Use of Adapted Diffusion Method as Preliminary Assay for Antifungal Biocides Efficacy Currently Used in Decontamination

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The study purpose was to assess the efficacy of four, commonly used biocidal commercial conditionings on fungi and yeasts strains, sampled in eight representative poultry and swine units from Romania. These were tested in the aim of Diffusion Qualitative Method (DQM), biologic material being represented by: Aspergillus niger, A. flavus, A. fumigatus, Rhodotorula rubra and Candida albicans. Assay revealed that out of four biocide products tested, the cidal activity was ascertained only in a share of 75% (3/4), in the case of one product, at the manufacturer's recommended concentration of 2%, the cidal action being absent to the filamentous strains of fungi.

Keywords: antifungal, biocides, efficacy, decontamination, diffusion method

In the last decades, significant progress has been made in the mechanisms of antibacterial action of biocides understanding. However, information on biocides action against fungi remained yet sparse. Studies of the biocides mode of action on fungi and yeasts, suggested that, unlike antibiotics, which act selectively on cellular targets; they act on one or more than one location, like: cell wall, plasma membrane, *thiol* groups of the proteins and enzymes, ribosomes and DNA [1-4].

An important issue, requiring multifaceted defence measures, is the presence of fungal or biofilm-related infections, more frequently signalled, and not easy to eradicate, became a significant threat, confirmed by the fungal resistance mechanisms [5].

In parallel with the rent animals intensive industrial systems expansion, an alarming increase in resistance occurrence and distribution of various microorganism species to one or more than one medicinal substances, antibiotics / antifungals, biocides / decontaminating products has been noticed, all followed by negative implications in the control of diseases both, in humans and animals [6-8]. Therefore, in order to survive to the biocides exposure, fungi act via activation of a number of resistance mechanisms to reduce the toxic concentrations of the chemical compounds [9].

Important steps, from non-standardized to standardized procedures, have been proposed in the last decade, into the specialists aid, antifungal susceptibility testing (AST) becoming an accepted methodology for human and veterinary mycology [10, 11].

The Clinical and Laboratory Standards Institute (CLSI), and the domain researchers published new methods for antifungal susceptibility testing. In this respect, the Diffusion Qualitative Method (DQM) for determining the susceptibility of fungi to biocides is based on principles used in antibiograms and antifungigrames and can be accomplished in conformity with the international standard CLSI-M2-A9. This technique could be useful in the laboratory or field evaluations offering the possibility of choosing the best antifungal products [12-14].

Present study is justified by growing of the scale and risks severity associated with exposure to fungi. It is proposed a comparative assay of four, commonly used biocidal commercial conditionings on filamentous fungi and yeasts strains, sampled in eight representative poultry and swine units from Romania, and tested in the aim of adapted Diffusion Qualitative Method (DQM), as preliminary assessment of these biocides efficacy and initial step in the fungal resistance prevention.

Experimental part

Materials and methods

As initial step, in our previous study, the mycological structure from four poultry and four swine intensive breeding units, from Romania it was ascertained, 250 samples of feed, air, water, bedding, floor and shelter's surface being sampled and analyzed in the respect of Romanian legislation methodology [15].

Results revealed a diverse mycoflora composed by filamentous fungi: *Aspergillus, Penicillium, Mucor, Absidia, Rhizopus, Alternaria, Ulocladium, Cladosporium, Fusarium* and two yeast strains: *Rhodotorula* and *Candida.* From a total of 544 fungi strains, 231 (42.5%) were represented by *Aspergillus sp,* 158 (29.1%) and *Candida sp,* 72 (13.4%), fact confirming that, these fungi were the most frequently and possible involved in the animal pathologies in the visited units. The association: *Aspergillus fumigatus, A. niger* and *A. flavus* and *Rhodotorula rubra* and *Candida albicans* were considered the most probable pathogen responsible fungi in our study, confirming the importance of continuous contact with the conidia of these fungi and their health and economic outcomes.

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Tested biocide/ concentration (%)					Composition of the commercial conditionings used
A					Glutaraldehide Alkyl dimethylbenzyl ammonium chloride, Didecyl dimethyl ammonium chloride Ethyl alcohol
5.0	4.0	**1.0** [a]	**0.5** [a]	**0.25** [b]	
B					Alkyl dimethylbenzyl ammonium chloride, Didecyl dimethyl ammonium chloride
4.5	3.5	**2.5** [a b]	1,0	0.5	
C					Chlorhexidine digluconate
3.0	**2.0** [a]	1.0	0.5	0.25	
D					Formaldehyde Alkyl dimethylbenzyl ammonium chloride,
6.0	**5.0** [a]	4.0	2.0	0.5	
a - recommended concentration by manufacturer for the necessity decontamination **b** - recommended concentration by manufacturer for prophylactic decontamination					

Table 1
CONCENTRATION OF COMMERCIAL BIOCIDES TESTED BY THE DISK DIFFUSION METHOD ADAPTED

Biocide conditionings

For the antifungal efficiency comparative study, four commercial biocide conditionings frequently used in the Romanian decontamination programs, abbreviated with letters A, B, C and D were used. The concentrations and composition of the tested biocide products are presented in table 1.

Biologic material

It was represented by the main five fungi strains isolated and identified in the visited units, respectively: *Aspergillus niger, A. flavus, A. fumigatus, Rhodotorula rubra* and *Candida albicans.*

Media cultures, reagents and equipments used were:

- Sabouraud Dextrose Agar with chloramphenicol, (MM1067) (HiMedia, India);
- RPMI 1640 Agar w/MOPS (Sigma-Aldrich),
- McFarland scale and glycerol (Merk, Germany),
- sterile distilled water, ethanol 95% (Chemical Company, Romania);
- sterilized tubes; sanitation wipes; microbiological loop; sterile Petri dishes 90 cm; Filter paper disks with a diameter of 0.5 mm;
- thermostate (Forma Scientific, Inc USA);
- laminar flow hood microbiological;
- densitometer (Bio-Den-1 Grant Instruments Ltd England);
- Vortex; semi-automatic multi-channel pipette etc.

Methodology

Principle of DQM consists in placing of discs containing known amounts of biocide on solid medium surface, previously seeded with standardized fungal inoculums. After incubation, lysis zones and diameter of developed fungal strains, should be observed and measured, the results being based on lysis zones intensity.

Positive dilutions were considered those producing the cultures' total lysis, without resistant colonies within them, regardless of their number. Since this method is used classically to determine the sensitivity of filamentous fungi and yeast to antifungal drugs in the absence of a punctual guidance on determining the sensitivity for biocidal products, researchers interpreting the results in a similar manner [14,16-18].

To prepare the inoculums, 0.5 McFarland suspensions corresponding to 106 CFU/mL in sterile distilled water were made from *Candida albicans* and *Rhodotorula rubra* 24-48 h old cultures and of 5-7 days old *Aspergillus fumigatus, A. niger, A. flavus* cultures on Sabouraud Dextrose Agar with chloramphenicol (HiMedia, India).

In the case of filamentous fungi spores and hyphae suspensions, these were vortexed and left to stand for coarse particles to settle, about 15 min after which the supernatants were transferred to other sterile tubes. The RPMI 1640 Agar w/MOPS (Sigma-Aldrich) plates were seeded with the aid of buffer sanitation with 200 µL / each inoculum and were allowed to dry. After drying, on the surface of culture medium, disks of filter paper soaked in different concentrations of the studied biocides were applied.

Plates incubation was done for 48-72 h at 27 ± 2 °C, for filamentous fungi and for 24-48 h at 36 ±1°C for yeasts.

Results and discussions

The evaluation of *cidal* activity on isolated fungi strains of *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus, Rhodotorula rubra* and *Candia albicans*, the diameter of inhibition zones revealing / each biocide product and every used concentration is presented in table 2 and figure 1.

Results confirmed the presence of a multifarious fungal biota in the intensive poultry and swine units and the potential determinant role of *Aspergillus* and *Candida* (or their association) in developing fungal patho-mechanisms.

The four commercial biocide products, currently used in the decontamination programs in the poultry and swine breeding units in Romania, tested by adapted DQM, have had a certain *cidal* activity in a share of 75% (3/4) of all strains of fungi in planktonic strains isolated and tested at the necessity concentrations recommended by the manufacturers, respectively 1% - A product; 2.5% - B product; 5% and respectively D - product.

In what concerns the C product, at the manufacturer's recommended concentration of 2%, it can be ascertained that *cidal* action was observed only on the yeast fungi strains *Rhodotorula rubra* and *Candida albicans* but did not have any *cidal* activity on the studied filamentous fungi strains *Aspergillus fumigatus, A. niger* and *A. flavus.*

In this aim, the knowledge of resistance phenomenon to some commercial biocidal products is particularly important, either in order to increase the concentration of active substance in the commercial biocidal product, or in order to give up its use in farms where the phenomenon is yet present. In parallel with the afore-mentioned measures, it is outlined the reaction of biocidal products manufacturers to keep up with market demands, respectively the livestock and food industry, by diversifying the range of biocides and continuous increase of their efficiency [19-21].

The practical aspects linked to the fungi isolation and identification, respectively to reveal the incidence per each fungi gender / species and livestock category, could be considered a helpful tool to understand and imagine the most appropriate and coherent steps in future antifungal

Biocide product	%	Diameter of inhibition zone (mm)				
		Aspergillus fumigatus	*Aspergillus niger*	*Aspergillus flavus*	*Rhodotorulla rubra*	*Candida albicans*
A	5.0	14.0	11.50	10.0	16.75	13,25
	4.0	11,50	10.50	7.75	15.0	11,25
	1.0[a]	9.25	6.50	6.0	12.75	9,25
	0.50[a]	6.75	5.0	5.50	11.0	8,25
	0.25[b]	5.0	5.0	5.0	8,50	7.0
B	4.50	11,5	9.50	9.25	15.5	11.50
	3.50	10	8.0	8.0	12.5	9,50
	2.50[a,b]	8.0	6.75	6,50	11.0	8.0
	1.0	6,25	5.0	5.0	9.50	7.0
	0.50	5	5.0	5.0	5.0	5.0
C	3.0	6,0	5.75	5.25	15.75	8.50
	2.0[a]	5,0	5.0	5.0	14.0	6.75
	1.0	5,0	5.0	5.0	12.50	5.50
	0.50	5,0	5.0	5.0	11.0	5.0
	0.25	5,0	5.0	5.0	10.0	5.0
D	6.0	24,0	13.75	10.0	15.25	11,50
	5.0[a]	16.50	11.75	8.50	13.50	9.50
	4.0	13,0	8.0	7.0	12.25	8.0
	2.0	9.50	6.50	5.50	9.50	6.75
	0.50	5,0	5.0	5.0	7.75	5.50

Table 2
EVALUATION OF CIDAL ACTIVITY / FUNGI STRAIN / CONCENTRATION

Legend :
a - Manufacturer's recommended concentration(s) for current and/or necessity decontamination
b - Manufacturer's recommended concentration for prophylactic decontamination

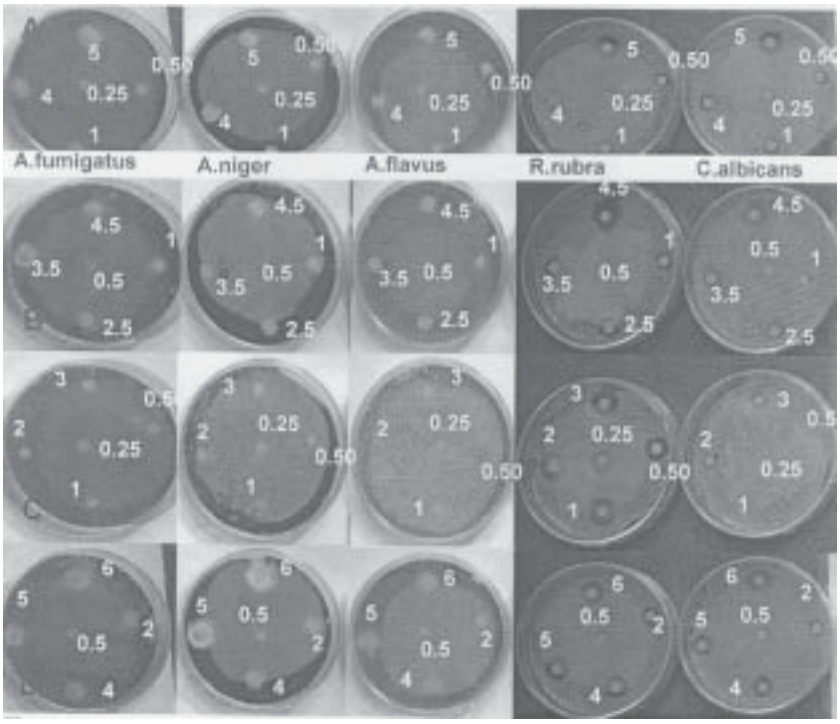

Fig. 1. The image of the incubated plates / biocidal products: A, B, C, D / used concentrations and inhibition areas / each studied fungal strains

strategies including here the efficiency assay. Agreeing that this could be an occult cause of the more often reported disinfection efficiency decline, it appears the necessity of an improved evaluation. For example, Théraud et al. (2004), demonstrated that antiseptics and disinfectants overall efficacy against yeast isolates was different, when the cells were grown under planktonic or in biofilm conditions. Authors have established that, eight out of nine agents investigated, were ineffective against *Candida* growing in biofilms, situation that can easily overlapped in the veterinary field [22].

Conclusions

The inactivation of fungal pathogens is impossible without ensuring active substances' optimal concentrations in the commercial biocidal product used for fungi, and especially those organized in biofilm. The adapted DQM has proven to be a simple and effective screening method, fast and cheap, to estimate the likely concentrations with *cidal* effect of products used in the decontamination programs in the poultry and swine units being in our opinion a helpful tool for the field specialists.

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