Scanning Electron Micrograph (SEM) of PHB (100mg) 48hrs treated oocyst at 3000X



The drugs which can inhibit sporulation process are the best choice as preventive mechanisms against coccidiosis. Due to the lack of effective and non-toxic disinfectants against coccidian and recent restriction of coccidiostatic drugs in poultry production, lead to the search for safe and effective alternatives for controlling coccidiosis. Various studies have been carried out to study the sporulation inhibition by using various products. In the present study, the polymer PHB was used at different doses (10, 20, 50 and 100mg) against the sporulation of *E. tenella* oocyst. The present study showed dose dependent inhibition of sporulation of *E. tenella* oocyst. At the dose rate of 100mg of PHB inhibited the sporulation at 62.40% followed by 50mg, 20mg and 10mg of PHB inhibited at 53.33%, 46.20% and 45.42% respectively, when compared to the Positive control and negative control, 8.60% and 29.05% respectively.

Hanan *et al.,* (2009) conducted a trial using *Xenorhabdus* and *Photorhabdus spp* on sporulation of *Eimeria* oocyst and reported promising results for controlling the *Eimeria* in deep litter system. Remmal *et al.,* (2013) used essential oil components against the chicken *Eimeria* oocyst and the number of oocyst decreased with 20mg/ml of essential oil. In the present study the sporulation was inhibited at 100mg of PHB followed by 50mg, 20mg and 10mg of PHB when compared to control groups. Similar results were by Zaman *et al.,* (2015). They performed an experiment using herbal extracts against *Eimeria tenella* oocyst and found out that these herbal extracts exhibited anti-sporulation effect by interfering in the physiological process necessary for sporulation. These extracts inhibited the sporulation at dose dependent manner from 500µg to 0.244 µg/ml.

Jitviriyanon *et al.,* (2016) used various essential oils collected from indigenous plants against the oocyst of *E. tenella.* Out of various oils, only two essential oils from *B. pandurata* and *O. basilicum* showed inhibition effect on sporulation of oocyst when compared with the positive control. By Comparing the present study with various studies of other products the PHB produced

by *Bacillus spp* was shown to have sporulation inhibition effect.

Mikail *et al.,* (2016) studied the anticoccidial activity of Methanolic extract of leaves of *Lanneaschimperi* against *E. tenella* oocyst. He studied the efficacy of these products against the cell wall of oocyst and found out that extracts at higher concentration (100mg) showed more efficacy on the lysis of cell wall of oocyst followed by 50mg and 25mg concentration when compared to the control groups. 100mg of extracts inhibited sporulation at 98% followed by 50mg and 25mg with sporulation inhibition at 89% and 68% respectively. The oocyst of coccidia are very resistant to physical and chemical treatment because of the presence of the two proteinaceous layers on its walls derived from the coalescence of wall forming bodies found in the macrogamete stage of parasite (Belli *et al.,* 2006). The present study revealed that PHB could be used to break the oocyst which was more helpful in controlling as well as preventing coccidiosis which is causing major economic loss to the poultry industry.

PHB extracted from *Bacillus spp* inhibited the sporulation of *E. tenella* oocyst under i*nvitro* condition

Conflict of interest

None declared

Acknowledgment

The work designed was carried out for the award of M.V.Sc degree in Animal Biotechnology in the academic year 2016–2018. The author wishes to thank the Tamil Nadu Veterinary and Animal Sciences University for funding the entire research project and Department of Animal Biotechnology for providing all assistance with equipment and chemicals

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How to cite this article:

Aadithya, T*, S. Meignanalakshmi, M. Raman, M. Parthiban and Vijayarani, K. 2018. A Preliminary *in vitro* Study to Evaluate Poly-3-Hydroxy Butyrate as Anticoccidial Agent against Oocysts of *E. tenella*. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 4364-4372.
doi: https://doi.org/10.20546/ijcmas.2018.708.458