RESISTANCE OF YEASTS AND ALGAE ISOLATED FROM COW MASTITIC MILK TO ANTIMICROBIAL AGENTS

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Abstract

The objective of this study was to determine the antimicrobial resistance of 319 yeast strains and 168 algae strains isolated from cows’ mastitic milk. The in vitro susceptibility to 10 antifungals and 10 antibiotics was examined by disc diffusion method. Above 75% of yeast strains were resistant to amphotericin B, 66% to fluconazole, and 40% to pimaricin and itraconazole. Algae strains were resistant to the most of the tested antifungals. Only nystatin, amphotericin B, and pimaricin were active against the most of these microorganisms. Algae showed the high resistance to antibiotics too. Only the aminoglycosides: gentamycin (75.5% of strains), kanamycin (71.7%), and neomycin (30.6%) inhibited the growth of these organisms in vitro. This study demonstrates that only a few antifungal and antimicrobial agents are active in vitro against algae; however, yeast strains show the high in vitro susceptibility to the most antifungals.

Key words: cow, mastitis, yeast, algae, resistance to antibiotics, resistance to fungicidals.

Yeasts from Candida genus and algae from Protothecata genus (mainly P. zopfii) are two groups of eukaryotic pathogens causing infections of cows’ mammary glands. Infections and mastitis occur when the high number of microorganisms exist on the teat, and break down the udder defences. A marked increase in the number of udder infections caused by yeasts and algae was observed in various countries during the last decade (1, 4, 6, 7, 11, 12, 16, 17).

With the rising frequency of fungal infections, an increase in the resistance to antifungal agents was reported (20). The resistance mechanisms to amphotericin B generally work through the modification of the membrane ergosterol, those to flucytosine - through mutation of metabolic enzymes, and those toazole derivatives - through modification of cytochrome P 450. Antifungal drug resistance is divided into two forms: the primary or innate resistance, present without a prior antifungal therapy, and the secondary or acquired one, developed in previously susceptible isolates during or after exposure to an antifungal drug (8).

A number of different antifungal drugs are currently in medical use: the polyenes (amphotericin B, nystatin), azoles (fluconazole, ketoconazole), allylamines (terbinafine) and antimetabolites (flucytosine). These agents vary in their mechanisms of antifungal action. Polyenes interact with ergosterol, azoles inhibit the synthesis of ergosterol by blocking the action of cytochrome P-450 14-α-demethylase, allylamines inhibit oxydosqualene cyclase, and antimetabolites inhibit the fungal protein synthesis by replacing uracil with 5-fluorouracil in the fungal RNA (9).

The in vitro susceptibility of microorganisms to antimicrobial agents is one of several factors that can increase the therapy success. A number of methods to evaluate the antifungal susceptibility are known at present. The standard CLSI (Clinical and Laboratory Standards Institute) M27-A method is a broth microdilution. It is very laborious, time-consuming, and cumbersome. The agar based antifungal susceptibility tests are a good alternative to the microdilution method. These tests are very attractive because they are simple, reproductible, and do not require any specialised equipment. While the E-test method (AB Biodisk Sweden) is relatively expensive, disc diffusion test is easy to perform, cheap, and well suited for routine use in laboratories.

The aim of the study was to determine the in vitro susceptibility of isolated from mastitis cows yeast strains to antifungals and algae strains to antifungals and antibiotics.

Material and Methods

A total of 319 strains of yeast and 168 strains of algae, isolated from inflamed cows’ udder secretions, were examined. In vitro susceptibility of the yeast to antifungals and algae to antifungals and antibiotics was assayed by disc diffusion method. Prior to testing, each isolate was grown on Sabouraud agar for 24-48 h at 37°C. The yeast inoculum suspensions were prepared in 0.85% saline to a density of 0.5 Mc Farland standard,
resulting in a concentration of $1 \times 10^6$ to $5 \times 10^6$ yeast cells/ml. The 0.5 ml volume of this inoculum was cultured on Yeast Nitrogen Base (YNB) agar. The plates were allowed to dry for at least 15 min before the discs were put on. The discs of amphotericin B (10 µg), itraconazole (10 µg), fluconazole (10 µg), tioconazole (10 µg), 5-fluorocytosine (0.5 µg), clotrimazole (10 µg), ketoconazole (10 µg), miconazole (10 µg), nystatin (100 J), and pimaricin (10 µg) were applied. The plates were incubated at 37°C for 24 h and the inhibitory zone diameters were measured and interpreted according to the manufacturer’s instructions (DHN PAN Kraków, Poland). Zone diameters of ≥ 18 mm indicated the susceptibility (S), zone diameters of 14-17 mm indicated the dose-dependent susceptibility (DDS), and that of ≤ 14 mm indicated the resistance (R) to nystatin, itraconazole, fluconazole, tioconazole, clotrimazole, ketoconazole, miconazole and pimaricin. The zones “S”, “DDS”, and “R” were ≥16 mm, 12-15 mm, and ≤ 12 mm for amphotericin B and ≥20 mm, 16-19 mm, and < 16 mm for 5-fluorocytosine, respectively. The susceptibility of algae isolates to antibiotics was tested on Mueller-Hinton agar and penicillin (10 µg), kanamycin (30 µg), gentamycin (10 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), amoxicillin (25 µg), lincomycin (15 µg), cephaloridine (30 µg), novobiocin (30 µg) discs were used. Zone diameters of complete growth inhibition were measured in millimetres. As resistant ones, there were assumed the strains with zones for: penicillin <28 mm, kanamycin <13 mm, gentamycin and neomycin <12 mm, streptomycin <11 mm, tetracycline, novobiocin, cephaloridine and amoxicillin <14 mm, and lincomycin <17 mm. As susceptible, there were recognised strains with zones for: penicillin >29 mm, kanamycin >18 mm, gentamycin and streptomycin >15 mm, neomycin and novobiocin >17 mm, tetracycline > 19 mm, cephaloridine, and amoxicillin and lincomycin >21 mm.

Results

Fig. 1 presents the resistance of yeast isolates to antimycotic drugs. The examined strains showed resistance to amphotericin B (75.2%), fluconazole (66.8%), pimaricin (40.8%), and itraconazole (38.9%). They were susceptible mostly to tioconazole (91.2%), clotrimazole (84.0%), and 5-fluorocytosine (82.1%). The resistance of algae strains to antifungals is presented on Fig. 2. More than 90% of the strains were resistant to itraconazole, fluconazole, tioconazole, clotrimazole, ketoconazole, miconazole, 5-fluorocytosine, and pimaricin. Only nystatin and amphotericin B showed activity against these microorganisms. The tested strains of algae were resistant to most of antimicrobial agents also (penicillin, tetracycline, amoxicillin, lincomycin, cephaloridine, novobiocin) and were susceptible only to aminoglycosides. Gentamycin and kanamycin turn out to be the most active against Prototheca: 73.8% and 68.5% of the strains were susceptible and susceptible-dose-dependent, respectively (Fig.3). Neomycin and streptomycin demonstrated some low ability to inhibit the in vitro growth of algae. Altogether, 41.1% and 16.1% isolates were susceptible and susceptible-dose-dependent, respectively.

Fig. 1. In vitro antifungal resistance of yeast strains. F - fluconazole, P - pimaricin, T - tioconazole, N - nystatin, C - clotrimazole, K - ketoconazole, - itraconazole, M - miconazole, Fl - 5-fluorocytosine, AmB - amphotericine B.
similar results were obtained by other authors, who examined the susceptibility of yeast and algae causing the cow’s mastitis. Sanchez et al. (20) proved high susceptibility of 36 yeast strains isolated from clinical cases of various animal diseases to tioconazole, clotrimazole, and ketoconazole. McDonald et al. (14) reported that clotrimazole, ketoconazole, nystatin, and miconazole were the most active antifungals against yeasts isolated from the mammary gland. They reported that only 6 of 27 antimicrobial agents were active in vitro against causing mastitis strains of \textit{P. zopfii} (15). All of the 48 strains were susceptible to mixin and nystatin, 45.8\% of the strains to amphotericin B, 43.8\% to polymixin B, 37.5\% to gentamycin, and only 2.1\% to kanamycin. Bexiga et al. (2) noted that \textit{Prototheca zopfii} strains were only sensitive to gentamycin, kanamycin, and polymixin B, and Buzzini (4) indicated the susceptibility of these microorganisms only to nystatin and amphotericin B (58 and 33 \% of total strains, respectively). In the study by Marquez (13) nystatin showed the better efficacy than amphotericin B in the inhibition of \textit{P. zopfii} growth. It is in agreement with our results.

It is important that the increased use of antibacterial and antifungal agents has resulted in the development of resistance to these drugs. Like these of other living organisms, fungal and algal cells may become resistant to toxic compounds. Many authors (3, 5, 18) reported that increase in the resistance among yeast may be a worldwide problem, especially in human medicine.

There are different opinions on therapy of mycotic mastitis. The treatment is generally complicated because of the lack of efficient antifungal drugs that can be used intramammary, but some authors observed that yeast mastitis ends with spontaneous recovery without therapy (17). Treatment of \textit{Prototheca} sp. is getting serious because of algae resistance to antibiotic and antifungal treatment (4, 10, 19). No spontaneous recovery from protothecal mastitis has been reported yet.

\textbf{Fig. 2.} \textit{In vitro} antifungal resistance of \textit{Prototheca zopfii} strains.

\begin{itemize}
  \item F - fluconazole, P - pimaricin, T - tioconazole, N - nystatin, C - clotrimazole, K - ketoconazole, I - itraconazole, M - miconazole, Fl - 5-fluorocytosine, AmB - amphotericine B.
\end{itemize}

\textbf{Fig. 3.} \textit{In vitro} susceptibility of \textit{Prototheca zopfii} strains to aminoglycosides.

\begin{itemize}
  \item Cn - gentamycin, K - kanamycin, N - neomycin, S – streptomycyn.
\end{itemize}
References