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EFFICIENCY OF FOUR DISINFECTANTS AGAINST *EIMERIA TENELLA* ISOLATES FROM EGYPTIAN CHICKENS (*IN VITRO* ASSESSMENT)

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ABSTRACT

Background: Control of coccidiosis depends, in addition to the commercial preparation of herbal extracts, on good sanitation and litter management, along with the use of medication or vaccination programmes. In addition to the chemotherapeutic treatment of coccidiosis early disinfection of the poultry houses should be done using various disinfectants for controlling presence of oocysts. The objective of this research was to evaluate in vitro the action of four different coccidicidal disinfectant including (Quaternary ammonium compounds QACs, chlorocresol, glutaraldehyde and kilcox) on oocysts of *Eimeria tenella*.
Methods: In this study the action of four coccidicidal disinfectant including (Quaternary ammonium compounds QACs, chlorocresol, glutaraldehyde and kilcox) on both sporulated and unsporulated oocysts of *Eimeria tenella* was evaluated in vitro. *E. tenella* Oocysts were obtained from naturally infected Egyptian native breed chickens. The oocysts were exposed to the disinfectants at different concentrations and different contact times. The efficacy of the disinfection was assessed by either destruction of sporulated oocyst or inhibition of sporulation. **Results:** It was observed that the most efficacious disinfectants against unsporulated and sporulated *E. tenella* oocysts was kilcox followed by chlorocresol, while QACs and, glutaraldehyde were less effective. kilcox sporulation inhibition reached 100 % on unsporulated *E. tenella* oocysts,and their destructive effect reached 99% on sporulated oocysts. furthermore, the results showed that both inhibitory and destructive activity of the tested disinfectants was significantly increased by increasing their concentrations and/or the contact time.

Key words: *Eimeria tenella*; chlorocresol; Chickens; sporulation

INTRODUCTION

Coccidiosis is one of the most serious apicomplexan parasites infecting domestic chickens as well as other fowls. Recent reports estimated the direct losses from the disease to exceed one billion us dollars globally in the poultry sector (Allen and Fetterer, 2002). The unicellular protozoa invade the enterocytes of the birds, propagated in a sexual and sexual stages leading to destruction of epithelial lining intestine, bloody diarrhoea, dehydration, poor feed conversion, weight loss and death.

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Chickens can be infected by any of the seven *Eimeria* species, including *Eimeria tenella* (*E. tenella*), *Eimeria maxima, Eimeria acervulina, Eimeria necatrix, Eimeria brunette, Eimeria mitis, and Eimeria praecox*. In Egypt, *E. tenella* was the most prevalent species among broiler flocks (Mahareek, 1992). In 2012, Abdel-Gawad *et al.,* found that (25.82%) of 711 samples from Native chickens were infected with *E. tenella.*

E. tenella oocyst sporulated rapidly outside the infected host. Sporulation can be completed as early as 18 hours after oocysts shedding (Reid *et al.,* 1991). The sporulation depends on many environmental factors, such as: temperature, humidity, and oxygen availability. Viability and infectivity of sporulated oocysts can also depend on the parasite's characteristics, like resistance of disinfectants and adverse field conditions (Fayer, 1980).

Disinfection plays the major role in control process of coccidiosis. Chemical disinfectants that are efficient in inactivating different micro-organisms such as bacteria, fungi or virus do not generally display sufficient coccidiocidal activity (Straberg and Daugschies, 2007). The wall of the oocyst consists of two chemically different layers and the combination of lipids and glycoproteins provides efficient protection of the germinal substance of the oocyst from the action of commonly used disinfectants (Greif *et al.,* 2011). Each chemical disinfectant requires multi-optimized conditions to reach its maximum effectiveness such as contact time, concentration and the presence of organic materials that cover and protect oocysts from direct contact, the majority of the effective disinfectants on oocysts are caustic or toxic, inducing dangerous corrosive and health threating side effects upon their use in poultry installations. Studies were carried out worldwide to assess the action of various active principles with disinfectant action over *Eimeria spp.* oocysts (Fayer, 1980).

The disinfection efficacy (DE) is defined as the sporulation inhibition percentage of oocysts (Daugschies *et al.,* 2002). After in vitro incubation, the coccidiocidal effect of the disinfectant is indicated by its capability of prevent sporulation (Williams, 1997).

This action is observed under two forms on the optic microscope: the oocysts present have lysed wall so, no sporulation occurs, or the wall keeps itself visually preserved, but the oocysts did not sporulate (Daugschies *et al.,* 2002). Though disinfection is widely recognized as an important component of hygiene management (Straberg and Daugschies, 2007), remarkably few papers have been published since then on how to reliably assess the suitability of disinfectants.

This study was conducted to estimate the efficacy of some widely used disinfectant in Egyptian market as coccidiocidal disinfectant on *E. tenella* oocysts in vitro.

MATERIALS AND METHODS

Eimeria Oocysts

Multiple oocysts isolates were obtained from ceci of clinically diseased native breeds chickens (25-40 days old) from different local farms in Assiut, Chickens suffered from depression, ruffled feathers, poor performance and with or without bloody faecal matter. The oocysts harvested from caecal contents and purified by using flotation technique. Oocysts were counted using MacMaster slide, and the number of oocysts was adjusted to 25000 oocysts per ml (Nematollahi *et al.,* 2008).

Morphological identification

Isolates used for morphological determination were chosen to be mostly consisted of oocysts that are morphologically similar to *E.tenella* Oocysts morphology and size were determined by measuring length and width

of 50 similar oocysts using ocular micrometer.

Molecular identification of Eimeria species

To confirm the presence of *E.tenella* in the examined isolates, the purified isolates containing aliquots of ~1 × 10^6 oocysts were taken and oocysts were centrifugated at 2,000 rpm for 2 min. The genomic DNA extracted as per kit protocol, using Gene jet DNA and RNA purification kit (thermo scientific). PCR with the SCAR primers for *E*. tenella; **F** CCGCCCAAACCAGGTGTCACG and **R** CCGCCCAAACATGCAAGATGGC were carried out with a thermocycler adjusted as follow: Standardized cycling conditions consisted of initial denaturation: 96 °C for 5 min, (denaturation: 94 °C for 1 min, annealing: 62 °C for 2 min, extension: 72 °C for 1 min) for 30 cycles and Final extension: 72 °C for 7 min. All PCR products were analyzed by separation on 1.5% agarose gel in TAE buffer at 100 V for 30 min, were stained with ethidium bromide, and examined under UV light.

Primer sequences (5-3)	Amplicon size (bp)	Annealing temperature
CCGCCCAAACCAGGTGTCACG CCGCCCAAACATGCAAGATGGC	539	60ºC

Disinfectants:

Four different disinfectants were obtained from Kilco company for veterinary disinfectants (Kilco International Ltd, Northern Ireland) with a different active principle each as follow:

Chlorocresol was obtained as 100% concentrated crystals, then two dilutions 10% and 20% were prepared by adding 10gm of chlorocresol to 100 ml Ether and by adding 20gm of chlorocresol to 100 ml Ether, respectively.

Quaternary ammonium compounds (QACs) was obtained as 100% concentrated solution, then two dilutions 2% and 4% were prepared by adding 2ml of QACs to 100 ml water and by adding 4ml of QACs to 100 ml water respectively.

Glutaraldehyde (50%) solution was used at concentrations 2% and 4%. kilcox Extra (a commercial product from Kilco company), it was used at concentrations 2% and 4%. Contains.

Para chloro meta Chlorocresol	100 g / kg.
Benzalkonium Chloride	100 g / kg.
Glutaraldehyde	150 g / kg.

Isolates were divided into two Parts, first was subjected to different concentrations and contact times of the disinfectants, and then allowed to sporulate in potassium dichromate (2.5%) in petri-dishes.

The second part was allowed to sporulate by adding amount of potassium dichromate (2.5%) in petri-dishes. The Petri-dishes were semi-covered and kept at room temperature (25-28ºC) for 3-5 days (Conway and McKenzie, 2007) and then subjected to different concentrations and contact times to the disinfectants after sporulation to evaluate the destructive activity of each disinfectant.

Both sporulated and unsporulated oocysts were subjected to the following protocols according to (Junior *et al.*, 2007).

Disinfectant	Concentration	Time of exposure
Chlorocresol	10%	30, 120 minutes
	20%	
Quaternary ammonium compounds	2%	
	4%	
Glutaraldehyde (50%)	2%	
	4%	
kilcox	2%	
	4%	

sporulation percentage and sporulation inhibition activity

Sporulation percentage (SP) was calculated after 7 days to get the percentage of Sporulation inhibition activity (IA) of the oocysts and were calculated according to (junior *et al.*, 2007)

IA% = {(sporulation % of control – sporulation % of disinfectant treated oocysts) X 100} sporulation % of control.

RESULTS

On gross lesion examination of the intestinal tract especially the two caecal lesions showed haemorrhages & clotted blood in caecal pouches. Figure (1)

figure legends, and tables:

Figure 1: Two caeci of infected chicken showing bloody contents.
Figure 2: *Eimeria tenella* oocysts in wet smear (10x objective lens).
Figure 3: *lane 4 control positive for E. tenella 539bp and lane 5 pool positive for (E. tenella 539bp).*
Figure 4: inhibitory activity of disinfectants on unsporulated oocysts.
Figure 5: effect of disinfectants on sporulated oocysts.
Table 1: The inhibitory activity of disinfectants on unsporulated oocysts.
Table 2: The destructive activity of disinfectants on sporulated oocysts.

Figure (1): Two caeci of infected chicken showing bloody contents.

Morphological identification:

Microscopical identification of *Eimeria* oocyst species revealed that 90.25% of collected samples were found infected with *E. tenella.* Figure (2)

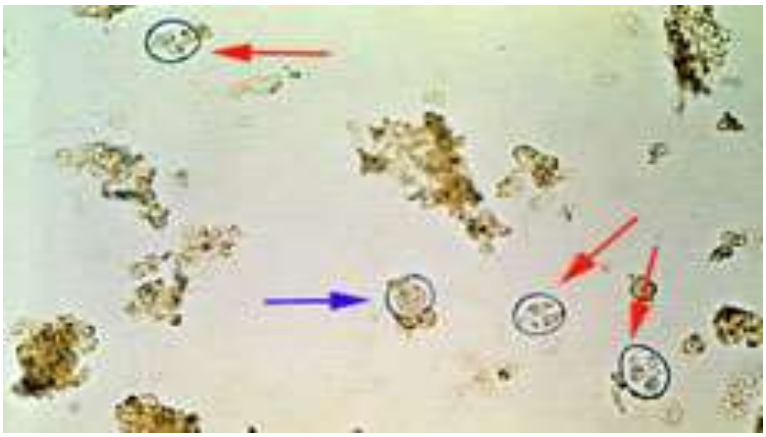

Figure (2): *Eimeria tenella* oocysts in wet smear (10x objective lens)

Molecular identification of Eimeria species:

The presence of *E. tenella* oocysts in the examined samples was confirmed by the detection of the amplified fragment (539 bp). Figure (3)

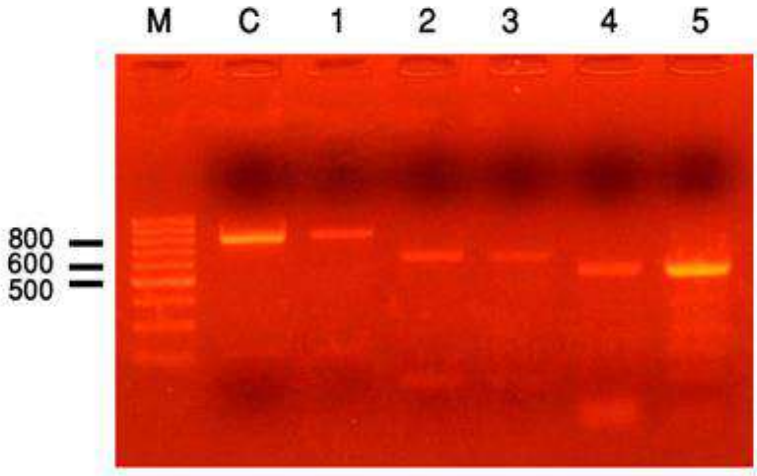

Figure (3): lane 4 control positive for E. tenella 539bp and lane 5 pool positive for (E. tenella 539bp)

The efficacy of disinfectants on E.tenella oocysts:

The inhibitory and destructive effect of different disinfectants on unsporulated and sporulated *E. tenella* oocysts were tabulated in tables 1 & 2.

Table 1: The inhibitory activity (IA) of disinfectants on unsporulated oocysts.

C.T.	kilcox				QACs				glutaraldehyde				chlorocresol				control	
	2%		4%		2%		4%		2%		4%		10%		20%			
	SP %	IA%	SP %	IA %	SP%	IA%	SP%	IA %	SP %	IA %	SP %	IA %	SP %	IA %	SP %	IA %	SP %	IA %
30 min.	27.6	97.3	2	99	24.7	76.3	11.9	88.1	38.3	61.7	18.2	81.8	13.3	86.7	7.3	92.7	99	0
120 min	20.1	98	0.3	100	8.2	91.8	6.2	93.9	23.4	76.6	11	94.8	9.3	90.7	6	94	99	0

C.T.= contact time **SP%=** sporulation percentage **IA%=** inhibitory activity

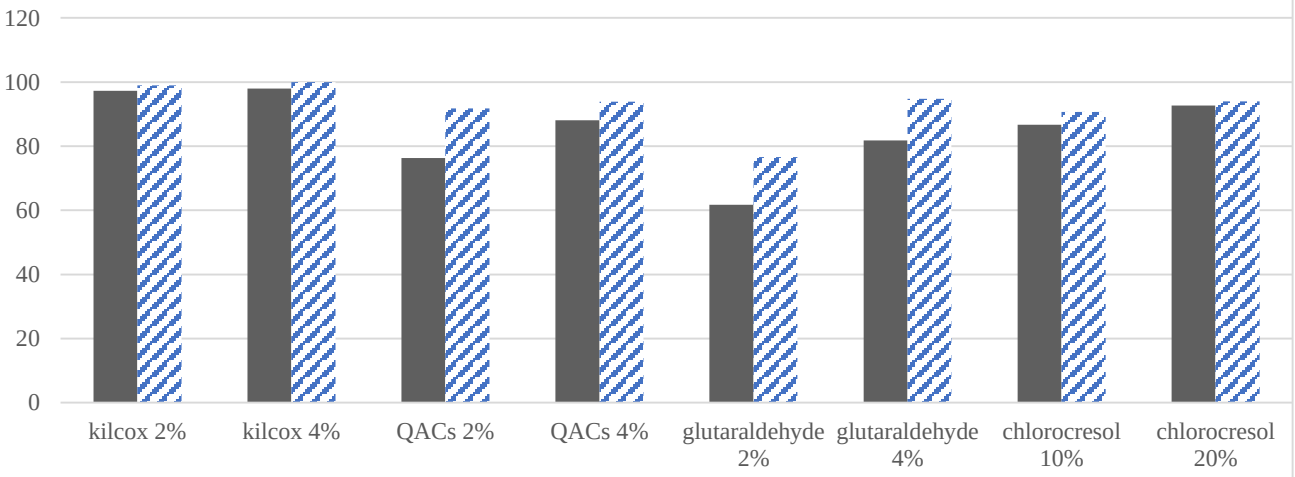

figure 4:inhibitory activity of disinfectants on unsporulated oocysts

Results in Table (1) illustrated that the IA of four disinfectants against unsporulated *E. tenella* oocysts, kilcox (4%) was highly effective against unsporulated *E. tenella* oocysts at C.T of 30 and 120 minutes where the IA reached 99 % and 100 %, respectively. While the effect of lower concentration (2 %) for C.T of 30 and 120 minutes reached 97.3 % and 98 %, respectively.

The IA of Quaternary ammonium compound (QACs) on *E. tenella* unsporulated oocysts at 2% solution for 30 and 120 minutes C.T. resulted 76.3% and 91.8% IA and when we used 4% solution of QACs recorded good efficacy 88.1% and 93.9% respectively.

The effect of 2% solution of Glutaraldehyde at 30 and 120 minutes C.T. against the unsporulated oocysts resulted 61.7% and 76.6% IA. And usage of 4% solution of Glutaraldehyde resulted 81.8% and 94.8%.

The usage of 10% solution of chlorocresol against unsporulated oocysts at 30 and 120 minutes C.T. resulted 86.7% and 90.7% IA respectively, at 20% conc. the results were 92.7% and 94%.

Table 2: The destructive activity of disinfectants on sporulated oocysts

C.T.	kilcox		QACs		glutaraldehyde		chlorocresol		control
	2%	4%	2%	4%	2%	4%	10%	20%	
30 min.	72.4	93	53.6	59.2	21.5	66.8	60	85	2
120 min.	79.9	99	66.4	69.2	49.2	89	77	98	2

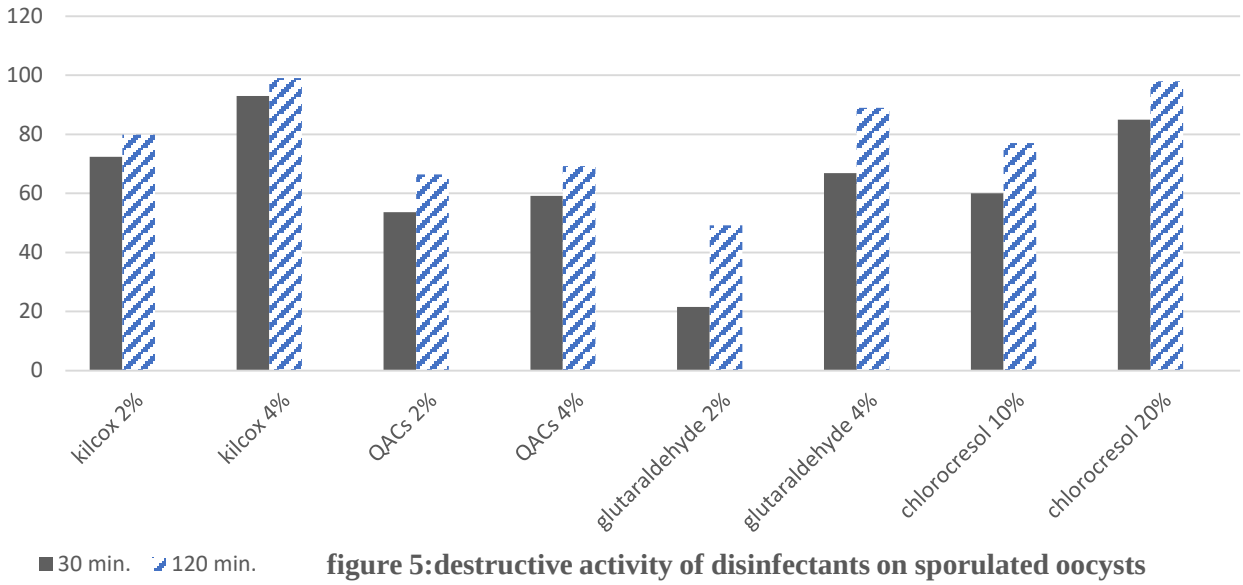

figure 5:destructive activity of disinfectants on sporulated oocysts

On other hand the obtained results in table (2) documented the destructive activity of the examined disinfectants on sporulated oocysts, the destructive effect of kilcox concentrations 4% against sporulated oocysts at 30 and 120 minutes reached 93 % and 99 %, respectively.

Usage of 2% QACs solution at 30 and 120 minutes C.T. their (DE) was 53.6% and 66%, consequently, while the usage of 4% solution of QACs at 30 and 120 minutes C.T. resulted in (DE) 59% and 69%, respectively.

2% solution of glutaraldehyde at 30 and 120 minutes C.T. able to induce lysis of 21.5% and 49% of treated oocysts, respectively. while at 4% conc. 30 and 120 minutes C.T. the result were 66.7% and 89%, respectively.

The usage of 10% solution of chlorocresol at 30 and 120 minutes C.T. resulted in destruction activity by 60% and 77%, respectively. At 20% conc. the results were 85% and 98%.

The findings obtained illustrated the powerful effect of Kilcox against sporulated oocysts.

DISCUSSION

From the viewpoint of coccidia control inactivation of exogenous stages of parasites is important for reducing infection pressure and protecting hosts from disease. Many commercial chemical disinfections eliminate bacteria or virus particles but don't have inadequate or unknown anticoccidial properties (Daugschies *et al.*, 2013).

The present research tested the ability of four chemical disinfectants to stop oocysts sporulation after in vitro incubation, and the effect of concentration and contact time (C.T) on efficacy of tested disinfectants against *E. tenella* oocysts.

Firstly, it was observed that the effect of different disinfectants on unsporulated oocycts was nearly convergent this may be due to absence of the double cell wall which facilitate the action of disinfectants, firstly Chlorocresol in high concentration was highly effective against unsporulated *E. tenella* oocysts at C.T of 30 and 120 minutes where the IA reached 99 % and 100 %, respectively. The obtained results revealed also the powerful destructive

effect of kilcox concentrations 4% against sporulated oocysts at 30 and 120 minutes reached 93 % and 99 %, respectively.

Concerning to Quaternary ammonium compound (QACs): its IA on *E. tenella* unsporulated oocysts at 4% solution recorded good efficacy 88.1% and 93.9% respectively. additionally, it was found that the efficacy was improved as the contact time increased. This result may be due to QACs were lipophilic compounds so; it may work on the oocyst wall so increasing concentration and/or CT leads to increasing of IA that agreed with (Bessems, 1998). Also, National Seafood HACCP Alliance, (2000) mentioned that QACs required a relatively long contact time to achieve significant kill.

In contrast, the disinfection efficacy (DE) of QACs on sporulated oocysts is much lower than other disinfectants.

Data tabulated in table (1) also showed that the effect of high concentration 4% solution of Glutaraldehyde against the unsporulated oocysts having good efficacy against the unsporulated oocysts. regarding its effect on sporulated oocysts, it was observed that the efficacy was increased by increasing the contact time, as the best result was obtained at 120 minutes contact time.

The usage of 20% solution of chlorocresol against both unsporulated and sporulated oocysts assessed good efficacy and powerful effect of chlorocresol. Similar results were obtained by (Straberg and Daugschies, 2007) and (You and Korean, 2014) who observed that cresol effectively inhibited sporulation up to 85.5%. While the obtained results were higher than that obtained by Oliveira, *et al.* (2004) who treated the oocysts of *E. tenella, E. acervulina and E. maxima* species with phenol 10.5% + Chlorocresol 10.5% at exposure time of 30 minutes and recorded only 7.7% IA. Daugschies *et al.* (2007) showed completely destroyed oocysts after a contact time of 90 min or more with the cresol-based products.

The present study cleared presence of differences between effect of the most tested disinfectants against unsporulated and sporulated oocysts. Williams (1997) indicated that unsporulated oocysts are more susceptible to disinfectants than sporulated one.

According to Daugschies *et al.* (2002) an inhibitory activity (IA) of at least 95% was required for certification of sufficient disinfecting efficacy by the German Veterinary Society. For which, Chlorocresol is considered the best chemical disinfectant against unsporulated and sporulated *E. tenella* oocysts in vitro in the present study.

CONCLUSION

From this study, it can be concluded that, a) disinfectant efficiency was conditioned by the disinfectant concentration and its exposure time to the oocysts. b) the most effective disinfectants against unsporulated and sporulated *E. tenella* oocysts was kilcox followed by chlorocresol, while QACS and, glutaraldehyde was less effective. c) in practical application of any disinfectant we should care to other environmental factors that can interact with the effect of disinfectants.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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كفاءة أربع مطهرات ضد معزولات الايميريا تينيلا من الدجاج المصري (تقييم معملي)

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يعتبر طفيل الكوكسيديا من أخطر الطفيليات التي تصيب الدواجن وكذلك أنواع الطيور الأخرى مسببة الكثير من الخسائر الاقتصادية في صناعة الدواجن. وتعتمد مقاومة الكوكسيديا على عدة طرق من أهمها الرعاية الجيدة داخل العنابر والاهتمام بالفرشة واستخدام التحصينات والأدوية المناسبة بالإضافة الى استخدام المطهرات المختلفة داخل عنابر الدواجن من اجل السيطرة على الطفيل.

الهدف من هذه الدراسة هو التقييم المعملي لكفاءة أربعة مطهرات وهي مركبات رباعي الامونيوم والجلوترالدهيد والكلوروكريسول والكيل كوكس ضد بويضات طفيل الكوكسيديا (الايميريا تينيلا) المعزولة من دجاج بلدي بمحافظة أسيوط.

تم تقييم كفاءة المطهرات باستخدامها ضد بويضات الايميريا الغير متحوصلة وكذلك ضد البويضات المتحوصلة وذلك بتعريض البويضات للمطهرات الاربعه كلا على حدى عند تركيزات مختلفة وكذلك عند زمن تلامس مختلف وتقييم التاثير التدميري للمطهر على البويضات وكذلك تقييم قدرة المطهر على منع التحوصل.

وكانت النتائج كلاتي أفضل النتائج حصلنا عليها عند استخدام الكيل كوكس ثم الكلوروكريسول يتبعه مركبات رباعي الامونيوم وأخيرا الجلوترالدهيد. ويتاكد من الدراسة ان زيادة التركيز وزمن التعرض تزيد من كفاءة المطهرات وتأثيرها على بويضات الطفيل.