Animal Health Research Journal Vol. 6, No. 2, June 2018 pp. 12-19

ISSN: 2356-7767

Using various types of stabilizers in different Lyophilization Programs for maintenance viability of *Eimeria tenella*

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Received in 6/5/2018
Accepted in 4/7/2018

Abstract

Lyophilization (Freeze drying) technique for preservation of sporulated *Eimeria tenella* oocysts was applied using four lyophilization programs I, II, III and V, with three different stabilizers (lacto albumin sucrose (A), peptone (B) and skimmed milk (C) with, addition of gelatin to each stabilizers) were used for the first time in this recent study. Good and reliable results were obtained especially on using the first lyophilization program (I) in comparison with the other three programs. Lacto-albumin sucrose stabilizer (A) provided satisfied results in keeping the number of oocysts with less reduction rate post lyophilization up to 9 months. Also it retained its pathogenicity degree of *Eimeria tenella.*

Key word: *Eimeria, tenella*, stabilizers, lyophilization

1. Introduction

Coccidiosis is one of the most important and serious diseases of poultry industry worldwide causing huge economic losses due to heavy infection which lead to high morbidity and mortality each year mainly in broilers. The disease generally affects chicks below 10 weeks of age with maximum incidence in 3 to 6 weeks old chicks (**Chauhan and Roy, 2008**).

Chicken flocks free from coccidia are extremely rare and at least three species (*Eimeria tenella, Eimeria necatrix and Eimeria acervulina)* are commonly found in all commercial chickens (**Williams, 2002).**

Most coccidia in poultry belongs to genus *Eimeria*. *Eimeria tenella* is the most common and pathogenic coccidia in chicks also called caecal *Eimeria* as it considered extremely host specific, where the predilection site of *Eimeria tenella* is in the two caeci and causes hemorrhagic caecal syndrome especially in young age and lead to high morbidity and mortality in chicks with serious economic losses to poultry farms. The clinical signs of the disease characterized by depression, bloody diarrhea, closed eye, lost weight gain, ruffled feathers, emaciation and loss of skin pigment. On post mortem examination,it is showed that the two caeci greatly enlarged and distended with clotted blood. On applying microscopic examination of the mucosal scraping of the two caeci of the infected birds, clusters of large schizonts and oocysts were detected (**Christensen and Allen, 2004).** Transmission of *Eimeria tenella* is occurred by ingestion of sporulated oocysts through contaminated feed and water which is the only natural method of transmission, also it can be spread mechanically through animals,

insects, equipment and movement of people between farms **(Vegad, 2008**).
Preservation of *Eimeria tenella* in lab is one of the very important steps to the workers in this field. So using potassium dichromate as a maintenance medium at refrigerator temperature is considered as one of the most suitable methods for storage this strain but unfortunately it has many disadvantages like the reduction of the number of oocysts and the pathogenicity of the coccidia by the long keeping, also the media become contaminated and toxic by long storage.

Also the sporulated oocysts preserved by using liquid nitrogen for about 3 months **(Kristensen *et al,* 2011**) but this type of preservation is expensive and need the regular supply of liquid nitrogen which is not always guaranteed and the electricity may be problematic especially in the developing countries. So, the lyophilization is considered the most economical, time consuming and sterile method for preserving biological agents for long time.The principle of lyophilization technique depends on the exposing of the frozen pure culture to higher vacuum pump that remove frozen water through a process called Supplementation (primary drying) in which frozen water is transformed directly from solid to gas to avoid the formation of ice crystals which damage the cells then adsorption (secondary drying), then the sealed vials stored at refrigerator temperature until be used for examination (**Buman *et al*, 2007 and Lilian *et al*, 2009**). So the Present work aimed to use lyophilization for preserving *Eimeria tenella* strain using three types of maintenance stabilizers as lacto albumin sucrose, peptone and skimmed milk, and four programs of lyophilization were used for the detection of the most suitable type of stabilizer and method of lyophilization used for preserving *Eimeria tenella* and maintain its viability for long time to be used in researches and vaccine production.

2. Material and Methods:

2.1. Strain of *Eimeria* used:

Eimeria tenella strain was previously characterized and kindly provided from Parasitological Vaccine Research Department, Veterinary Serum and Vaccine Research Institute, Abbasia – Cairo-Egypt.

2.2. Chicken used

A total number of 260 young susceptible chicks 3-5 weeks age Saso strain have no history of infection or vaccination against *Eimeria tenella* or any other *Eimeria* were used in this study, twenty chicks were used for the purpose of the propagation, multiplication and increasing the number of oocysts shedding The remaining 240 chicks were used for the detection the effect of lyophilization programs on the counting, viability and pathogenicity of *Eimeria tenella* oocysts post lyophilization.

2.3. Propagation of oocysts

Eimeria tenella was propagated by inoculating the strain orally into young susceptible chicks, their dropping were examined daily using the microscope, till reached the high maximum of shedding vegetative oocysts (over 50100% oocysts\ml), these chicks were slaughtered and the oocysts were collected from the two caeci for further sporulation.

2.4. Purification *of Eimeria tenella*:

Eimeria tenella vegetative oocysts either collected from the droplets or the two caeci were washed using tap water and suspended in super saturated sodium chloride using the flotation technique to obtain pure and clean oocysts **(Ryler *et al,* 1976)** and **(Lilian *et al* , 2014).**

2.5. Sporulation of *Eimeria tenella*:

Purified collected vegetative oocysts. They were put in potassium dichromate 2.5% and incubated at 26 cº - 28 cº till complete sporulation within 48 – 72 hours (**Duncan *et al* 1987, Eskander and Germine, 2013).**

2.6. Stabilizers:

Three types of stabilizers were used as lacto

albumen sucrose, peptone and skimmed milk with adding of gelatin to each one at concentration of 10% .They were prepared according to **Woodward** and **Tudor (1975), Odds *et al* (1978), Christine (2008) and Cody *et al* (2008).**

2.7. Lyophilization technique:

The lyophilization process was carried out using a freeze-dryer apparatus (**Tofflon, China 2014-033 CA)**. Sporulated *Eimeria tenella* oocysts suspended in the potassium dichromate counted to be 50100 oocysts/ml solution and mixed with the each stabilizer as volume / volume and then aliquot in lyophilized bottles and put in lyophilizer as the routine work of the Lyophilization Department, Serum and Vaccine Research Institute. (**Li *et al,* 2003 and Wang and Zhan, 2007**) using the following programs**.**

2.8. The lyophilization programs used

Four types of lyophilization programs (Freeze drying programs) were used in this study as shown in table (1).

Table (1). The lyophilization programs used

Types of programs	The temperature shelf pre-cooled	The actual cooling Period of the bottles by hours	The temperature of primary drying	The temperature of secondary drying	The secondary drying period
The first program (I)	-50°C	6	−32 °C	25 °C	8
The second program (II)	-50°C	8	−32 °C	25 °C	8
The third program (III)	−40 °C	6	−32 °C	30 °C	8
The fourth program (IV)	-45°C	8	−32 °C	30 °C	8

After lyophilization, the bottles were sealed and kept at refrigerator .

2.9. Evaluation of the lyophilized *Eimeria tenella* :

The lyophilized dick of *Eimeria tenella* oocysts was rehydrated with 1ml sterile saline and examined microscopically and counted to record the approximate reduction rate of the number of oocysts at 0, 3, 6 and 9 months post lyophilization at the same time they were inoculcated in groups of susceptible chicks 3-5 weeks of age to detect its pathogenicity**.**

Experimental chicks: A total number 240 chicks were treated as follow

Table (2). Groups of chicks used for detection of viability and pathogenicity of lyophilized sporulated *Eimeria tenella* oocysts

Groups	Program of lyophilization	A		B		C		control
		Subgroups	No. of chicks	Subgroups	No. of chicks	Subgroups	No. of chicks	No. of chicks
1st group	I	IA	20	IB	20	IC	20	-
2nd group	II	IIA	20	II B	20	II C	20	-
3rd group	III	IIIA	20	IIIB	20	IIIC	20	-
4th group	IV	IV A	20	IV B	20	IV C	20	-

A = Lacto albumen sucrose with gelatin stabilizer.
B = Peptone with gelatin stabilizer.
C = Skimmed milk with gelatin stabilizer

3. Results

3.1. Counting of sporulated oocysts

Fixed number of sporulated *Eimeria tenella* oocysts (50100/ml) was adjusted before adding the three types of the stabilizers and applying the four lyophilization programs. Then the number of the lyophilized *Eimeria* oocysts was counted at 0, 3, 6and 9 months post lyophilization **Augustine (1999).**

Results illustrated in tables (3-6) indicating reduction of number of sporulated *Eimeria* oocysts using different stabilizers were between 7% and 86.2% during different periods .Also the tables indicated that using of lacto- albumin sucrose with gelatin as stabilizers with the first lyophilization program was the best, comparing to using of other stabilizers and lyophilization programs in which the reduction of sporulated oocysts was the least till 9 months.

3.2.The pathogenicity of lyophilized *Eimeria tenella* in experimental chicken

Tables (7-10) indicated the results of pathogenicity of lyophilized *Eimeria tenella* oocysts in different storage periods from 0 time to 9 months it was clear that the first program of lyophilization using lacto albumin sucrose started with 100% pathogenicity at zero time and then reduced till it reached 60% at 9 months compared to other programs and other stabilizers. The controls lyophilized samples without stabilizers showed no pathogenicity in all lyophilized programs.

Table (3). Counting of sporulated oocysts (*Eimeria tenella*) post lyophilization using the 1st program of lyophilization

Types of stabilizer	No. of oocysts / ml at different periods							
	Zero time	The % of reduction	3rd month	The % of reduction	6th month	The % of reduction	9th month	The % of reduction
AI	46500	7%	42300	15.4 %	40500	19 %	36300	27%
BI	39900	20.2 %	36900	26.2 %	27100	45.8%	26700	46.6 %
CI	38100	23.8 %	30000	40 %	24900	50.2 %	21000	58 %
Control	3600	92.8%	-	-	-	-	-	-

AI: Lacto albumen sucrose with gelatin.
BI: Peptone with gelatin.
CI: Skimmed milk with gelatin.
Control: lyophilized *Eimeria tenella* sporulated oocysts without stabilizer.

Table (4). Counting of sporulated oocysts (*Eimeria tenella*) post lyophilization using the 2nd program of lyophilization

Types of stabilizer	No of oocysts / ml at different periods							
	Zero time	The % of reduction	3rd month	The % of reduction	6th month	The % of reduction	9th month	The % of reduction
A II	45000	10 %	42000	16 %	36300	27 %	29100	41.8 %
B II	38700	22.6 %	35700	28.6 %	30300	39.4 %	24600	50.8 %
C II	31200	37.6 %	27000	46 %	22800	54.4 %	20700	58.6 %
control	3900	92.2%	-	-	-	-	-	-

A II: Lacto albumen sucrose with gelatin
B II; Peptone with gelatin
C II: Skimmed milk with gelatin
Control: lyophilized sporulated *Eimeria tenella* oocysts without stabilizer.

Table (5). Counting of sporulated oocysts (*Eimeria tenella*) post lyophilization using the 3rd program of lyophilization

Types of stabilizer	No. of oocysts / ml at different periods							
	Zero time	The % of reduction	3rd month	The % of reduction	6th month	The % of reduction	9th month	The % of reduction
A III	42900	14.2 %	36000	28 %	30000	40 %	25200	49.6 %
B III	40200	19.6 %	32700	34.6 %	27000	46 %	21000	58 %
C III	30000	40 %	24900	50.2 %	21000	58 %	15000	70 %
control	2400	95.2%	-	-	-	-	-	-

A III: Lacto albumen sucrose with gelatin.
B III: Peptone with gelatin
C III: Skimmed milk with gelatin
Control: lyophilized sporulated *Eimeria tenella* oocysts without stabilizer.

Table (6). Counting of sporulated oocysts (*Eimeria tenella*) post lyophilization using the 4th program of lyophilization

Types of stabilizer	No. of oocysts / ml at different periods							
	Zero time	The % of reduction	3rd month	The % of reduction	6th month	The % of reduction	9th month	The % of reduction
AIV	37200	25.6 %	31500	37 %	29100	41.8 %	26400	47.2 %
BIV	33000	34 %	29700	40.6 %	24600	50.8 %	19300	61.4 %
CIV	27000	46 %	19500	61 %	14400	71.2 %	6900	86.2 %
control	3000	94.0%	-	-	-	-	-	-

AIV: Lacto albumen sucrose with gelatin
BIV: Peptone with gelatin
CIV: Skimmed milk with gelatin
Control: lyophilized sporulated *Eimeriatenella* oocysts without stabilizer.

Table (7). Pathogenicity of sporulated *Eimeria tenella* oocysts using 1st lyophilization program (group I)

Groups of experimental chicks	Pathogenicity percentage			
	Zero time	3rd month	6th month	9th month
IA	100%	80%	80%	60%
IB	80%	60%	40%	40%
IC	60%	40%	20%	20%
Control	-	-	-	-

IA: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Lacto albumen sucrose and gelatin stabilizers
IB: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Peptone and gelatin stabilizer
IC: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Skimmed milk and gelatin stabilizers
Control chicks showed no pathogenicity

Table (8). Pathogenicity of sporulated *Eimeria tenella* oocysts using 2nd lyophilization program (group II)

Groups of experimental chicks	Pathogenicity percentage			
	Zero time	3rd month	6th month	9th month
IIA	80%	80%	60%	40%
IIB	60%	60%	40%	20%
IIC	60%	40%	20%	20%
Control	-	-	-	-

IIA: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Lacto albumen sucrose and gelatin stabilizers
IIB: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Peptone and gelatin stabilizer
IIC: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Skimmed milk and gelatin stabilizers
Control chicks showed no pathogenicity

Table (9). Pathogenicity of sporulated *Eimeria tenella* oocysts using 3rd lyophilization program (group III)

Types of stabilizer	Pathogenicity percentage			
	Zero time	3rd month	6th month	9th month
III A	80%	60%	60%	40%
III*B*	60%	60%	40%	20%
III C	40%	40%	20%	0%
Control	-	-	-	-

IIIA: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Lacto albumen sucrose and gelatin stabilizers
IIIB: Chicks ingested lyophilized *Eimeria tenella*oocysts mixed with Peptone and gelatin stabilizer
IIIC: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Skimmed milk and gelatin stabilizers
Control chicks showed no pathogenicity

Table (10). Pathogenicity of sporulated Eimeria tenella oocysts using4thlyophilization program (group IV)

Types of stabilizer	Pathogenicity percentage			
	Zero time	3rd month	6th month	9th month
IVA	60%	60%	40%	40%
IV B	60%	40%	20%	20%
IVC	40%	20%	0%	0%
Control	-	-	-	-

IVA Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Lacto albumen sucrose and gelatin stabilizers
IVB: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Peptone and gelatin stabilizer
IVC: Chicks ingested lyophilized *Eimeria tenella* oocysts mixed with Skimmed milk with gelatin stabilizers
Control chicks showed no pathogenicity

4. Discussion

Avian Coccidiosis is the most common poultry disease causing great economic losses. *Eimeria tenella* is one of the major pathogenic species of *Eimeria* effecting the epithelial cells of ceca resulting high morbidity and mortality and easily diagnosed **(long *et al,* 1976 and Bowman *et al,* 2013).**

Preservation of *Eimeria tenella* or any types of *Eimeria* species in lab for long time is the aim of every researcher work in this field. So using of refrigerator for preserving of coccidia was the usual and first choice, although this method is easy but unfortunately have many disadvantages as mentioned before. Four lyophilization programs were used in the study to determine the most suitable program could be applied to produce satisfied results as tabulated in table (1). Also a total of 240 susceptible chicks were used in this work divided into four groups each group consist of 80 chicks, (20) chicks \group and 5 chicks/subgroup as declared in table (2).

So this study aimed to use lyophilization of speculated *Eimeria tenella* oocysts to maintain it for longer time. Three different stabilizers were used in this study which were lacto-albuminsucrose (A), peptone (B) and skimmed milk (C) were used in recent work using the four lyophilization programs, According to the results illustrated it was clear that the viability of lyophilized sporulated *Eimeria tenella*oocystsremained viable till 9 months with gradual reduction of its counting number (tables 3-6).also it could be indicated that using of lacto-albumin sucrose with gelatin stabilizer in first lyophilization programs was the best than using other stabilizers with other lyophilization-programs. **Zdenek (2003)** mentionedthat gelatin act as non-permeating and non-penetrating compound that cause extracellular protection when present at concentration of 10%-40%, and the peptone is not preferred in preservation of protozoa. **Allison *et al,* (1999) and *Jang et al,* (2013)** stated that using lacto- albumin sucrose as stabilizers was required for sporulation of the oocysts and survival of sporozoites, as the coccidia have poly saccharides in their structure so this statement was proved in this study.

Regarding the pathogenicity of lyophilized *Eimeria tenella* oocysts in susceptible young chicks which come in parallel with result of its viability as illustrated in tables (7-10) indicated that using of lacto- albumin sucrose with gelatin in the first lyophilization program induced 100% pathogenicity at zero time post lyophilization if it compared with using other stabilizers and lyophilization programs, using skimmed milk as stabilizers sharp reduction in number and the pathogenicity of sporulated *Eimeria tenella* oocysts was recorded ,through this study control lyophilized sporulated oocysts without stabilizer lost its pathogenicity as the number of its viability at a zero time post lyophilization ranged between 2400 – 3900 oocysts \ ml and this number is not enough to produce infection and pathogenicity in the susceptible chicks. From results obtained in this study it could be concluded that lyophilization is a reliable method for preserving sporulated *Eimeria tenella* oocysts using lacto-albumin sucrose stabilizer with first lyophilization program which is used for the first time and further studies should be done for using other stabilizers which may keep the oocysts for more longer period as this method is more economic than other types of preservation and important in vaccine production**.**

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