Environmental Monitoring of Zoonotic Fungal Infection in Broiler Chickens: Novel Approach to Control using Nano-fungicide Composite

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ABSTRACT

Control of fungal infections has not taken much attention compared to bacterial and viral pathogens inflicting significant economic losses to the poultry sectors as well as direct harms to human health due to their zoonotic implication. This study aimed to investigate the existence of fungal pathogens in broiler chicks dropping and their environment. As well, evaluate the efficiency of Terminator disinfectant (coco-benzyl-dimethyl ammonium chlorides and glutaraldehyde), nano copper oxide (CuO NPs), and Terminator/ nano copper oxide (Terminator/CuO NPs) on the fungal growth inhibition to control resistant fungus to antifungal agents. All samples (n= 320) were collected from chicks dropping, their environment (air, water, feeds, litter, drinkers, and feeders) as well, the attendant's hand swabs for isolation and identification of fungal pathogens. The susceptibility pattern of 65 strains of fungal isolates to antifungal agents, terminator disinfectant, and nano-fungicide composites was determined by the disc diffusion assay and broth micro-dilution method. All isolates were highly resistant to voriconazole antifungal drugs, whilst *Aspergillus fumigatus (A. fumigatus)* was resistant (100%) to fluconazole. Furthermore, the sensitivity of *Aspergillus terreus (A. terreus)* and *Penicillium corylophilum (P. corylophilum)* was 0.0% to fluconazole, and amphotericin-B. Whilst the antifungal activity of Terminator/CuO NPs against fungal pathogens proved its lethal effect (100%) against all fungal isolates at 0.5 mg/mL compared to the efficiency of both Terminator at 1:50 and CuO NPs at 2.0 mg/mL was not exceeded 84.6% and 76.9%, respectively against all fungal strains. In conclusion, nano-fungicide is a promising product for the prevention of fungal pathogens in broiler chickens and their environment. The control of zoonotic resistant fungus using novel nano-fungicide composite (Terminator/CuO NPs) at 0.5 mg/mL concentration was efficiently achieved compared to nano copper oxide at 2.0 mg/mL.

Keywords: resistant fungus; chick's environment; nano-fungicide; antifungal agents; disinfectants

INTRODUCTION

Fungal diseases cause a direct/indirect infection through the production of mycotoxins that have potential economic losses. Some fungi are harmful to birds, and humans are being induced serious diseases (Hyde *et al.,* 2018). In poultry farms, fungal diseases involve aspergillosis, candidiasis, favus, and cryptococcosis. Furthermore, the most important and impactful one was both aspergillosis, and candidiasis (Dhama *et al.,* 2013; Girma *et al.,* 2016). Besides, mycotoxins are one of the main causes that leading to produce immune suppression in birds, which makes them exposed to many viral and bacterial infections and subsequent cause economic losses to the poultry sectors (Asfaw & Dawit, 2017).

Monitoring and/or the surveying of fungal infections in poultry flocks as well as following the prudent use of antifungal agents incorporate with strict biosecurity measures; sanitation, disinfection, and proper hygiene, are considered the essential elements in an effective prevention and control program (Dhama *et*

al., 2013). Interestingly, the resistance of *C. albicans* to fluconazole and itraconazole drugs was observed while it was sensitive to amphotericin-B and ketoconazole at 0.5 μg/mL and 1 μg/mL (Zaidi *et al.,* 2018). Quaternary ammonium surfactant compounds (QACs) are broadly used as antimicrobial and/or antifungal both alone and in combination with other chemical agents (Perinelli *et al.,* 2019). Oppositely, there are various generations of quaternary compounds (Pernak *et al.,* 2001), whereas benzalkonium chlorides are the primary generation obtained from quaternary salts with alkyl distributions. Moreover, compounds with long alkyl chains of C_{12} were most effective against fungi and yeast. Meanwhile, the second-generation form was synthesized through aromatic ring substitution in alkyl-benzyl dimethyl ammonium chlorides (Brycki *et al.,* 2011). In previous literature, Marek *et al.* (2015) found that alkyl chains of C_{14} and C_{16} analogs were more effective against yeast fungus, whilst C_{16} was non-sensitive to filamentous fungi. On the other hand, none of the compounds evaluated had a higher level of sensitiv-

ity compared to the benzalkonium C_{12} analog. Using nanoparticles and/or nanomaterials as novel agents for micro-organisms growth inhibition has been developed due to the existing antimicrobial resistance (Eshed *et al.*, 2012). Oppositely, the surrounding of Candida cells by exo-polymeric substance matrix can save the yeast cells against the antifungal agents. Among nanomaterial compounds, nanometals have been extensively used due to their being less toxic (Lara *et al.*, 2015). Nano copper oxide (CuO NP) has a range of potential physical properties, high surface areas, and unusual crystal morphologies. Copper element is essential for all living organisms. It could be easily mixed with polymers to provide the composites with unique physio-chemical characterizations and be used in biomedical applications (Chang *et al.*, 2012). The present work was designed to monitor the existence of fungi and yeast in broiler chicks dropping and their environment. The sensitivity pattern of isolated fungi and yeast to various antifungal agents, Terminator disinfectant, and nano copper oxide (CuO NPs) were assessed. The lethal effect (%) of novel nano-fungicide (Terminator/CuO NPs) to resistant fungi and yeast were evaluated to achieve an efficient control strategy in poultry sectors in the investigated area.

MATERIALS AND METHODS

Ethical Statement

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB), reference number [IORG-0009255] of Beni-Suef University.

Study Area

The study was carried out on four private small commercial poultry farms during the period from August to December 2019 in Beni-Suef (coordinates: 29° 04′ N–31° 05′ E) province, Egypt. Each farm contained three building units. Broiler chickens were reared in a deep litter system and kept on wood shaving litter. The level of hygienic measures was fair in the investigated farms, whereas the average environmental conditions including in-door temperature (35.5±0.21°C), relative humidity (65.3±3.5%), and the speed of airflow (0.31±0.2 Knots/h) were recorded in all investigated buildings during this study.

Samples Collection and Preparation

Samples were collected randomized from broiler chicks dropping and their environment (*n*= 320). Sampling sites included chicks dropping (*n*= 50), air (*n*= 40), water source (Tap water *n*= 40), feeds (*n*= 40), drinkers (*n*= 40), feeders (*n*= 40), litter (*n*= 40), as well attendants hand swabs (*n*= 30) were obtained from investigating farm workers who were close to chicken flocks. Samples were kept in sterilized screw-capped bottles and bags. Feeds, chicks' litter, and droppings samples were prepared (10 g from each sample was diluted in 100 mL sterilized distilled water) and agitated well for

30 min at 1000 rpm. Air samples were collected at the one-meter height of chicks' litter onto malt extract agar supplemented with chloramphenicol and gentamycin at 40 and 500 µg/mL, respectively, using settle plate method, and after an incubation period, all qualitative and quantitative (colony-forming units; CFU/m³) results were obtained. As well, swabs were collected on a sterile screw-capped tube containing 9 mL Sabouraud dextrose broth, then labeled and sent to the laboratory in an icebox. All samples were examined directly within 24 h after sampling to investigate the presence of fungal species as a method described by Barnett & Hunter (1998); Aliyu *et al.* (2016).

Fungal Pathogens Isolation and Identification

From each sample dilution, 0.2 mL was cultured on Sabouraud dextrose agar (SDA: Oxoid®, CM0041, Ltd, UK), and malt extract agar supplemented with chloramphenicol and gentamycin at 40, and 500 µg/mL, respectively using a spread plate technique (Davis *et al.*, 1990). Thereafter, all cultured fungal pathogens are incubated at 25°C for 5-7 days. Fungal isolates were identified based on macroscopic and microscopic features of the isolates obtained from pure cultures using lactophenol blue staining (De Hoog *et al.*, 2000). The features involved shape, color, size, rapidity of growth, and septation of hyphae (Barnett & Hunter 1998). Isolated fungi were sub-cultured onto SDA slants, incubated at 25°C for 5-7 days, then stored in a refrigerator for further mycological studies (Saleemi *et al.,* 2010).

Susceptibility of Fungal Isolates to Antifungal Agents

Susceptibilities of 65 strains of fungal pathogens [*A. niger* (*n*=13), *A. fumigatus* (*n*=13), *A. terreus* (*n*=13), *P. corylophilum* (*n*=13), and *C. albicans* (*n*=13)] to common antifungal drugs used (Hi-Media Laboratories Pvt. Ltd. A-516, India): Itraconazole (30 mcg), fluconazole (10 mcg), amphotericin-B (100 units), nystatin (100 units), and voriconazole (1 mcg) were evaluated using disc diffusion method. The fungal colonies were suspended in 5 mL of 0.85% sterile saline, and the turbidity was adjusted using 0.5 McFarland standard. A swab from the suspension of the inoculum was streaked onto Muller-Hinton agar supplemented with 2% Glucose and 0.5mcg/mL methylene blue dye, then left for 3 min. Antifungal disks were applied to the surface of the inoculated plates. The plates, in an inverted position, were incubated at 30°C for 24-48 h for fungal growth. The inhibition zone was measured and recorded. The obtained results were interpreted according to CLSI (2009).

Antifungal Activity of Terminator and Nanocomposites *In-Vitro*

Efficacy of Terminator disinfectant (Coco-benzyldimethyl ammonium chlorides (QAC) 10% and Glutaraldehyde 15%, Bomac Laboratories Ltd, New Zealand) at concentrations of (1:200, 1:100, and 1:50 mL), CuO NPs (0.5, 1.0, and 2.0 mg/mL), and Terminator/CuO NPs composite (0.25, and 0.5 mg/mL) against 65 strains

of different fungal isolates from chicks dropping and their environment were evaluated using broth microdilution method and disc diffusion assay as the methods described by CLSI (2008; 2009) and Amiri *et al.* (2017).

Preparation of Testing Compounds

The solutions of the Terminator disinfectant were serially diluted with distilled water to obtain the required concentrations: 1:50, 1:100, and 1:200 mL. Whilst CuO NPs were synthesized using the chemical precipitation method as described by Phiwdanga *et al.* (2013). One hundred mM copper (II) sulfate pentahydrate ($CuSO_4$ $5H_2O$) was dissolved in 50 mL of distilled water with continuous stirring on a magnetic stirrer for 30 min at room temperature. Moreover, a solution of NaOH (100 mM) was added dropwise into the $CuSO_4$ solution with continuous high-speed stirring. The resultant precipitate was washed with deionized water and ethanol 70%. Thereafter, it was centrifugated at 6000 rpm for 15 min then dried in a hot air oven at 60°C for 24 h, followed by calcination at 400°C for 4 h. The test solutions of CuO NPs were 0.5, 1.0, and 2.0 mg/mL. Thereafter, to prepare Terminator/CuO NPs composite, 1 mL of Terminator disinfectant was dissolved in 100 mL of distilled water to obtain (1:100 mL) then added to CuO NPs concentrations (0.25, and 0.5 mg/mL). The mixture was shaken and stirred well using the magnetic stirrer to avoid agglomeration and the settlement of nanoparticles over the incubation period (4 h). Thereafter, it was sonicated for 30 min. The nano-solution was heated in a water bath at 40-90°C for 2.5 h, then filtered and washed several times with distilled water then, dried in a hot air oven at 50°C for 1 h.

Characterization of Testing Nanocomposites

Nanomaterials (CuO NPs and Terminator/CuO NPs) were characterized using high-resolution transmission electron microscopy (HR-TEM, JEOL-JEM 2100) and Fourier-transform infrared spectrum (FT-IR, VERTEX 70) were used to examine the nanomaterials microstructures and description of solid morphologies, respectively (Figures 1 and 2).

Broth Microdilution Assay

One microliter of fungal strains (1×10^6) colony-forming units (CFU/mL) was inoculated with different concentrations of Terminator disinfectant (1:50, 1:100, and 1:200 mL), CuO NPs (0.5, 1.0, and 2.0 mg/mL), and Terminator/CuO NPs (0.25, and 0.5 mg/mL) in Sabouraud dextrose broth onto a 96-well plate (Sarstedt, Nümbrecht, Germany) using broth micro-dilution assay according to CLSI (2008) and Amiri *et al.* (2017). Furthermore, 1 µl of broth culture added to Sabouraud dextrose broth without testing compounds served as negative control while testing nanocomposites in Sabouraud dextrose broth (positive control). All treatments and control were incubated at 25-28°C for 48 h. The trial was carried out in a triplicate. To determine the minimum inhibitory concentration (MIC) of disin-

fectant and nanocomposites, one loopful of each well was streaked on Saparoud dextrose agar supplemented with chloramphenicol and gentamycin at 40 and 500 µg/mL, respectively, using the spread plate technique to detect the fungal colony growth at different concentrations of Terminator, CuO NPs, and Terminator/CuO NPs composite. Meanwhile, the minimum fungicide concentration (MFC) detected the least concentration of Terminator disinfectant and tested nanocomposites indicating the absence of fungal growth (CFU/mL).

Disc Diffusion Assay

One hundred discs of a standard size of filter paper (13 mm diameter) were obtained then saved in screws capped bottles and to ensure sterilization. Bottles were placed in a hot air oven at 180°C for 20 min. Sterilized discs were impregnated overnight with the different tested concentrations of Terminator (1:50, 1:100, and 1:200 mL) according to recommended concentrations of product meanwhile, CuO NPs (0.5, 1.0, and 2.0 mg/mL), and Terminator/ CuO NPs concentrations (0.25 and 0.5 mg/mL) were chosen as preliminary study. Fungal isolates were diluted in normal saline at a concentration of 10^6 (CFU/mL), according to reference to McFarland 0.5. Thereafter, 100 µL of a suspension was streaked on Muller-Hinton agar supplemented with 2% Glucose and 0.5 mcg/mL methylene blue dye, then left for 3 min. Impregnated discs were placed with sterilized forceps on agar plates. All plates were inculcated at 28°C for 48 h then the diameter of growth inhibition zones around each disc of all tested fungus was measured by caliper and recorded. The readings were taken in a triplicate. This procedure was done according to CLSI (2009) and Amiri *et al.* (2017).

Statistical Analyses

Collected data were assembled in Microsoft Excel Spreadsheets, then analyzed using SPSS version 26 (Statistical Package for Social Sciences Software). The prevalence of fungal pathogens in chicks' dropping, its distribution in their environment, and antifungal activity of Terminator disinfectant and novel nano-fungicide composite were statistically analyzed by Chi-Square Test (non-parametric test). To detect the inhibition zone diameter of testing compounds against isolated fungi, the One-Way ANOVA test was applied.

RESULTS

The Existence of Different Fungal Pathogens in Poultry Flocks and Their Environment

The prevalence of fungal pathogens in the investigated small commercial poultry flocks was 52.8% (169/320). The highest prevalence of fungus in chicks' droppings was 56.0 CFU/g (28/50), followed by their environment and attendants hand swabs (55.8%; 134/240 and 23.3%; 7/30, respectively) (Table 1). Oppositely, the highest prevalence of fungal pathogen isolates, *A. niger*, *C. albicans*, *A. fumigatus*, *A. terreus* , and *P. corylophilum*

were 26.6% (45/169), 22.5% (38/169), 20.7% (35/169), 14.8% (25/169), and 15.4% (26/169), respectively at χ^2 = 14.7, $P<0.05$.

The frequency (%) of fungal pathogens in examined samples revealed that the highest rate of fungal isolates was detected in chicks' litter and fecal droppings followed by drinkers, feeders, air, and feeds (80.0 CFU/g; 32/40, 56.0 CFU/g; 28/50, 60.0%; 24/40, 60.0; 24/40, 55.5 CFU/m³; 22/40, and 45.0 CFU/g; 18/40, respectively) at χ^2=19.62, p<0.05 (Table 2). Furthermore, *A. niger* was detected in the highest percentage in chicks dropping (35.7 CFU/g; 10/28) followed by feeds, feeder swabs, and chicks' litter (33.3 CFU/g; 6/18, 33.3%; 8/24, and 31.2 CFU/g; 10/32, respectively). Meanwhile, the rate in air samples and drinkers' swabs was 18.2 CFU/m³; 4/22 and 16.7%; 4/24 compared to the least rate recorded in tap water and attendants hand swabs (14.3%; 2/14 each). In contrast, *A. fumigatus* distribution in air samples and feeds was detected in the highest rate (36.4 CFU/m³; 8/22 and 33.3 CFU/g; 6/18) followed by chicks dropping and their litter (21.4 CFU/g; 6/28 and 18.7 CFU/g; 6/32) respectively. In addition, other *A. terreus* was found at a higher rate (42.8%; 6/14) in tap water than other environmental samples. Oppositely, *P. corylophilum* was not detected in tap water (0.0%). Meanwhile, the highest rate was found in air, feeder, and feeds (27.3 CFU/m³; 6/22, 25.0%; 6/24, and 22.2 CFU/g; 4/18, respectively). *C. albicans* yeast was discovered at the highest rate in attendants hand swabs and tap water (54.1%; 4/7, and 42.8%;

6/14) compared to drinkers and litter (33.3%; 8/24, and 25.0 CFU/g; 8/32, respectively) (Table 2).

Susceptibility of Fungal Pathogens to Antifungal Agents *In Vitro*

Susceptibility testing of fungal isolates to antifungal agents *in vitro* revealed that all isolated fungus and yeast were highly resistant to voriconazole antifungal drugs except *A. fumigatus*. Moreover, the susceptibility of both *A. terreus* and *P. corylophilum* were 0.0% to fluconazole, and amphotericin-B. On the other hand, all fungal isolates clarified the susceptibility to itraconazole drug (100%). Furthermore, the susceptibility of all *Aspergillus* spp. (*A. niger*, *A. fumigatus*, and *A. terreus*) to fluconazole was 0.0%. *A. niger* was highly resistant to nystatin (100.0%) on contrary the other fungal species were highly susceptible (Table 3).

Characterization of Testing Nanocomposites

Nanomaterials (CuO NPs and Terminator/CuO NPs) were characterized by HR-TEM (Figure 1). It was evident that HR-TEM micrographs of CuO NPs (Figure 1a) showing spherical uniformly NPs distribution in the field. Moreover, the NP size was ranged from 1.39 to 12.5 nm in diameter (Figure 1b). HR-TEM micrographs of Terminator/CuO NPs clarified the nanoparticles' distribution in nanocomposite (Figure 1c) as NPs appeared

Table 1. Prevalence rate of fungal pathogens in chicks fecal dropping and their environment

Collected samples	Total		Prevalence rate of fungal pathogens No. (%)				
			Aspergillus spp.			*Penicillium corylophilum*	*Candida albicans*
	Examined (No.)	Positive No. (%)	*Aspergillus niger*	*Aspergillus fumigatus*	*Aspergillus terreus*		
Chicks fecal dropping	50	28 (56.0)	10 (35.7)	6 (21.4)	4 (14.3)	2 (7.1)	6 (21.4)
Environment	240	134 (55.8)	34 (25.4)	28 (20.9)	20 (14.9)	24 (17.9)	28 (20.9)
Workers hand swabs	30	7 (23.3)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0.0)	4 (54.1)
Total	320	169 (52.8)	45 (26.6)	35 (20.7)	25 (14.8)	26 (15.4)	38 (22.5)

Note: The association between the prevalence rate of different fungal pathogens is statistically significant at χ2 = 14.7 (p<0.05).

Table 2. Frequency (%) of fungal pathogens in different examined samples during study periods

Collected samples	Total		Prevalence rate of fungal pathogens No. (%)				
			Aspergillus spp.			*Penicillium corylophilum*	*Candida albicans*
	Examined (No.)	Positive No. (%)	*Aspergillus niger*	*Aspergillus fumigatus*	*Aspergillus terreus*		
Chicks fecal dropping	50	28 (56.0)	10 (35.7)	6 (21.4)	4 (14.3)	2 (7.1)	6 (21.4)
Air	40	22 (55.0)	4 (18.2)	8 (36.4)	2 (9.1)	6 (27.3)	2 (9.1)
Tap water	40	14 (35.0)	2 (14.3)	0 (0.0)	6 (42.8)	0 (0.0)	6 (42.8)
Feeds	40	18 (45.0)	6 (33.3)	6 (33.3)	2 (11.1)	4 (22.2)	0 (0.0)
Litter	40	32 (80.0)	10 (31.2)	6 (18.7)	4 (12.5)	4 (12.5)	8 (25.0)
Drinkers	40	24 (60.0)	4 (16.7)	4 (16.7)	4 (16.7)	4 (16.7)	8 (33.3)
Feeders	40	24 (60.0)	8 (33.3)	4 (16.7)	2 (8.3)	6 (25.0)	4 (16.7)
Workers hand swabs	30	7 (23.3)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0.0)	4 (54.1)
Total	320	169 (52.8)	45 (26.6)	35 (20.7)	25 (14.8)	26 (15.4)	38 (22.5)

Note: The association between frequency of different fungal isolates in investigated data is statistically significant at χ2 = 19.62 (p<0.05).

Table 3. *In vitro* susceptibility testing of fungal isolates to antifungal agents

Antifungal agents	Tested conc. (mcg/disc)	Susceptibility of fungal isolates (%)									
		Aspergillus spp.						*Penicillium corylophilum*		*Candida albicans*	
		Aspergillus niger		*Aspergillus fumigatus*		*Aspergillus terreus*					
		S	R	S	R	S	R	S	R	S	R
IT	30	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
FLC	10	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	100.0	0.0
AP	100	100.0	0.0	100.0	0.0	0.0	100.0	0.0	100.0	100.0	0.0
NS	100	0.0	100.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
VRC	1.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0	100.0	0.0	100.0

Note: S= Susceptible (absence of fungal growth) on agar plates; R= resistant (presence of fungal growth) on agar plates; IT= Itraconazole; FLC= Fluconazole; AP= Amphotericin-B; NS= Nystatin; VRC= Voriconazole.
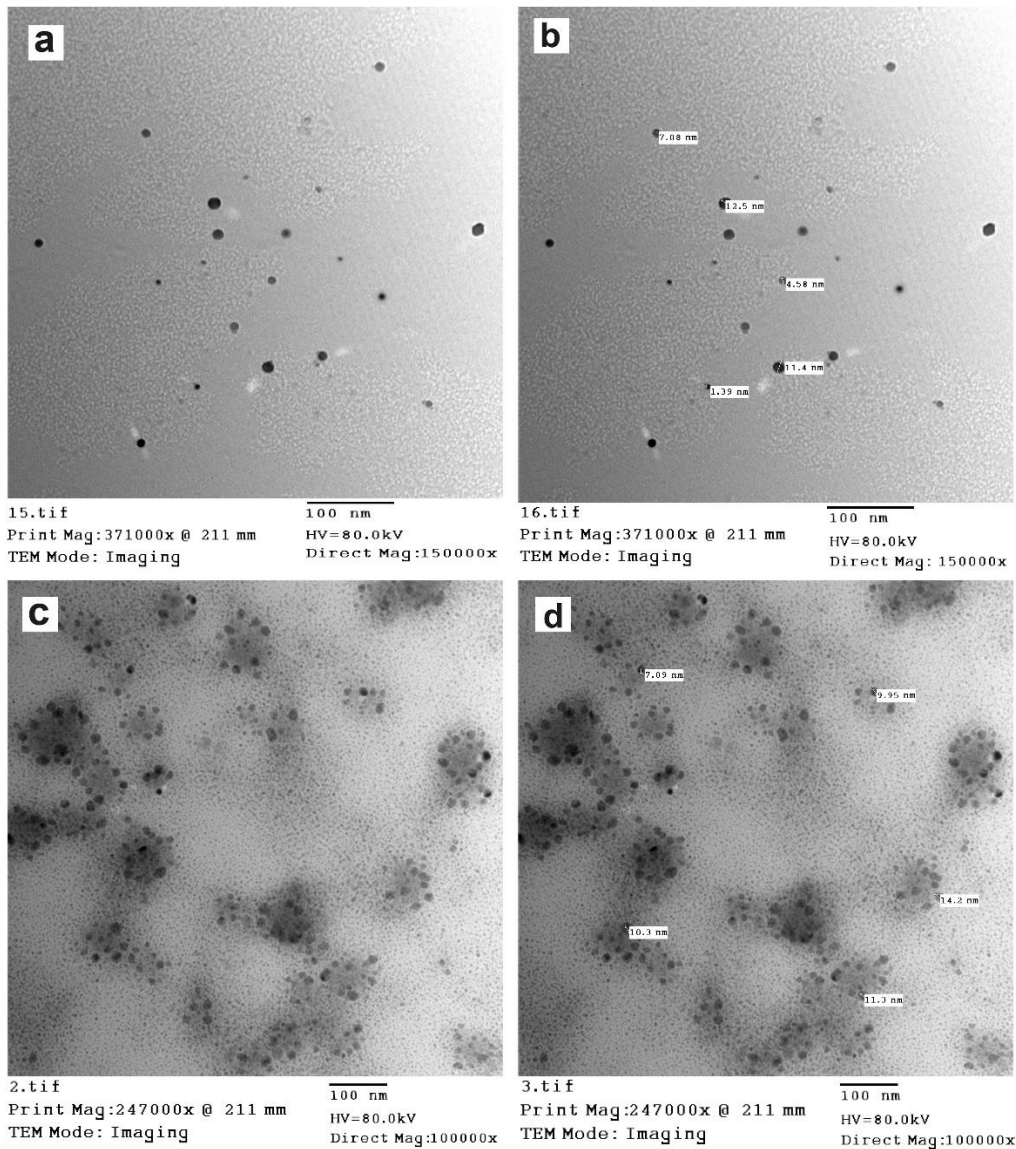

Figure 1. HR-TEM micrographs of CuO NPs (a) clarified morphological spherical uniformly shape of NPs distribution besides the diameter of NPs size (b) was ranged from 1.39 and 12.5 nm. Whilst Terminator/CuO NPs Micrographs (c) clarified irregular spherical and elongated shape of NPs. Nanoparticles size exhibited variation in its diameter (d) ranged up to 14.2 nm.

in irregular spherical and elongated shapes. Oppositely, NPs diameter varied in size (Figure 1d) ranged up to 14.2 nm. FT-IR spectroscopy of nano copper oxide was obtained in the wavelength range between 500 to 3500 cm^{-1} (Figure 2a). The intensity of absorption peaks appeared in the range of 3417 to 521 cm^{-1}. Furthermore, the absorption band at 3417 cm^{-1} corresponded to hydroxyl groups (O–H), whilst the band at 2379 cm^{-1} represented C–H stretching of an aromatic compound. In addition, the FT-IR spectra of Terminator disinfectant (Figure 2b) revealed a strong absorption peak at 3331 cm^{-1} that

attributed to the absorption of methyl groups in the testing disinfectant. As well, there were characteristic peaks that appeared at 2943, 2539, 2349, 1640, 1361, 1147, and 606 cm^{-1}, respectively. Otherwise, Terminator/CuO NPs (nano-fungicide composite) showed the strongest peak was moving at 3288 cm^{-1} (Figure 2c), indicating the strong interaction between nano copper oxide and both hydroxyl and methyl groups. Furthermore, there were intense peaks observed at 2431, 2350, 1638, 1477, 1151, and 603 cm^{-1} that proved the biosynthesis of the nano-fungicide composite.
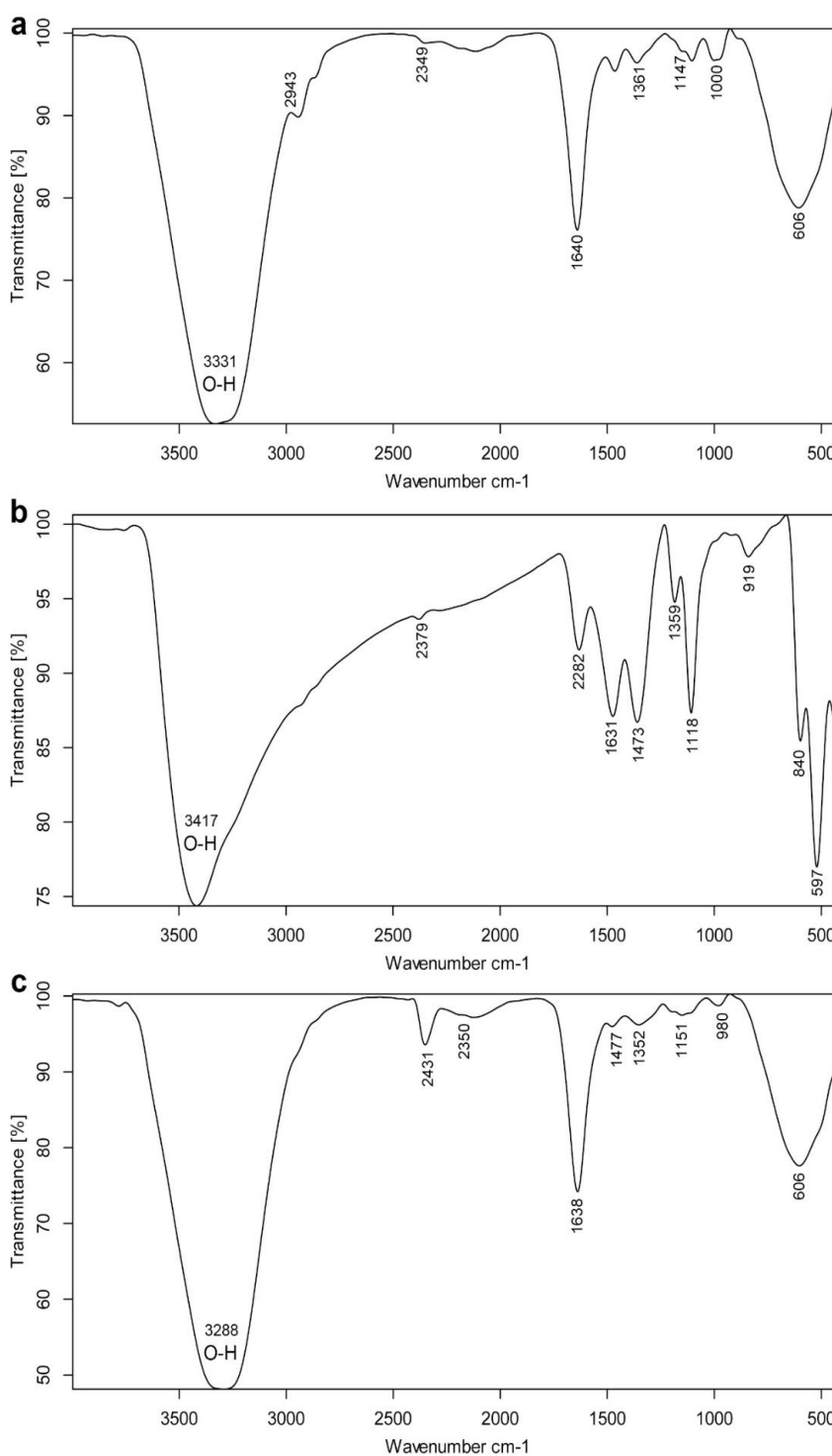

Figure 2. FT-IR spectroscopy of Terminator disinfectant (a), CuO NPs (b), and Terminator/CuO NPs composite (c).

Antifungal Activity of Terminator Disinfectant and Nanocomposites

The MIC value of Terminator disinfectant against all isolated fungus and yeast (*A. niger*, *A. fumigatus*, *P. corylophilum*, and *C. albicans* yeast) was 1:100 mL except for *A. terreus* fungus was 1:50 mL. In contrast, the minimum concentration of disinfectant that led to the absence of fungal growth (MFC) of all isolated fungi and yeast was 1:50 mL at $p \leq 0.05$. Furthermore, the inhibition zone was ranged between 17.0±0.04 and 22.5±0.01 mm, respectively. Oppositely, the MIC value of CuO NPs against *A. niger*, *A. fumigatus*, *P. corylophilum* fungus was 0.5 mg/mL. Meanwhile, both *A. terreus* and *C. albicans* were 1.0 mg/mL. The MFC value of nano copper oxide against fungal isolates was 2.0 mg/mL. In addition, the

inhibition zone was ranged from 6.0±0.08 to 25.0±1.5 mm. This study is the first to determine the MIC and MFC values of Terminator/CuO NPs composite against fungal pathogens. The values of nano-fungicides were 0.25 mg/mL and 0.5 mg/mL, respectively to all fungal isolates. Moreover, the inhibition zone diameter ranged from 26.0±0.7 to 45.0±2.1 mm (Table 4 and Figure 3).

The susceptibility pattern of fungal isolates to Terminator, CuO NPs, and Terminator/CuO NPs. It has been revealed that *P. corylophilum* susceptibility to Terminator was 84.6%, followed by *A. niger*, *A. fumigatus*, *C. albicans* (76.9% each) at 1:50 mL concentration (Table 5). Meanwhile, the susceptibility of *A. terreus* did not exceed 61.5% compared to other tested concentrations of 1:200 mL and 1:100 mL, respectively. Regarding

Table 4. Distribution of MIC and MFC of terminator disinfectant, and nanocomposites against fungal isolates

Tested compounds		Aspergillus spp.			Penicillium corylophilum	Candida albicans
		Aspergillus niger	Aspergillus fumigatus	Aspergillus terreus		
Terminator	MIC (mL)	1:100	1:100	1:50	1:100	1:100
	MFC (mL)	1:50	1:50	1:50	1:50	1:50
Diameter of inhibition zone (mm)		20.0±0.11[b]	17.0±0.04[ab]	21.5±3.0[a]	21.5±0.26[b]	22.5±0.01[b]
CuO NPs	MIC (mL)	0.5	0.5	1.0	0.5	1.0
	MFC (mL)	2.0	2.0	2.0	2.0	2.0
Diameter of inhibition zone (mm)		12.0±2.0[ab]	25.0±1.5[b]	6.0±0.08[b]	23.0±1.5[b]	11.0±0.13[ab]
Terminator/CuO NPs	MIC (mL)	0.25	0.25	0.25	0.25	0.25
	MFC (mL)	0.5	0.5	0.25	0.5	0.25
Diameter of inhibition zone (mm)		30.0±0.0[a]	35.0±0.5[a]	26.0±0.7[a]	40.0±0.03[a]	45.0±2.1[a]

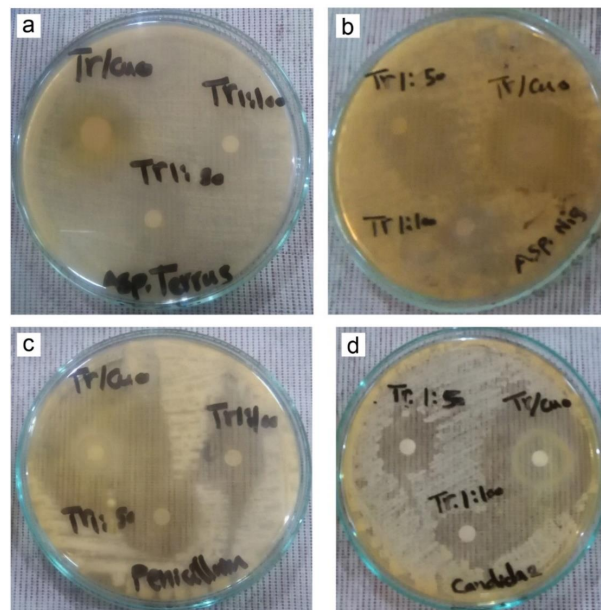Note: Means in the same column with different superscripts differ significantly (p≤0.05).

Figure 3. Sensitivity of different fungal pathogens to Terminator disinfectant and nano-fungicide composite (Terminator/ CuO NPs) using the disc diffusion assay. The highest sensitivity rate of all Aspergillus spp. [A. terreus (a), and A. niger (b), P. Corylophilum (c), and C. albicans (d)] was found to nano-fungicide composite at 0.5 mg/mL, whereas zone of inhibition was ranged from 26.0 ± 0.7 to 45.0 ± 2.1 mm compared to testing Terminator disinfectant.

Table 5. Susceptibility pattern (%) of fungal pathogens to Terminator, CuO NPs, and Terminator/CuO NPs composite

Tested compounds		Susceptibility pattern (%) of fungal pathogens to disinfectant and nanocomposites. No. of tested isolates (n = 65 strains)										p-value
		Aspergillus niger		Aspergillus fumigatus		Aspergillus terreus		Penicillium corylophilum		Candida albicans		
		S	R	S	R	S	R	S	R	S	R	
Terminator (mL)	1:200	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100	N
	1:100	69.2	30.8	61.5	38.5	46.1	35.8	76.9	23.1	69.2	30.8	0.05
	1:50	76.9	23.1	76.9	23.1	61.5	61.5	84.6	15.4	76.9	15.4	0.03
CuO NPs	0.5 mg/mL	15.4	84.6	23.1	76.9	0.0	100.0	15.4	84.6	0.0	100	0.01
	1 mg/mL	53.8	46.1	61.5	38.5	53.8	46.1	53.8	46.1	38.5	38.5	0.05
	2 mg/mL	69.2	30.8	76.9	23.1	61.5	38.5	69.2	30.8	61.5	38.5	0.05
Terminator/ CuO NPs	0.25 mg/mL	92.3	7.7	84.6	18.2	100.0	0.0	92.3	7.7	100.0	0.0	0.01
	0.5 mg/mL	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	N

Note: S= Susceptible % (absence of fungal growth) on agar media; R= resistant (presence of fungal growth) on agar media; N= means no statistics are computed.

the efficiency of CuO NPs at a concentration of 2.0 mg/mL against fungus and yeast isolates clarified that its efficiency wasn't exceeded 76.9% for *A. fumigatus*, as well *A. niger* and *P. corylophilum* (69.2% each) meanwhile, the sensitivity of *A. terreus* and *C. albicans* was 61.5% each compared to the other tested concentrations. Oppositely, the promising nano-fungicide composite (Terminator/CuO NPs) exhibited its lethal effect (100%) against all fungus and yeast isolates at 0.5 mg/mL compared to its efficiency at 0.25 mg/mL was significantly high against *A. terreus,* and *C. albicans* (100%) whilst *A. niger, P. corylophilum,* and *A. fumigatus* were 92.3%, 92.3%, and 84.6%, respectively at p≤0.01 (Table 5).

DISCUSSION

The mycotic infection is considered one of the most serious hygienic problems in broiler flocks due to their high morbidity and mortality rate in young chicks that cause immunosuppression and lowering the resistance to various bacterial and viral diseases (Abd El Tawab *et al.,* 2015). Therefore, the current study revealed that the highest prevalence rate of fungal isolates as *A. niger, A. fumigatus,* and *C. albicans* in a broiler poultry farm. Oppositely, fungal isolates were detected in chicks' litter and chick droppings at the highest rate, followed by drinkers, feeders, air, and feeds, respectively. Increasing fungal infections in chick's environment could be attributed to the increase of environmental contamination with organic materials and sewage, indicating improper hygienic measures and poor ventilation indoor of poultry building. Arne *et al.* (2011) clarified the most pathogenic fungi affected poultry was *A. fumigatus* that cause brooder's pneumonia as the spores of this fungal species are smaller than another *Aspergillus* spp. The overriding factors for the flaring of spore dissemination in the air and poultry environment are poor ventilation rate and improper sanitation measures (Gitika *et al.,* 2019). The environmental humidity in the investigated broiler chicken farms was noticed its association with increase the fungal infection during our study period, whereas the average temperature and relative humidity were 35.5±0.21°C, and 65.3±3.5%, respectively. Furthermore, Kunkle (2003) found both temperature and humidity promoted the rapid growth of fungal hyphae that might be dispersed and inhaled by chickens. In addition, Walker & White (2017) noticed that environmental and nutritional conditions play a role in reproduce of fungi asexually. The current study was in line with Aliyu *et al.* (2016), who found that the most prevalent *Aspergillus* species in poultry feeds were *A. fumigatus* (58.8%) followed by *A. flavus* (41.2%). Oppositely, farmworkers are at great risk of contracting respiratory infection as they are exposed to dust loaded with spores of *Aspergillus* spp. (*A. fumigatus*) during their work on a poultry farm. Furthermore, the prevalence of aspergillosis was significantly higher in sawdust litter (70.0%) as compared with rice husk litter (40.0%) that used in poultry farms (Sabino *et al.,* 2012; Salem & Ali, 2014) and spreading of those litters in agricultural lands has a potential public health concern, as well toxigenic fungi such as *Aspergillus* and *Penicillium* were isolated from poultry litter. Meanwhile, wood shavings litter tended to load higher fungi compared to rice hulls litter (Viegas *et al.,* 2012). Moreover, a huge number of spores are found in wet litter, whereas birds affected by *A. terreus,* and *A. niger* may show dyspnea, fever, gasping, yellowish diarrhea, and mortality rate ranged from 5% to 50% especially during initial 1-3 weeks of age (Dhama *et al.,* 2013). In contrast, Ezekwueche *et al.* (2018) found that the most predominant *Aspergillus* spp. was A. *niger* in poultry dropping samples (48.0%). Regarding *C. albicans* yeast, it was discovered in the highest percentage in attendants hand swabs and tap water compared to drinkers and chicks' litter during this study. Oppositely, Answar *et al.* (2012) clarified that the high prevalence of *C. albicans* was reported on the environment. Besides, domestic chicken droppings are a possible reservoir and infection source (72.13%) compared to soil (27.87%). So, humans could acquire candidiasis through inhaling spores from chicken droppings (Kemoi *et al.,* 2013).

Developing a control strategy of the toxigenic mycoflora of poultry feeds needs regular monitoring and surveillance of the existence of fungal pathogens

in their environment (Saleemi *et al.*, 2010; Aliyu *et al.*, 2016). Susceptibility testing of fungal pathogens to antifungal agents should be periodically evaluated to avoid the problem of antifungal resistance patterns. The current study was found that all *Aspergillus* spp. isolates were completely resistant to both fluconazole and voriconazole antifungal drugs, except *A. fumigatus* fungus. Moreover, *A. niger* revealed a resistance profile to nystatin. As well, the susceptibility of *A. terreus* and *P. corylophilum* were 0.0% to fluconazole and amphotericin-B. Concomitantly, Chowdhary *et al.* (2014) clarified that *Penicillium* spp. are among the common fungi worldwide, causing an economic impact as well as various infections in both animals and humans. The same results were reported by Sutton *et al.* (2004) and Steinbach *et al.* (2004). In contrast, Wiederhold (2017) found that *A. fumigatus* was resistant to the azole group: voriconazole, itraconazole, and fluconazole, as well as *Aspergillus* isolates, can cause a high mortality rate. The current study revealed that *C. albicans* was highly sensitive to most of the tested antifungals (itraconazole, fluconazole, amphotericin B, and nystatin) while it exhibited resistance pattern to voriconazole. Flaring up of *C. albicans* occurred when the host immune system became depressed (Kullberg & Erendurp, 2015; Zaidi *et al.*, 2018).

Prevention and control of aspergillosis in poultry sectors area complicated and difficult problem that depends on maintenance conditions (Ziółkowska & Tokarzewski, 2006). The current study implicated that the efficiency of Terminator disinfectant was not exceeded by 84.6% against all isolated fungi and yeasts at the MIC value 1:50 mL. The MIC value of Terminator disinfectant against all isolated fungus and yeast was 1:100 mL. In contrast, the minimum concentration of disinfectant that led to the absence of fungal growth (MFC) of all isolated fungi and yeast was 1:50 mL at $p \leq 0.05$. Jeffrey (1995) and Howett *et al.* (1999) showed that quaternary ammonium compounds were low potent against *Aspergillus* species. On the contrary, Perinelli *et al.*, (2019) revealed that all synthesized quaternary ammonium surfactant compounds were more effective against *C. albicans* yeast.

The lethal effect of nano copper oxide (CuO NPs) was not exceeded 76.9% to all fungal isolates at a concentration of 2.0 mg/mL with NPs size was ranged from 1.39 to 12.5 nm in diameter. Furthermore, the FT-IR spectra of nano copper oxide clarified the strongest absorption peak at 3417 cm^{-1} that in line with Ghareib *et al.* (2019). As well, Khan *et al.* (2014) pointed to the shape and size of NPs influencing the antifungal activity. Amiri *et al.* (2017) found that CuO NPs had a weak influence on *Candida* spp. and it showed a decrease in yeast growth between 30% to 40% at 1-1000 μg/mL besides the NPs size was 40 nm in diameter. In addition, Karimiyan *et al.* (2015) noticed that the MIC50 value of nano copper oxide for *C. albicans* was 400 μg/mL. In the present work, the conjugation of Terminator disinfectant to CuO NPs surface was proved through the characterization by HR-TEM and FT-IR spectroscopy. Moreover, the NPs size in nano-fungicide composite ranged from 7.09 to 14.2 nm. As well, characteristic peaks of the nano-fungicide composite were observed at 3288, 2431,

2350, 1638, 1477, 1151, and 603 cm^{-1}, respectively, that ensured the biosynthesis of the nanocomposite**. In addition, NPs diameter in nano-fungicide varied in size and ranged up to 14.2 nm that enhanced the penetration power of nanocomposite to the cell wall of all fungal, yeast isolates and destructive or damage it.

CONCLUSION

Terminator/CuO NPs composite is the future promising nano-fungicide product to eradicate the fungal pathogens from chicken flocks and their environment at a concentration of 0.5 mg/mL. Periodical monitoring of the antifungal efficiency against fungal pathogens is key in detecting and controlling resistant fungus. The novel nano-fungicide compound could be used in a biosecurity program as a strong disinfectant and/or fungicide due to its lethal effect against isolated fungal pathogens.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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