Alexandria Journal of Veterinary Sciences 2014, 42: 28-35
ISSN 1110-2047, www.alexjvs.com
DOI: 10.5455/ajvs.154693

Sporicidal Activity of some Disinfectants Against *Clostridium Perfringens* Isolated from Broiler Poultry Litter

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Key words

Sporicidal Activity,

Disinfectants

Clostridium

Poultry Litter

ABSTRACT:

Necrotic enteritis and the subclinical form of Clostridium perfringens infection in poultry are caused by C. perfringens type A, producing the alpha toxin, and to a lesser extent type C, producing both alpha toxin and beta toxin *C* and have become serious threats to poultry health.This study was undertaken to determine the activity of 4 commonly available disinfectants against C. perfringens spores after 5,7.5,10,15,30 and 60 minutes of contact under dirty conditions, Of the 4 products tested, calcium hypochlorite 1% and Germicidan KOK 4% achieved the required reduction in microbial viability ($\geq 10^3$-fold) for relatively long contact times of 30 min, under dirty (3% yeast) conditions, One product (Biosentury 904, 2% conc) achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 10 minutes, while Prophyl 75,1% concentration. achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 5minutes.Upon addition of formic acid 2% and urea 1% the required reduction improved for (Biosentury 904, 2% conc) and Prophyl 75,1% concentration and achieved after 5 minutes and after 7.5 minutes for Germicidan KOK 4% while calcium hypochlorite was not improved by addition of formic acid 2% or urea 1%. Application of surface test using the four disinfectants was used alone and in combination with formic acid 2% or urea 1% showed nearly the same results obtained in the suspension test

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1. INTRODUCTION

Enteric disorders are one of the most important groups of diseases that affect poultry industry and are continuing to cause high economic losses in Egypt and almost all countries due to increased mortality rates, decreased weight gain, increased medication costs, and decreased feed conversion rates.

Several pathogens (viruses, bacteria, fungi and parasites) are incriminated as possible causes of enteric disorders (Hafez 2011).

In broiler flocks with high levels of *Clostridium perfringens* performance reduction can range from 25-43%, these losses were attributed to lowered feed conversion, caused by damage to the intestine, which result in reduced weight at slaughter (Lovland and Kaldhusal 2001). Infection with *C. perfringens* type A can cause gangrenous dermatitis, a disease diagnosed by necrotic lesions of the skin and subcutaneous tissues. Gangrenous dermatitis is commonly associated with older broilers and can cause sudden mortality; this can lead to severe profit losses depending on the severity of the outbreak (Opengart 2008). Alexander *et al*. (1968) isolated *Clostridium spp*. from 60% of litter samples, and found *C. perfringens* in 8 out of 44 litter samples. Lu *et al*. (2003) found that 7.78% of potentially pathogenic bacteria in litter were some type of *Clostridium spp*. Moreover, the diminished use of growth promoting antibiotics makes *C. perfringens*-induced necrotic enteritis and subclinical infections important threats to poultry health (Gholamiandekhordi et al. 2006). *Clostridial infections* in humans account for nearly 965,000 illnesses a year. All of them have been found to be food-borne in origin (Scallan *et al*. 2011).These bacteria are characterized as being Gram positive rods, motile by peritrichous flagella, having a strict anaerobic metabolism and able to produce numerous toxins (John. et al. 1994). The bacterial spore is a complex entity which is resistant to inactivation by many chemical and physical agents (Bloomfield 1998). Several suspension-type tests can be used for evaluating sporicidal activity but the advent of the European Test should provide a consistent approach as to whether a test chemical

possesses sporicidal activity. Improvements are still needed in producing suitable practical and in-use tests of sporicidal activity (Russell1998). Disinfection with a solution of per formic acid has an intensive bactericidal and sporicidal effect. This method is simple and prompt in service, is free from danger, inexpensive and can be used everywhere (Szechy et al. 1977).

This study was undertaken to assess the efficacy of a range of sporicidal agents, to determine the activity of 4 commonly available disinfectants against *C. perfringens* spores after 5, 7.5, 10, 15, 30 and 60 min of contact under dirty conditions.

2. MATERIALS AND METHODS

The following disinfectants were included which were available as a concentrate:

1. Calcium hypochlorite (80%chlorine), it was used in a concentration of 1%.

2-Biosentury 904, it was consisted of: Didecyldimethylammonium Chloride 9.2% ,dimethyl benzyl ammoniumchloride 9.2%, Alkyl (C12-40%, C14-50%, C16-10) , dimethyl benzyl ammoniumchloride 4.6% bis(tributyltin) oxide 1% , It was used in a concentration of 2%.

3- Prophyl 75. It was consisted of: Chloro 4 Méthyl 3 Phénol: 100 g/l (CMP), 2 Benzyl 4 ChloroPhénol 50 g/l (Chlorophène), It was used in a concentration of 1%.

4- Germicidan KOK, it was consisted of: phenols and halogenous phenols 15 – 30 %, non-ionic surfactants 5 – 15 %, it was used in a concentration of 4%.

5- Formic acid (85%) used in 2% conc.

6-Urea crystal used in 1% conc.

Tested organism:

Field strains of *Clostridium perfringens* were isolated from litter samples which were taken and inoculated directly into cooked meat broth medium (Oxoid) and were incubated anaerobically in anaerobic Gas pack jar for 24 h at 37 ºC.

A loopful of growth was then streaked onto 5% sheep blood agar supplemented with neomycin sulphate. The plates were incubated anaerobically for 24 h at 37 ºC. The suspected isolates were identified by biochemical tests according to Effat et al. (2007).

Several confirmed *C. perfringens* isolates were pooled and inoculated into Duncan-Strong sporulation medium (Duncan and Strong 1967.) and incubated anaerobically overnight at 42ºC to produce spore stocks. Gram staining and light microscopy were performed on dilutions of these suspensions to determine the amount of spore aggregation as well as the proportion of vegetative bacteria versus spores.

Spore preparations used in disinfection experiments were free of visible aggregates and contained at least 90% spores.

For disinfection experiments, stock spore suspensions were added to a final concentration of 10^6- 10^7spores/ml in test disinfectant solutions. Control and disinfected samples were serially diluted for analyses using *C. perfringens*agar, Concentrations of *C.perfringens* in disinfected and control samples were expressed as CFU per ml.

Sterile hard water

0.304 (g) anhydrous calcium chloride and 0.065 g anhydrous magnesium chloride were dissolved in glass-distilled water, and made up to one liter. The final concentration is 2.7 mM CaCl2, 0.7 mM MgCl2.Were dispensed into glass containers and was sterilized by autoclaving at 121 ± 1º C for 15 minutes.

Killed yeast suspension

200 g of moist compressed baker's yeast were Weighed, Creamed by the gradual addition of sterile hard water using a heavy glass rod for stirring.

the creamed portion was decanted into a flask, more water was added to any lumpy residue remaining and the creaming and decantation were repeated until no residue remains and 500 (mL) of water has been used.

The contents of the flask were shaken vigorously and were strained through a 100-mesh sieve; any remaining lumps were braked down, 500 mL sterile hard water was added shake vigorously and the pH was adjusted to 6.9–7.1 with 1N sodium hydroxide. 50 mL, 100 mL or 200 mL of the yeast solution were transferred into screw-capped bottles. Autoclaved at 121 ± 1 ºC for 15 minutes and the autoclave was allowed to cool without releasing pressure. Stored at 4–8 ºC. Two Petri dishes were dried to constant weight. Into each, 25 mL of sterilized yeast suspension was added, and was dried to constant weight at 100 ºC, the average solids content of the suspension was calculated. Before use, 25 mL of the sterilized yeast suspension was added into a beaker. The pH was determined using the glass electrode, and the volume of 1N sodium hydroxide solution needed to adjust the pH to within the range 6.9 to 7.1 was determined.

Immediately before use, a volume of sterile hard water was added to sterilized yeast, to adjust the concentration of dry yeast to 5.0%, and a pre-determined volume of 1N NaOH to adjust the pH to within the range 6.9–7.1.

Disinfectant dilutions

A sample of the disinfectant was diluted to the specified extent, using sterile hard water as diluents. Not less than 10 mL or 10 g of sample for the first dilution was used and not less than 1 mL of any dilution to prepare subsequent dilutions. All dilutions in glass containers on the day of testing were made. The glass containers must be twice rinsed in glass-distilled water, and sterilized.

Temperature

The containers were hold at21 ± 1ºC in which the test is to be carried out

Testing for sporicidal activity:

A dilution–neutralization method was used according to preliminary European Standard prEN EN 13704, 2002. Disinfection testing was performed by thoroughly mixing 0.2 mL of the spore suspension with 0.8 mL of disinfecting agent previously prepared with hard water.

Samples (0.1 mL) were taken from the reaction mixture after 5, 7.5, 10, 15, 30, 60 minutes in 0.8 mL of neutralizer (For calcium hypochlorite, the neutralizerwas 1.0% (wt/vol) sodium thiosulfate in normal saline). For phenol, cresol and QAC (0.1% final concentration) of Tween-80 and 0.1 mL of water to prevent further inactivation, and left for 5 minutes. Samples of the final mixture and 10- fold dilutions then seeded on blood agar and incubated anaerobically at 35°C for 48 hours. Colonies from germinated spores were counted and expressed as colony-forming units (CFU)/mL. The test was performed at high-level soiling conditions (5 % yeast). To describe the sporicidal effect of the disinfecting agents, the results were given as the 3 log10 CFU reduction of the viable count from the initial inoculums. According to the Standard, $\geq 10^3$ reduction in spore viability was required for a product to be considered effective.

Surface disinfection tests

According to European Committee for standardization (2002): European standard **EN13697: (**EN 13697 Quantitative Surface Test of Bactericidal Activity)

A film of bacterial cells about 50 ul inoculums was placed onto the surface of a steilr Microscopic glass slide and after 1 hour drying at 37 C, the inoculums was covered by 100ul of the disinfectant for a specified contact time. After defined exposure time, the bactericidal and/or bacteriostatic activity was immediately suppressed by submerging the carrier in a container with neutralizer fluid. The number of surviving bacteria in each sample was determined by standard culture plate and incubation. The reduction in viable counts was calculated.

3. RESULTS AND DISCUSSION

The results of assessment of the efficacy of the different products are shown in Tables I,2,3,4 and figures 1, 2, 3, 4 of the 4 products tested, calcium hypochlorite 1% and Germicidan KOK 4% achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 30 minutes, under dirty (5% yeast) conditions,

One product (Biosentury 904, 2% concentration) achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 10 minutes, while Prophyl 75,1% concentration achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 7.5minutes.

Upon addition of formic acid 2% and urea 1% the required reduction improved for (Biosentury 904, 2% conc) and Prophyl 75,1% concentration and achieved after 5 minutes and after 7.5 minutes for Germicidan KOK 4% while calcium hypochlorite not improved by addition of formic acid 2% or urea 1%. application of surface test using the four disinfectants used alone and in combination with formic acid 2% or urea 1% showed nearly the same results obtained in the suspension test .

Table 5 showed that, 2% formic acid and 1% urea alone not achieved the required reduction even after long time exposure time 60 minutes and not considered sporicidal agent

Clostridium perfringens associated necrotic enteritis in poultry causes significant loss and increased morbidity in the industry. In addition, *C.perfringens* type A has been showed to cause food poisoning in humans (Løvland and Kaldhusdal 2001, McClane *et al*. 2006; Novoa-Garrido *et al*. 2006). Spores *of Cl. perfringens A*, are one of the most resistant pathogenic clostridial species tested (Stockinger et al. 1989)

Based on data generated using the sporicidal suspension test, of the 4 products tested, tables (1-5) and figures (1-5) showed that ,calcium hypochlorite 1% and Germicidan KOK 4% achieved the required reduction in microbial viability ($\geq 10^3$-fold) for relatively long contact times of 30 min, under dirty (5% yeast) conditions.

Table 1: Log10 reduction of spores of *Clostridium perfringens* due to addition of 1% Ca.hypochlorite and with addition of 2% formic acid and 1% urea

Time	**Ca.hypochlorite 1%**		**+ 2% formic acid**		**+1%urea**	
	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction	Spores count log	Spores count Log reduction
zero	6.94		6.94		6.94	
5m	5.2	1.74	5.52	1.42	5.97	0.97
7.5m	4.72	2.22	4.85	2.09	5.68	1.26
10m	4.7	2.24	4.78	2.16	5.28	1.66
15m	3.11	2.49	4.45	2.49	5.04	1.9
30m	3.83	3.11	4.38	2.56	4.76	2.18
60m	3.7	3.24	3	3.94	3.58	3.36
Surface test after 60m	3.7	3.24	3.52	3.42	3.82	3.66

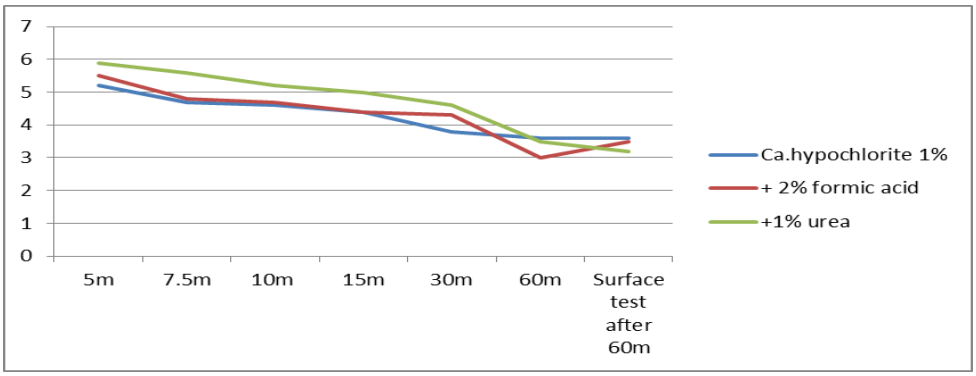

Fig. 1 : Log10 reduction of spores of *Clostridium perfringens* due to addition of 1% Ca.hypochlorite and with addition of formic acid 2% and urea 1%

Table 2: Log10 reduction of spores of *Clostridium perfringens* due to addition of 2% Biosentury and with addition of 2% formic acid and 1% urea

Time	**Biosentury2%**		**+ 2% formic acid**		**+1% urea**	
	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction
Zero	6.94		6.94		6.94	
5m	4.6	2.34	3.7	3.24	3.9	3.04
7.5m	4.6	2.34	3.6	3.34	3.48	3.46
10m	3.48	3.46	3.6	3.34	3.3	3.64
15m	3.48	3.46	3.34	3.6	3.18	3.76
30m	3.08	3.86	3.32	3.62	3.18	3.76
60m	3.0	3.94	3.3	3.64	3.08	3.86
Surface test after 60m	3.0	3.94	3.38	3.56	3.08	3.86

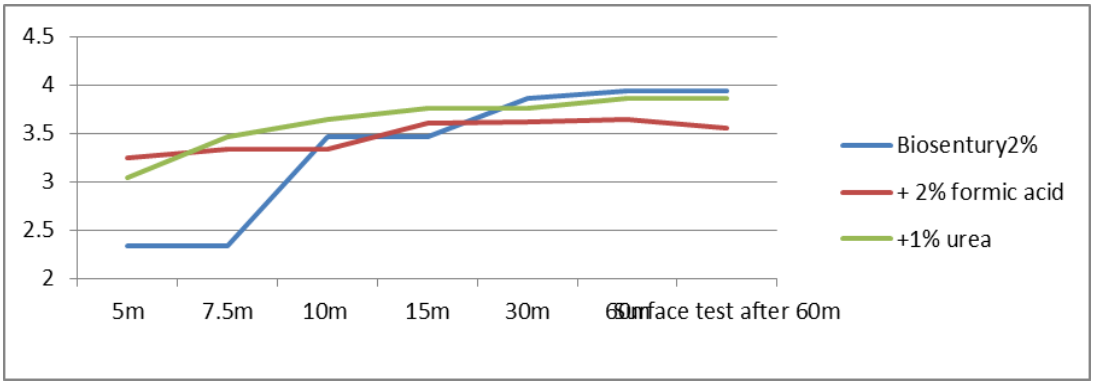

Fig. 2: Log10 reduction of spores of *Clostridium perfringens* due to addition of 2% Biosentury and with addition of 2% formic acid and 1% urea

Table 3: Log10 reduction of spores of *Clostridium perfringens* due to addition of 1% Prophyl and with addition of 2% formic acid and 1% urea

Time	**Prophyl 1%**		**+ 2% formic acid**		**+1% urea**	
	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction
Zero	6.94		6.94		6.94	
5m	3.9	3.04	3.78	3.16	3.3	3.64
7.5m	3.7	3.24	3.6	3.34	2.48	4.46
10m	3.7	3.24	3.6	3.34	2.48	4.46
15m	3.3	3.64	3.48	3.46	2.36	4.58
30m	3.3	3.64	3.3	3.64	2.23	4.71
60m	3.3	3.64	3	3.94	2.23	4.71
Surface test after 60m	3.3	3.64	3.2	3.74	2,9	4.04

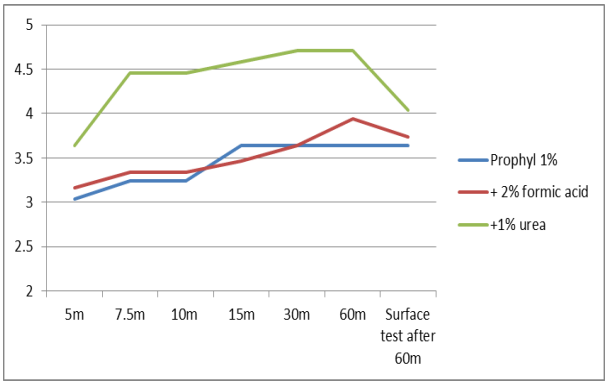

Fig. 3. Log10 reduction of spores of *Clostridium perfringens* due to addition of 1% Prophyl and with addition of 2% formic acid and 1% urea

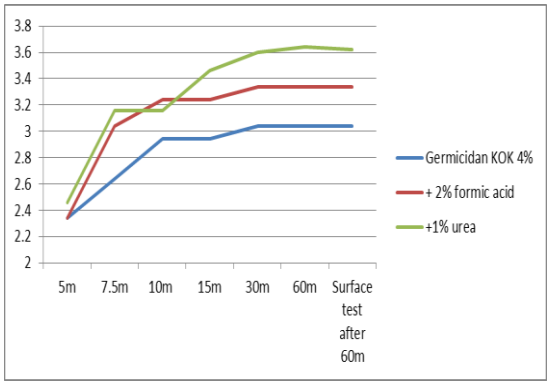

Fig. 4. Log10 reduction of spores of *Clostridium perfringens* due to addition of 4% Germicidan KOK and with addition of 2% formic acid and 1% urea

Table 4. Log10 reduction of spores of *Clostridium perfringens* due to addition of 4% Germicidan KOK and with addition of 2% formic acid and 1% urea

Time	Germicidan KOK 4%		+ 2% formic acid		+1% urea	
	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction	Spores count log	Spores count log reduction
Zero	6.94		6.94		6.94	
5m	4.6	2.34	4.6	2.34	4.3	2.46
7.5m	4.3	2.64	3.9	3.04	3.78	3.16
10m	4	2.94	3.7	3.24	3.78	3.16
15m	4	2.94	3.7	3.24	3.48	3.46
30m	3.9	3.04	3.6	3.34	3.34	3.6
60m	3.9	3.04	3.6	3.34	3.3	3.64
Surface test after 60m	3.9	3.04	3.6	3.34	3.32	3.62

Table 5: Log10 reduction of spores of *Clostridium perfringens* using 2% formic acid and 1% urea

Time	2% formic acid		+1% urea	
	log	log reduction	log	log reduction
zero	6.94		6.94	
5m	5.85	1.09	5.3	1.64
7.5m	5.85	1.09	5.3	1.64
10m	5.7	1.24	5.18	1.76
15m	5.2	1.74	5.18	1.76
30m	5	1.94	4.94	2.o
60 m	4.6	2.34	4.43	2.51
surface test after 60 m	4.6	2.34	4.43	2.51

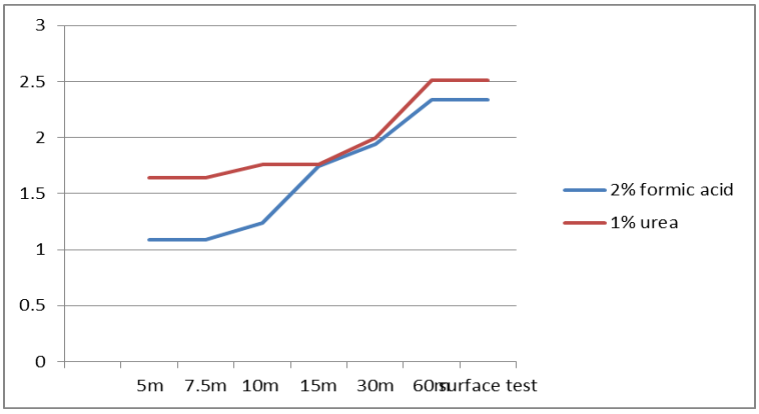

Fig. 5 : **Log10 reduction of spores of *Clostridium perfringens* using 2% formic acid and 1% urea**

One product (Biosentury 904, 2% conc) achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 10 min, while Prophyl 75,1% conc. achieved the required reduction in microbial viability ($\geq 10^3$-fold) for contact times of 5min. The result agreed with DYE and MEAD 1972 who found that *Clostridium welchii* was the most resistant to chlorine , the QAC-based and lower concentration of bleach-based products did not reveal sporicidal activity (Smith et al. 2008)

These data can be classified as valid for two reasons. First, The sporicidal activity is dependent on active type, concentration and exposure times. (Kyle et al. (2008); Douglas and Kamp (2011). The proportion of spores within the total cell population was determined prior to testing and was found to be at least 90%. This is a relevant detail because lack of efficient spore preparation may result in favorable efficacy data which represent partly the effect against the vegetative cells (Wullt et al. 2003).

Upon addition of formic acid 2% and urea 1% the required reduction improved for (Biosentury 904, 2% conc) and Prophyl 75,1% conc and achieved after 5 min and after 7.5 min for Germicidan KOK 4% while calcium hypochlorite was not improved by addition of formic acid 2% or urea 1%. Disinfection with a solution of performic acid has an intensive bactericidal and sporocidal effect. This method is simple and prompt in service, free from danger, inexpensive and can be used everywhere (Szechy et al. 1977)

As shown in tables (1-5) and Figures (1-5) application of surface test using the four disinfectants used alone and in combination with formic acid 2% or urea 1% showed nearly the same results obtained in the suspension test .

Surface disinfectants test determine the antimicrobial activity of commercial formulations or active substances on bacteria in the conditions in which they are used. The practical tests under real-life conditions are performed after measuring the time-concentration relationship of the disinfectant in a quantitative suspension test. The objective is to verify whether the proposed use dilution is still adequate in the conditions under which it would be used (Joan et al. 1991).

Table 5showed that the effect of formic acid 2% and urea 1% alone not achieved the required reduction even after long time exposure time 60 min and not considered sporicidal agent. Vinnerås (2002),found that, the spore-forming bacteria *Clostridium spp* in its dormant state was resistant to urea treatment. As the urea has to be degraded to ammonia before it functions as a disinfectant, as additional effects, urea increases the fertilizer value of the treated material and there is no risk of microbial regret (Vinneras et al. 2003).

The results of our study concluded that, some disinfectants when used alone were not achieved the required log reduction and were not considered sporicidal but upon addition of formic acid 2% or urea 1% , the sporicidal effect was improved, on the other hands some other disinfectants were not affected.

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