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Antimicrobial susceptibility of *Prototheca zopfii* isolated from bovine mastitis

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Abstract

The aim of the study was to evaluate the susceptibility of algae of the genus *Prototheca* to antifungal and antibacterial antibiotics. The study involved 27 isolates of *Prototheca zopfii* obtained from milk of mastitis-affected cows kept in a detached cowshed in the North-Eastern part of Poland. Analysis of *P. zopfii* susceptibility has demonstrated low effectiveness of both antifungal and antibacterial antibiotics. All algae isolated from milk were resistant to clotrimazole, fluconazole, econazole, flucytosine, cefoperazone, cephalexin, enrofloxacin, lincomycin, and oxytetracycline (100% of resistant isolates), as well as miconazole (92.6% of resistant isolates). Nystatin, ketoconazole, and amphotericin B showed the highest activity amongst the antifungal antibiotics (88.9% and 0.0%, 51.9% and 22.2%, 0.0% and 48.1% of susceptible and intermediate susceptible isolates, respectively). In the group of antibacterial antibiotics, the high activity against *P. zopfii* was observed only in the case of gentamicin, kanamycin (96.3% and 92.6% of susceptible isolates, respectively), and polymyxin B (59.3% of susceptible and 33.3% of intermediate susceptible isolates).

Key words: cows, mastitis, *Prototheca,* antibiotic susceptibility.

Introduction

Algae are ubiquitous, unicellular, achlorophyllic organisms existing in the natural environment (19). They belong to the genus of *Prototheca*, the family *Chlorellaceae*. They are one of few plant-like organisms, which can cause infections in humans and animals (11-13, 18, 21). The species isolated from affected humans and animals include *P. zopfii, P. wickerhamii*, and *P. blaschkeae* (12, 20, 22, 23, 25, 27). The remaining species were considered as non-pathogenic.

Mastitis in dairy cows is one of the most important infections caused by *Prototheca* pathogens (17). The disease results in substantially decreased milk production and increased somatic cell count (8). It even can lead to cow culling, causing high financial losses.

Protothecosis of the udder is a serious therapeutic problem. According to the literature data, *Prototheca* sp. does not respond to the routine therapy of mastitis (9, 15, 16, 21). The studies concerning *in vitro* and *in vivo* susceptibility of algae indicate their high resistance to antibacterial and antifungal antibiotics (4-6, 11).

The algae in natural environment occur in places characterised by high humidity and high organic matter content (bedding, wastes, and animal faeces) (6, 16, 28). Therefore, the development of bovine udder infections induced by *Prototheca* pathogens is considered to be highly dependent on predisposing factors, *i.e.* unsuitable upkeep conditions and inappropriate hygiene of milking (8, 9, 11, 12, 21, 23). Spontaneous recoveries have not been noted.

The objective of the study was to evaluate antifungal and antibacterial antibiotic susceptibility of *Prototheca* algae isolated from mastitis-affected dairy cows.

Material and Methods

The study involved 27 *Prototheca zopfii* species isolated from milk of mastitis-affected cows. The study was carried out in the summer 2011. The cows

originated from one herd in the North-Eastern part of Poland. The animals were kept in a detached cowshed.

The affected cows did not respond to routine antibiotic treatment. Milk was sampled according to the accepted procedure. Once the udder skin was cleansed, washed, and dried, and the teats disinfected with 70% alcohol, milk was collected to sterile, labelled test tubes without preservatives, chilled to 4°C and delivered to the laboratory for further examinations.

Bacteriological and mycological testing was carried out according to standard procedures: milk culture, using the agar medium plus 5% mutton blood, and Sabouraud agar with the addition of chloramphenicol, followed by 24-72h incubation of plates under aerobic conditions at 37°C, and evaluation of morphology of colonies of isolated pathogens and Gram stained specimens. The isolated *Prototheca* sp. were identified using API 20C AUX (Biomerieux, France).

Antibiotic susceptibility of the pathogens was analysed using the disc diffusion method on the Mueller-Hinton agar according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).

The testing of *Prototheca zopfii* susceptibility was performed with the following drugs: antimicotic - amphotericin B (20 µg), econazole (10 µg), fluconazole (10 µg), flucytosine (1 µg), ketoconazole (10 µg), clotrimazole (10 µg), miconazole (10 µg), nystatin (100 U), and antibacterial - amoxicillin with clavulanic acid (30 µg), cephalexin (30 µg), cefoperazone (75 µg), enrofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), colistin (50 µg), oxytetracycline (30 µg), lincomycin (15 µg), and polymyxin B (300 U).

Plates with discs were left at the room temperature for 30 min and incubated at 35°C for 48 h. The susceptibility of the algae was determined by measuring the zone of growth inhibition around the antibiotic-soaked disc (Oxoid, England). According to the size of inhibition zone, the strains were divided into 3 categories: susceptible (+), intermediately susceptible (+/-), and resistant (–).

Results

P. zopfii strains showed substantial *in vitro* resistance to antibiotics (Tables 1 and 2). All algae isolated from milk were unsusceptible to clotrimazole, fluconazole, econazole, and flucytosine (100.0% of resistant strains) (Table 1). Moreover, miconazole was also found to be ineffective (92.6% of resistant strains). The highest activity was demonstrated by nystatin (88.9% of susceptible strains), ketoconazole (51.9% of susceptible and 22.2% of intermediate susceptible strains), and amphotericin B (48.1% of intermediate susceptible strains).

The highest effectiveness amongst antibacterial antibiotics was demonstrated by gentamicin, kanamycin, and polymyxin B (Table 2). The percentages of strains susceptible and intermediately susceptible to these antibiotics were as follows: 96.3% and 3.7%, 92.6% and 3.7%, 59.3% and 33.3%, respectively. The remaining antibiotics, *i.e.* amoxicillin with clavulanic acid, enrofloxacin, cephalexin, cefoperazone, lincomycin, and oxytetracycline, were found to be ineffective *in vitro* (100.0% of resistant strains). The percentage of strains intermediately susceptible to colistin was high (29.6%).

Discussion

The analysis of antibiotic susceptibility of *P. zopfii* strains confirmed low effectiveness of antimicotic and antibacterial drugs. All algae isolated from milk were unsusceptible to clotrimazole, fluconazole, econazole, flucytosine, cefoperazone, cephalexin, enrofloxacin, lincomycin, and oxytetracycline (100.0% of resistant strains) as well as to miconazole and colistin (92.6.0% and 63.0% of resistant strains, respectively). Similarly, the combination of amoxicillin with clavulanic acid was found ineffective (100.0% of resistant strains). The results presented by Milanov *et al.* (17) were comparable; all algae isolated from milk were also unsusceptible to enrofloxacin, amoxicillin, tetracycline, penicillin, lincomycin, and novobiocin.

Table 1. Susceptibility of *Prototheca zopfii* to antimicotic drugs

	AMF		CLOT		ECO		FLC		FLU		KCA		MIC		NYST	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
S	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	14	51.9	1	3.7	24	88.9
I	13	48.1	0	0.0	0	0.0	0	0.0	0	0.0	6	22.2	1	3.7	0	0.0
R	14	51.9	27	100.0	27	100.0	27	100.0	27	100.0	7	25.9	25	92.6	3	11.1

S–susceptible; I–intermediately susceptible; R–resistant
AMF–amphotericin B; CLOT–clotrimazole; ECO–econazole; FLC–fluconazole; FLU–flucytosine; KCA–ketoconazole; MIC–miconazole; NYST–nystatin

Table 2. Susceptibility of *Prototheca zopfii* to antibacterial drugs

	AMC		CFP		CEF		COL		ENR		G		KAN		LINCO		T		POLI B	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
S	0	0.0	0	0.0	0	0.0	2	7.4	0	0.0	26	96.3	25	92.6	0	0.0	0	0.0	16	59.3
I	0	0.0	0	0.0	0	0.0	8	29.6	0	0.0	1	3.7	1	3.7	0	0.0	0	0.0	9	33.3
R	27	100.0	27	100.0	27	100.0	17	63.0	27	100.0	0	0.0	1	3.7	27	100.0	27	100.0	2	7.4

S–susceptible; I–intermediately susceptible; R–resistant
AMC–amoxicillin with clavulanic acid; CFP–cefoperazone; CEF–cephaleksin; COL–colistin; ENR–enrofloxacin; G–gentamicin; KAN–kanamycin; Linco–lincomycin; T–oxytetracycline; POLI B–polymyxin B

The obtained findings revealed that the highest antimicotic activity was demonstrated by nystatin, ketoconazole, and amphotericin B, which is consistent with the results of other authors confirming a high effectiveness of nystatin and amphotericin B against algae isolated from milk of mastitis-affected cows (5, 7, 14, 17). Moreover, the results reported by Malinowski *et al.* (10) were similar. In their study, the highest percentage of *Prototheca* species isolated from cow's milk was susceptible to amphotericin B (35.7% of susceptible and 7.1% of intermediate susceptible strains) and nystatin (21.4% of susceptible and 64.3% of intermediate susceptible strains). The growth of some species was also inhibited by pimaricin (17.9% of susceptible strains). Marques *et al.* (11) demonstrated different profiles of susceptibility of individual *Prototheca* species to cystatin and amphotericin B. Nystatin proved to be more effective against *P. wickerhamii* than amphotericin B. The susceptibility of *P. zofii* strains to these antibiotics was comparable. According to Srimuang *et al.* (24), amphotericin B combined with rifampicin was more effective than each antibiotic used separately. On the contrary, the combination of amphotericin B with 5-fluorocytosine did not result in the expected therapeutic outcome. The literature data regarding the activity of itraconazole is inconsistent. Blaschke-Hellmessen (2) isolated *P. zopfii* strains resistant to this antibiotic, while according to Tortorano *et al.* (26) itraconazole was one of the three effective drugs used.

Furthermore, our findings have indicated that only three antibacterial drugs demonstrated high activity against *P. zopfii*, *i.e.* gentamicin, kanamycin, and polymyxin B. Their effectiveness was confirmed by other authors (1, 3, 10, 14, 17). According to McDonald *et al.* (14) and Bexiga *et al.* (1), the isolated algae were susceptible only to three antibacterial drugs: gentamicin, kanamycin, and polymyxin B. In opposition, Malinowski *et al.* (10), who evaluated the susceptibility of 28 *P. zopfii* species, have observed low *in vitro* effectiveness of gentamicin, neomycin (12.5% of susceptible and 29.2% of intermediate susceptible strains), and streptomycin (4.2% of susceptible and 25.0% of intermediate susceptible strains). The strains susceptible to streptomycin, gentamicin, and polymyxin B were also isolated by Bodenhoff and Madsen (3). According to Milanov *et al.* (17), algae were intermediately susceptible to gentamicin and neomycin; no strains susceptible to kanamycin were found.

The results of the study and previously published data (9, 15, 17) have revealed high resistance of *P. zopfii* to antibiotics which affects the efficacy of mastitis treatment.

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