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Original Article

Antifungal activity of germicide combinations against arthroconidia of *Microsporum gallinae*

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Abstract

Microsporum gallinae is the fungus responsible for avian dermatophytosis. Arthroconidia are the infective spores that transmit the disease in endemic areas through direct contact. This study investigated germicide combinations for enhanced antifungal activity against *M. gallinae* arthroconidia. The checkerboard method revealed that benzalkonium chloride with chlorhexidine, benzalkonium chloride with glutaraldehyde, chlorhexidine with glutaraldehyde, and formaldehyde with glutaraldehyde combinations showed partial synergistic effects against *M. gallinae* arthroconidia with fractional inhibitory concentration indices (FICI) in the range of 0.625-0.750. In contrast, the benzalkonium chloride with formaldehyde and the chlorhexidine with formaldehyde combinations showed an indifferent effect. Time-kill assays of the synergistic combinations showed that concentrations from 50 to 500 × MIC eradicated fungal arthroconidia within 1 min. Environmental scanning electron microscopy demonstrated destruction of the cell membrane and cell wall of arthroconidia. The results of this study show the enhanced potential of germicide combinations for controlling avian dermatophytosis caused by *M. gallinae*.

Keywords: arthroconidia, avian dermatophytosis, germicide combinations, *Microsporum gallinae*

1. Introduction

Avian dermatophytosis is a fungal skin disease in poultry caused by dermatophytes. In particular, *Microsporum gallinae* is a zoophilic fungus that can cause acute skin inflammation in humans who come into contact with infected animals. This condition is prevalent in gallinaceous birds, ducks, and pigeons. It can also affect mammals such as dogs, cats, monkeys, mice, cattle, and squirrels (Ahmadi *et al*., 2016; Dahlhausen, 2006; de Hoog *et al*., 2017). Arthroconidia, or arthrospores, are produced when fungal hyphae fragment under stress conditions. They are the infective stage of dermatophytes and play a crucial role in the transmission of the disease. Arthrospores can be spread through direct contact with infected animals or contaminated objects, such as cages, bedding, brushes, and litter (Baumgardner, 2017; Nenoff, Kruger, Ginter-Hanselmayer, & Tietz, 2014). They can also lead to recurrent infections in humans (Hammer, Mucha, & Hoefer, 2011; Mancianti, Nardoni, Corazza, D'Achille, & Ponticelli, 2003).

Germicides are substances that can kill pathogenic microorganisms. They are divided into two categories: disinfectants and antiseptics. Disinfectants are used to kill microorganisms on non-living objects, while antiseptics are used to kill microorganisms on living tissue (Weber, Rutala, & Sickbert-Bennett, 2019). Some germicides have both disinfectant and antiseptic properties. The effectiveness of germicides can be influenced by a number of factors, including the type of microorganism, the concentration of the germicide, the contact time, and environmental conditions (Rutala, Weber, & Healthcare Infection Control Practices Advisory Committee, 2019). Our previous study found that benzalkonium chloride, chlorhexidine, formaldehyde, and

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glutaraldehyde are effective germicides against *M. gallinae* when used at 100- to 1,000-fold the minimum inhibitory concentration (MIC) for 5-10 minutes (Thongkham, Junnu, Borlace, Uopasai, & Aiemsaard, 2022). However, high concentrations of disinfectants can be harmful to users, animals, and the environment (Amy *et al*., 2000; Olson, 2017).

Recently, there has been a growing interest in the concept of germicide combinations. This approach can enhance the antimicrobial activity of agents while also reducing the amount used and preventing the development of side effects and microbial resistance (Boyce, 2016; Robertson, Barrell, & Maillard, 2019). The present study investigates the antifungal efficacy of some germicide combinations against arthroconidia of *M. gallinae*.

2. Materials and Methods

2.1 Fungal culture

The fungus *M. gallinae* ATCC 90749 was obtained from the American Type Culture Collection (ATCC), ATCC-Corporate Office, University Boulevard Manassas, Virginia. Arthrospore production was induced by subculturing *M. gallinae* on Sabouraud dextrose agar (SDA; Becton Dickinson, France) at 37ºC with 5% CO_2 and 80% relative humidity (Esco CelCulture®, Esco Micro Pte. Ltd., Singapore) for 14 days. Phosphate-buffered saline (PBS) pH 7.2 was added to the inoculated plates. The fungal fragments were then collected using a triangle-shaped glass rod spreader. The arthroconidia were separated from the hyphae by double filtering through 10 layers of folded sterile gauze. The presence of arthroconidia was confirmed by observation via a light microscope (Olympus Optical Co., Ltd., Japan) at 400-1000 × magnification (arthroconidia appeared ≤ 5 µm in length with conspicuous detachment scars at both ends). Aerobic plate counts were used to verify the concentration of the fungal suspensions utilized in the susceptibility tests (Thongkham *et al*., 2022).

2.2 Antifungal effect of the combination of germicides

In this study, we selected the germicides that showed effectiveness against *M. gallinae* based on our previous study (Thongkham *et al*., 2022), namely benzalkonium chloride (Sigma-Aldrich, Germany), chlorhexidine (Sigma-Aldrich, Germany), formaldehyde (Loba Chemie Pvt. Ltd., India), and glutaraldehyde (Loba Chemie Pvt. Ltd., India). These germicides were tested in binary combinations against *M. gallinae* arthroconidia using the checkerboard method according to a previous study by Aiemsaard, Kamollerd, Seubsasana, Thongkham, and Vonghataipaisarn (2021) and the Clinical and Laboratory Standard Institute (CLSI, 2008) guidelines with some modifications. Briefly, 50 µl of germicide A was serially diluted two-fold with Roswell Park Memorial Institute (RPMI)-1640 medium broth (Sigma-Aldrich, Germany) across the columns in a 96-well round-bottomed microtiter plate (Corning Incorporated, USA). Fifty microliters of each concentration of two-fold diluted germicide B were then added to each row. One hundred microliters of arthroconidia suspension ($1x10^4$ CFU/ml) were added to all tested wells. Wells containing only RPMI-1640 medium and RPMI-1640 with fungal suspension were used as negative and positive growth control wells, respectively. After 96 h at 30ºC, the minimum inhibitory concentrations (MIC) of the germicides A and B alone and in combination were determined. The fractional inhibitory concentration index (FICI) was calculated using the following formula:

$$FICI = (MIC_A \text{ in combination}/MIC_A \text{ alone}) + (MIC_B \text{ in combination}/MIC_B \text{ alone})$$

FICI values of no more than 0.5 indicate a synergistic effect, values greater than 0.5 and less than 1 indicate a partial synergistic effect, values of 1.0 indicate an additive effect, values greater than 1 and less than 4 indicate an indifferent effect, and values of 4.0 or greater indicate an antagonistic effect.

2.3 Time-kill assay

The germicide combinations showing synergism or partial synergism were selected to study the time-kill kinetics. One hundred microliters of diluted arthroconidial suspension (5×10^6 CFU/ml) were homogeneously mixed with 900 µl of each germicide combination to give final concentrations of 1, 5, 10, 50, 100, 500, and 1,000 × their respective combination MIC. After incubation at 30ºC for 1, 2, 3, 4, 5, 10, 15, and 20 min, the mixture was immediately ten-fold diluted with a neutralizing solution (0.6% w/v sodium thiosulfate, 0.5% w/v polysorbate 80, and 0.07% w/v lecithin in PBS). One hundred microliters of 10^{-1} to 10^{-4} dilutions were inoculated onto SDA and incubated at 30ºC for 96 h. The recovered fungal colonies were recorded. The fungal suspension with the neutralizing solution was used as a control (Aiemsaard, Kamollerd, Butudom, Worawong, & Thongkham, 2020; Association of Official Analytical Chemists, 1990).

2.4 Scanning electron microscopy

The synergistic or partially synergistic germicide combinations were studied for their morphological effects on arthroconidia using an environmental scanning electron microscope (Thermo Scientific™ Quattro-S E-SEM, Thermo Fisher Scientific Inc., USA). After being treated with the germicide combinations for 10 min at the minimum concentration that caused a 5-$\log_{10}$ reduction, the arthroconidia were washed 3 times with sterile distilled water and centrifuged at 3,000 × g for 5 min (Chen *et al*., 2020). The arthroconidia were then transferred to carbon conductive tabs and allowed to air dry. The samples were observed under the E-SEM at a magnification of 10,000 × in a high vacuum environment with 5-15 kV.

2.5 Leakage study

Ten milliliters of arthroconidia suspension ($1x10^8$ CFU/ml) were centrifuged at 3,500 × g for 10 minutes. The sediment was washed 3 times with sterile distilled water and added into each germicide combination solution for 1, 2, 5, 10, and 20 min at the concentration that caused a 5-$\log_{10}$ reduction. One minute before each time point, the mixtures

were centrifuged at 5,000 × g for 1 min (centrifuged immediately for the test time of 1 min). The UV absorbance of the supernatant was then measured at 260 nm to determine the levels of nucleic acids and proteins as indicators of membrane integrity using an Epoch™ 2 microplate spectrophotometer, BioTek Instruments, Inc., USA. The germicide solution without arthroconidia was used as a blank (Li, Cai, Liu, Sun, & Luo, 2019).

3. Results and Discussion

3.1 Antifungal efficacy of germicide combinations

The checkerboard method demonstrated that four of the tested germicide combinations showed a partial synergistic effect (0.5 < FICI < 1.0) and the other two showed an indifferent effect (1.0 < FICI < 4.0) against *M. gallinae* ATCC 90749 arthroconidia (Table 1). The partially synergistic combinations were benzalkonium chloride with chlorhexidine (FICI 0.75), benzalkonium chloride with glutaraldehyde (FICI 0.625), chlorhexidine with glutaraldehyde (FICI 0.75), and formaldehyde with glutaraldehyde (FICI 0.625). In combination, benzalkonium chloride and chlorhexidine showed four-fold and two-fold reductions in their relative FICs compared to their MICs, as did chlorhexidine and glutaraldehyde, leading to FICI of 0.75. The combination of benzalkonium chloride and glutaraldehyde showed eight-fold and two-fold decreases in their relative FIC and MIC values, which was the same for formaldehyde and glutaraldehyde, leading to FICI of 0.625. The indifferent combinations showed very different effects among the individual components. In the benzalkonium chloride and formaldehyde combination, the benzalkonium MIC in combination was the same as the MIC alone while the formaldehyde MIC in combination was reduced four-fold compared to the MIC alone (FICI 1.25). For the chlorhexidine and formaldehyde combination, the chlorhexidine MIC in combination was reduced four-fold compared to the MIC alone, but the formaldehyde MIC in combination was twice the MIC alone resulting in the FICI of 2.25 (Table 1).

3.2 Time-kill kinetics

The time-kill kinetics of the four partially synergistic germicide combinations are shown in Figure 1. All tested combinations were able to elicit 5-$\log_{10}$ reductions in the number of viable arthroconidia at various concentrations and times. The formaldehyde with glutaraldehyde combination (Figure 1A) eradicated the arthroconidia after 1 minute at 50 × MIC and after 15 minutes at 10 × MIC. The chlorhexidine with glutaraldehyde combination (Figure 1B) showed a 5-$\log_{10}$ reduction in the number of arthroconidia after 1 minute at 50 × MIC and after 2 minutes at 10 × MIC. The benzalkonium chloride with glutaraldehyde combination (Figure 1C) was effective after 1 minute at 50 × MIC and after 10 minutes at 10 × MIC. Finally, the benzalkonium chloride and chlorhexidine combination eliminated viable arthroconidia after 1 minute at 500 × MIC, after 10 minutes at 100 × MIC and after 20 minutes at 50 × MIC (Figure 1D).

3.3 Morphological effects of germicide combinations

After treatment with the germicide combinations, the arthroconidia exhibited distinctive morphological changes characterized by the destruction of surface integrity (Figure 2A-2D). Untreated arthroconidia showed a regular rod-shaped morphology and intact surface features (Figure 2E). Formaldehyde combined with glutaraldehyde (50 × MIC in combination) and chlorhexidine combined with glutaraldehyde (100 × MIC in combination) both caused cell membranes to shrivel and detach from cell walls (Figure 2A and 2B, white arrows). Arthroconidia treated with the benzalkonium chloride and glutaraldehyde (50 × MIC in combination) and benzalkonium chloride and chlorhexidine (500 × MIC in combination) combinations showed more severe morphological changes. Both the cell membrane and cell wall had irregular surfaces (Figure 2C and 2D, red arrows).

3.4 Effect of germicide combinations on cell membrane integrity

Figure 3 shows the absorbance$_{260\ nm}$ values of arthroconidial suspensions after treatment with the germicide combinations. The absorbance indicates the relative amount of nucleic acids and proteins present in the media resulting from cell membrane damage to *M. gallinae* arthroconidia. At contact times of 5 and 10 min, formaldehyde combined with

Table 1. Synergistic effect of combinations of the most effective disinfectants against *M. gallinae* arthroconidia

No.	Germicide	MIC (µg/ml)		FIC	FICI [a]	Outcome
		Alone	Combination			
1	Benzalkonium chloride	1.563	0.391	0.250	0.750	P
	Chlorhexidine	0.195	0.098	0.500		
2	Benzalkonium chloride	1.563	1.563	1.000	1.250	I
	Formaldehyde	6.250	1.563	0.250		
3	Benzalkonium chloride	1.563	0.195	0.125	0.625	P
	Glutaraldehyde	25.000	12.500	0.500		
4	Chlorhexidine	0.195	0.049	0.250	2.250	I
	Formaldehyde	6.250	12.500	2.000		
5	Chlorhexidine	0.195	0.049	0.250	0.750	P
	Glutaraldehyde	25.000	12.500	0.500		
6	Formaldehyde	6.250	0.781	0.125	0.625	P
	Glutaraldehyde	25.000	12.500	0.500		

[a] Fractional inhibitory concentration index (FICI) was interpreted as synergy (S) at ≤ 0.5, partial synergy (P) at > 0.5 but <1.0, Additive effect (A) at 1.0, indifferent (I) at >1.0 but <4.0, and antagonistic (Ant) when values were ≥ 4.

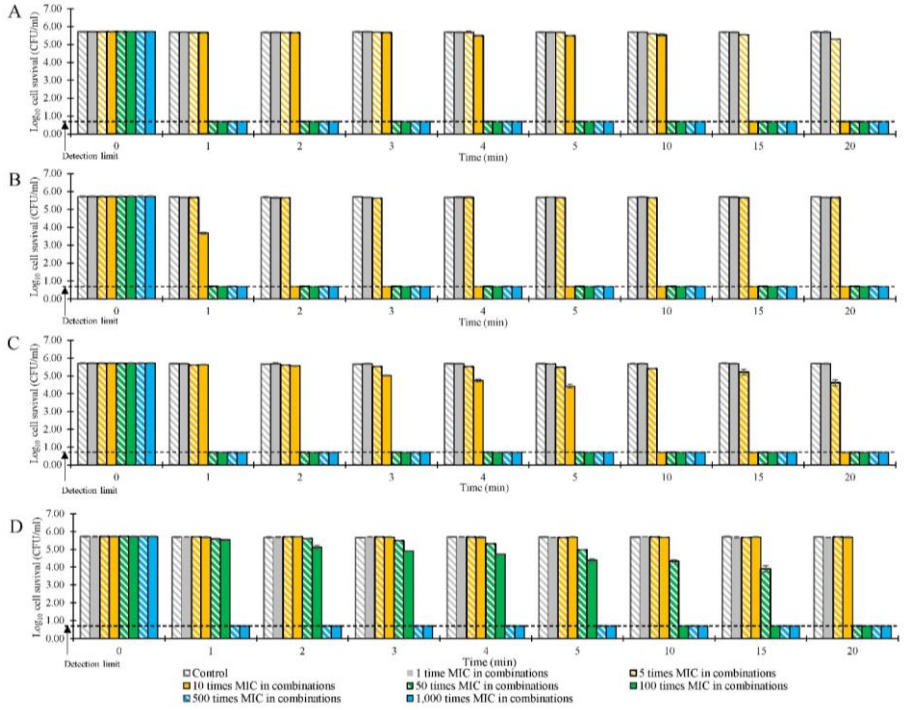

Figure 1. The effect of partial synergistic germicide combinations against *M. gallinae* ATCC 90749 arthroconidia (1 to 1,000 × MIC); formaldehyde and glutaraldehyde (1 × MIC in combination = 0.781 and 12.500 µg/ml, respectively) (A), chlorhexidine and glutaraldehyde (1 × MIC in combination = 0.049 µg/ml and 12.500, respectively) (B), benzalkonium chloride and glutaraldehyde (1 × MIC in combination = 0.195 and 12.500 µg/ml, respectively) (C), and benzalkonium chloride and chlorhexidine (1 × MIC in combination = 0.391 and 0.098 µg/ml, respectively) (D). Control = neutralizing solution (0.6% w/v sodium thiosulfate, 0.5% w/v polysorbate 80, and 0.07% w/v lecithin in PBS pH 7.4). The graph presents the means of triplicate experiments with error bars (SD).

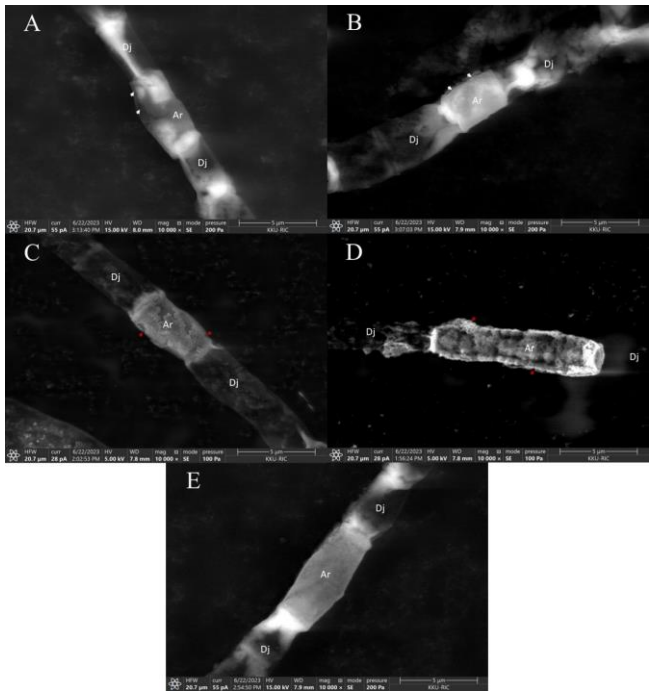

Figure 2. Morphological changes of arthroconidia under E-SEM at 10,000 × magnification after being treated with germicide for 10 min; formaldehyde (39.05 µg/ml) and glutaraldehyde (625 µg/ml) (A), chlorhexidine (4.9 µg/ml) and glutaraldehyde (1,250 µg/ml) (B), benzalkonium chloride (9.75 µg/ml) and glutaraldehyde (625 µg/ml) (C), benzalkonium chloride (195.5 µg/ml) and chlorhexidine (49 µg/ml) (D), and Control = distilled water (E). Ar = arthroconidium, Dj = disjunctor cell

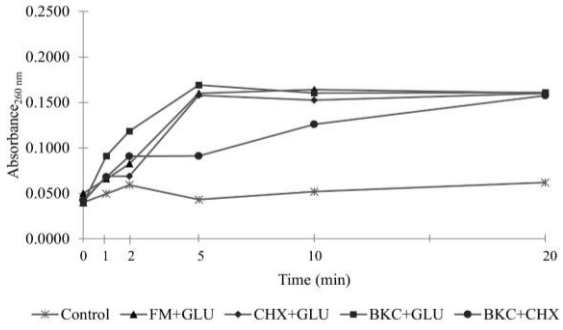

Figure 3. The effect of germicide combinations on the integrity of cell membranes of *M. gallinae* arthroconidia; FM+GLU = formaldehyde (39.05 µg/ml) and glutaraldehyde (625 µg/ml), CHX+GLU = chlorhexidine (4.9 µg/ml) and glutaraldehyde (1,250 µg/ml), BKC+GLU = benzalkonium chloride (9.75 µg/ml) and glutaraldehyde (625 µg/ml), BKC+CHX = benzalkonium chloride (195.5 µg/ml) and chlorhexidine (49 µg/ml), and Control = distilled water

4. Discussion

The antimicrobial mechanisms of germicides are broad and nonspecific, often meaning that these compounds show less synergistic activity and that their mechanisms of action may differ by microorganism (Noel, Keevil, & Wilks, 2021). However, the results of this study show that four out of six tested germicide combinations showed partial synergistic effects against *M. gallinae* arthroconidia. The use of combinations of disinfectants and antiseptics can reduce the required concentrations of individual compounds, depending on how their mechanisms combine and if there are any shared sites of action. Chlorhexidine and benzalkonium chloride are both cationic surfactants, but chlorhexidine is a synthetic biguanide while benzalkonium chloride is a quaternary ammonium compound. They both have the ability to damage cell walls and cell membranes of microorganisms by bonding with their negative ions as well as to form complexes with enzymes, proteins, and nucleic acids, causing abnormal cell function. However, chlorhexidine has high lipophilic properties that enable it to target both cell walls and membranes, while benzalkonium chloride is more hydrophilic, preferring to target cell membranes. Thus, when used with chlorhexidine, the penetration of benzalkonium chloride through the cell wall is enhanced while the anion-binding sites of these compounds appear to be dissimilar (Jiao *et al*., 2017; Kwaśniewska, Chen, & Wieczorek, 2020; Poppolo Deus & Ouanounou, 2022). A previous report by Hiller, Wenzl, Forster, Cieplik, and Maisch (2023) showed that a combination of chlorhexidine and benzalkonium chloride had a FICI against *Escherichia coli* in the range of 0.52-0.75, indicating partial synergy, and this is consistent with the findings in the current study. Similarly, Salvatico, Feuillolay, Jabbour, Gouhier-Kodas, and Roques (2018) found that chlorhexidine digluconate combined with benzalkonium chloride was more effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* than using either individual disinfectant.

Formaldehyde and glutaraldehyde are both aldehyde disinfectants, but formaldehyde is a monoaldehyde, with a single aldehyde functional group, whereas glutaraldehyde is a dialdehyde containing two aldehyde functional groups. Both formaldehyde and glutaraldehyde cross-link proteins as their antimicrobial mechanism of action. Formaldehyde has a smaller molecular size and can penetrate cells better. This allows it to interact with RNA and DNA inside of the cell although it has weaker cross-linking abilities. On the other hand, glutaraldehyde has slower penetration but forms stronger interactions with proteins and chitin, a vital structure of the fungal cell wall. It may be for this reason that the combination of these two compounds results in partial synergy (McDonnell & Russell, 1999). In addition, glutaraldehyde not only affects chitin, but also plays a role in enhancing the effectiveness of chlorhexidine and benzalkonium chloride, creating a partial synergistic effect.

The morphological characterization results under E-SEM confirmed that all tested germicide combinations caused noticeable changes to the cell surface of *M. gallinae* arthroconidia. This is consistent with the results of the leakage study, which found that increasing amounts of proteins and nucleic acids leaked out of arthroconidia treated with the germicide combinations over time. Formaldehyde with glutaraldehyde resulted in minimal structural changes to the arthroconidia because cell structures remain stable even when proteins are denatured, which is why these compounds are used for cell fixation (Singh *et al*., 2019). The combinations of germicides containing chlorhexidine or benzalkonium chloride showed much more serious changes to the surface structure of arthroconidia with the destruction of cell walls and cell membranes.

5. Conclusions

In conclusion, the benzalkonium chloride with chlorhexidine, benzalkonium chloride with glutaraldehyde, chlorhexidine with glutaraldehyde, and formaldehyde with glutaraldehyde combinations each showed a partial synergistic effect against *M. gallinae* arthroconidia reducing the minimum inhibitory concentration of each agent in the combinations by between 2 and 8 fold. At a concentration of 50 × MIC, the benzalkonium chloride with glutaraldehyde, chlorhexidine with glutaraldehyde, and formaldehyde with glutaraldehyde combinations eliminated arthroconidia by 99.999% within 1 minute, and the benzalkonium chloride with chlorhexidine combination eliminated arthroconidia within 1 minute at 500 × MIC. Scanning electron microscopy and leakage studies confirmed that the antimicrobial mechanism of these germicides against *M. gallinae* arthroconidia was due to the disruption of cell wall and cell membrane integrity. Future studies should develop combination germicide formulations and examine their application in the control of *M. gallinae* in poultry farming.

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