The Effect of Iodine Based Products on Unicellular Algae from Genus *Prototheca*

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Abstract

Iodine based products have been and are still used in medicine as disinfectant or antiseptic substances, because of their bactericidal, sporicidal, protocidal and disinfection effect. Algaecide effect has been less studied and represents a strong motivation for this study.

This paper is aiming to test the sensitivity to iodine based products of unicellular algae of the genus *Prototheca*. Such products might be used for the treatment of diseases involving these pathogens.

A total number of twenty-two *Prototheca zopfii* strains isolated from cows with mastitis or bovine shelters, were tested within this study. The algal strains were identified based on morphological characteristics (shape, size, presence of endospores), cultural (liquid and solid media characteristics) and biochemical (fermentation of sugars). *Prototheca wickerhamii* ATCC 16529 reference strain was also included in the evaluation. Iodinated products used were represented by: Lugol solution, iodine tincture, betadine, videne and potassium iodide. Determination of the inhibitory effect was measured by diffusion technique in agarose gel and by liquid medium dilution method. For the tincture of iodine and betadine, the inhibitory effect was also appreciated in relation with the time of contact (5, 10, 15, 30 and 60 minutes).

By the agar diffusion technique, it was found that inhibition zones varying sizes were correlated with the composition of those products. For the 22 *Prototheca zopfii* tested strains, the size of inhibition areas was within the following values (average diameter): iodine tincture 25.71 mm, betadine 26.4 mm, videne 25.61 mm, Lugol's solution 11.33 mm and potassium iodide 11.14 mm. For *Prototheca wickerhamii* strain inhibition areas had the following values: betadine 26 mm, iodine tincture 24 mm, videne 24 mm, Lugol's solution 10 mm, potassium iodide 14 mm. In case of different contact times meant to establish the algaecide effect - occurred in the first 5 minutes of contact, for both iodine tincture and betadine.

Iodine based products tested showed inhibitory effect on algal strains. The best efficacy was found for betadine and iodine tincture (pharmaceutical/commercial products). The inhibitory effect occurs in the first 5 minutes of contact. There was no resistance phenomenon nor the presence of resistant colonies in the inhibition zones.

Keywords: iodine based products, *Prototheca*, unicellular algae

INTRODUCTION

Because of its chemical properties, iodine is used in various fields of science and technology, including medicine. It can be used as the self, or as I_3 anion dissolved in water. Examples of such preparations are: iodine tincture (iodine in ethanol or iodine and sodium iodide in a mixture

of ethanol and water), Lugol's solution (iodine and potassium iodide in water). Because iodine is only slightly soluble in water, the solutions are prepared in the form of dyes in ethanol. Iodoform, iodochlorhydroxichinolina, iodophors, sodium iodide and potassium, exercise also bactericidal effects by releasing iodine [25]. Iodine can come from

Iodophors containing iodine complexed with a surfactant and solubilizing agent, povidone iodine is popular (polyvinylpyrrolidone and iodine) and iodine cadexomer (polysaccharide complex and iodine) (Cooper, 2004; Bootham, 2009; Sibbald *et al.*, 2011).

Since the phenomenon of antibiotic resistance has spread and amplified in recent years, alternatives for finding other preparations which exhibit antimicrobial effects have been frequently approached. Among them preparations extracted from plants or based on different chemical elements (Ag, Cu, Au, Zn, Fe, I and others). In the case of iodine and iodine-based preparations there are mentions that the resistance phenomenon is not installed or it is exceptionally uncommon (Boothman, 2009).

Iodine and iodine based products were processed and used as a disinfectant or antiseptic in medicine, especially in the preparation of the operative field before surgery, known to have bactericidal, sporicidal and virulicidal activity. The effect is especially microbicide against bacteria and viruses, but less intense against some forms of parasitic cysts such as *Giardia* and *Cryptosporium* [26]. Algaecide effect has been less studied, good reason for the research within this study.

Unicellular algae of the genus *Prototheca* began to be increasingly popular, with implications causing fatal chronic granulomatous lesions localized in different tissues. Illness are seen more frequently in cows (as mastitis) in dogs and several other species of warm-blooded and poikilothermic animals but humans too. It is known that the treatment with antibiotics and chemotherapeutics is frequently ineffective, animal slaughter or euthanasia is in some cases the final solution (Buyukmihci *et al.*, 1975). Contaminated milk is a major risk to public health (Krukowski *et al.*, 2013). Algae are considered as opportunistic pathogens with a low virulence, but can also indicate the pathogenicity of the host for various forms of immunosuppression such aspects being found in natural (Woolorich *et al.*, 1994; Wirth *et al.*, 1999) and experimental infections (Răpunțean *et al.*, 2001).

The paper aimed to test the in vitro effectiveness of iodine based products against 22 *Prototheca zopfii* strains isolated from bovine mastitis cow milk or in bovine shelters and ATCC 16529 *Prototheca wickerhamii* strain. The strains

were isolated for several years (2008-2014), and have been preserved in the collection of Microbiology Department, Faculty of Veterinary Medicine from Cluj-Napoca. The examination also covered a standardized strain: *Prototheca wickerhamii* ATCC 16529.

MATERIALS AND METHODS

We tested 22 *Prototheca zopfii* strains isolated from milk from cows with mastitis or cattle sheds. *Prototheca* strains have been identified based on several characteristics: *morphological* (shape, size, presence of endospores - for which the size and arrangement constitutes an important criterion for identification), *cultural* (growth on glucose culture medium, liquid and solid, incubated at 37°C; the colonies were small initially, gradually increasing in size, reaching 3-4 mm, white, matte, with slightly lobed edges and grainy/cauliflower-like surface) and *biochemical* (fermenting glucose, glycerol and fructose, variable at other sugars; oxidase and catalase positive reaction; indole and H₂S negative). Strains are preserved in soft agar. Iodinated products testing were: Lugol, iodine (SC Hippocrates SRL, Bucharest), Betadine® (EGIS Pharmaceuticals PLC, Budapest Hungary), Videne® (Adams, Gorforth Leeds, England) and 10% potassium iodide (GL Pharma GmbH).

The work technique was based on the 2013 CLSI vet. method (26) with some modifications listed below.

The inhibitory effect established by disc diffusion method. The tested strains were grown in glucose broth and incubated at 37° C for 48 hours. The prepared suspension was used to flood 9 cm diameter Petri dishes containing glucose agar having a thickness of 3 mm. The suspension was evenly spread using a Drigalski spatula, then the excess liquid removed. The plates were placed in thermostat for 30 minutes with the lid half open. After this time, in the agarose gel, wells were cut out using a 4 mm diameter mold, according to a radial pattern. This size, considering the thickness of 3 mm of the agar layer enables a well with a narrow diameter, which ensures a good distribution of the iodinated product in the surrounding gel. The wells were filled with 20 µl of the tested medium. The plates were placed in a thermostat at 37°C and examined at 24 and 48 hours.

The inhibition areas were appreciated, measuring their diameter (mm). To establish the

algaeicide effect, inoculations were performed on glucose broth.

In a different working method, from a thick suspension, prepared from *Prototheca* colonies developed on the agar, dilutions were performed in saline to densities according to McFarland standards: 0.5; 1; 2; 3; 4 and 5. Each dilution was used to inoculate two Petri dishes (1 ml per plate). After homogenization and removal of excess, the plates were placed in a thermostat for the surface to dry (30 minutes), then cut wells with a diameter of 4 mm filled with 20 µl of each iodinated product. The plates were incubated in a thermostat at 37°C and examined at 24 and 48 hours.

Establishing the efficiency of iodized products (tincture of iodine and Betadine) by dilution method. A quantity of 4.5 ml of glucose broth was distributed in a series of five tubes. To the first tube was added 0.5 ml of the iodinated product which, after mixing, was passed in succession 0.5 ml in the following tubes, in the end removing 0.5 ml of the tube no. 5. The resulted iodinated product dilution rate x10 is (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}). Subsequently, each tube was inoculated with a fixed amount of 5 µl (~2100 UFC/1 ml) of *Prototheca zopfii* (strain 22) and *Prototheca wickerhamii* (ATCC 16529). Control tubes, without iodized products: 1) tube that allows the development of culture (positive control); 2) non-inoculated tube to check the culture medium (negative control).

The tubes were incubated at 37°C and maintained for 24 hours. To verify the inhibitory effect of each tube in the dilution series set, glucose agar plates were streaked using one plate for each tested strain, and the plates were divided into 5 sectors of a triangular shape corresponding to each dilution. The plates were incubated at 37°C and examined at 24 and 48 hours, but kept under observation a further 5 days. The control tubes checking was done separately on another glucose agar plate.

Evaluation of the inhibitory at different contact times. The tested strains were *Prototheca zopfii* (22 strain) and *Prototheca wickerhamii* (ATCC 16529), and the testing was done to the iodine tincture and Betadine. In a 10/110 mm tube, 0.9 ml iodine tincture or Betadine was dispensed, followed by 0.1 ml culture two strains tested in glucose broth. Contact times were 5', 10', 15', 30' and 60'. After the end of each contact time

streaking's were performed on solid medium (glucose agar), previously distributed in Petri dishes, and divided into 5 triangular sectors. The plates were incubated in a thermostat at 37°C. The interpretation was made at 24 and 48 hours, with the final examination after 5 days.

RESULTS AND DISCUSSION

The results for disc diffusion method test. Iodine based products revealed inhibitory effect against *Prototheca* strains. Inhibition areas were correlated with products composition and the ability of iodine release.

For the 22 *Prototheca zopfii* tested strains, the average size of inhibition areas were as following: Betadine 16 to 30 mm (26.4), tincture of iodine 20 to 30 mm (25.71), Videne 16 to 30 mm (25.61), Lugol's solution 10 to 14 mm (11.33) and potassium iodide 10 to 24 mm (11.14) (Fig. 1).

For *Prototheca wickerhamii* strain, the inhibition areas were the following: Betadine 26 mm, tincture of iodine 24 mm, Videne 24 mm, Lugol's solution 10 mm, potassium iodide 14 mm (Fig. 2). There was no presence of drug resistant or resistant colonies in the inhibition zones. The inoculations carried out from the inhibition zones in glucose broth tubes remained sterile, confirming that the induced effect was algaeicide.

The results obtained at the test of different inoculum density according to McFarland standards. The aim of this test was to evaluate whether the inoculum density is influencing the size of the inhibition areas. Two strains were initially tested, *Prototheca zopfii* (strain 22) and *Prototheca wickerhamii* (ATCC collection strain).

It was found that the size of the inhibition zones has been less affected by the density of the inoculum, the recorded differences being of 2-4 mm between the smaller densities (0.5 to 1) and the larger (2, 3, 4 and 5) (Tab. 1, *P. zopfii* strain 22).

The results were similar for *P. wickerhamii* strain, as well as other 5 strains of *P. zopfii* subsequently tested.

Pictures with the inhibition areas obtained for the plates with the inoculum of different densities are observed below Fig. 3 (a and b).

The results obtained for diluted iodinated product. Algaeicide type inhibitory effect was found only in 10^{-1} dilution. In that dilution tube environment it remained clear, with a light purple colour (due to iodine). The other dilutions/tubes,

the medium also remained clear, for the first 24 hours, with the presence of reduced sediment, which easily disperse when stirring. During the examination at 48 hours was found a thin pellicle on the surface and the deposit became more abundant, meaning that algae multiplied.

The inoculations on glucose agar plates made from each dilution (after 48 hours) revealed that only 10^{-1} dilution have not developed colonies (algaecide effect). On the other dilutions (10^{-2} to 10^{-5}) *Prototheca* typical colony developed; at 24 hours they were small (less than 0.5 mm in diameter), circular, opaque, white, at 48-72 h, they grow in size (2-4 mm), having an irregular surface, the edges slightly lobed, white. A good characterization of colonies can be done by examining using the magnifying glass, where typical structure and other algae details (cauliflower surface aspect) were revealed and can differentiate in the situation of contamination by other bacteria colonies mainly staphylococci, micrococci and yeast.

When examining the wet smears (dispersion in a drop of Lügol), made of tubes that *Prototheca* dilutions remained viable (10^{-2} to 10^{-5}), it was found that the cells have undergone morphological changes, which reflected in the growth and formation of typical colonies on solid culture media. This behaviour was seen in both tested strains (*P. zopfii* and *P. wickerhamii*), both for iodine tincture and Betadine (Fig. 4 (a and b), Fig. 5 (a and b)). This means that at higher dilutions of 10^{-1} , algaecide effect is no longer achieved

due to dilution of the iodide product resulting in subinhibitory values.

Such results reveal that in order to obtain good efficiency in prevention or treatment of protothecosis, iodine based products must not be diluted more than 10^{-1} .

The inhibitory effect observed at different contact times. When examining the inoculated plates (glucose agar) after each contact time - 5, 10, 15, 30 and 60 minutes, it was observed that no colonies have developed in any of the tested strains after incubation at 37°C for 24 and 48 hours (Fig. 6 and 7). Algaecide effect manifested itself in the first 5 minutes of contact, for both iodine tincture and Betadine. The effect was algaecide, because the microorganisms presented no growth in culture plates even if maintaining the plates for the following 5 days at laboratory temperature.

The inoculations made in glucose broth from the areas corresponding to each contact time, and examined at 24 and 48 hours, remained sterile, confirming the induction of inhibitory effect. The experiment was repeated for another 5 strains, but the result was the same - total inhibition. The researches revealed that the iodine based products (Betadine and iodine tincture), have an inhibitory effect - algaecide, which is observed within the first five minutes of contact with the products. The choice of 5 minutes time as first stage of contact between the iodide products and a suspension of prothotecs, has been based on the comparison of the sensitivity of bacteria, which are usually inactivated in 15-30 seconds (according to

Fig. 1. Inhibition areas, *Prototheca zopfii* (strain 22)

Fig. 2. Inhibition areas, *Prototheca wickerhamii* (16529 ATCC)

the literature) (23). Knowing that prototecile are larger and have thicker cell wall, we considered that five minutes can be taken as the minimum time necessary to manifest inhibitory effect. In literature, we found no information in this regard.

DISCUSSION

The antimicrobial properties of iodine are known for long time, but research on the inhibitory effect of iodine and/or various iodine based products were amplified as a result of the increasing expansion of the phenomenon of antibiotic resistance. Numerous studies confirmed that iodine based products have bactericidal, sporicidal, cisticid and virulicid effect. Iodine exact mode of action is not fully known, but it is complex (Cooper, 2004; Bootham, 2009; Sibbald *et al.*, 2011).

It is believed that the iodine based products are able to quickly penetrate the cell wall of different microorganisms, causing structural and functional changes of the cell membranes and the nucleic acids by denaturation of proteins and enzymes, which results in rapid death of various microorganisms (Gottardi, 1999; Bootham, 2009; Sibbald *et al.*, 2011). The activity is immediately, but also over time due to persistence. The efficacy and stability of the aqueous solutions is influenced by the values of pH (Gottardi, 1999).

A broad use of these preparations is mentioned in the treatment of various types of wounds in which the antibiotic-resistant bacteria frequently colonized (Giacometti *et al.*, 2002). Direct application on wounds caused some controversy

about possible toxic effects on cells (Burck, 1988; Drosou *et al.*, 2003). It is believed that cytotoxicity effects are reduced and occur only in the event of prolonged treatments (Sibbald *et al.*, 2011). On the other hand, it is shown that the iodine preparations (povidone iodine and cadexomer iodine) reduced the bacterial load, which promotes epithelial regeneration and healing (McLure *et al.*, 1992; Lamme *et al.*, 1998; Mertz *et al.*, 1999).

Some products, such as iodins may even penetrate biofilms, which are formed on different surfaces, including wounds (Bootham, 2009; Phillips *et al.*, 2010). *In vitro*, microbicide effect has been demonstrated by various study methodologies. It shows that povidone iodine causes deadly alterations > 99% of microbial cells (methicillin susceptible and resistant *Staphylococcus aureus* strains) within 10 seconds of exposure (Lacey *et al.*, 1993). Iodine is active

Tab. 1. The size of inhibition areas at different densities of the inoculum

Density	Inhibition areas (ø mm), strain 22				
	Lugol sol.	Iodine tincture	Betadine	Videne	Potassium iodide
0,5	10	24	20	20	12
1	10	22	20	20	12
2	10	22	18	18	12
3	8	22	18	18	10
4	8	22	18	16	10
5	6	22	16	16	10

Fig. 3. Inhibition areas at different inoculum densities (*P. zoppii* strain 22)

against many pathogenic bacteria, most being killed in 15-30 seconds of contact [24].

It is important to note that iodine preparations do not develop resistance phenomena. However, in the case of *S. aureus* strains isolated from mastitic cow milk, a variation of the iodine minimum inhibitory concentration was described (Azizoglu *et al.*, 2013), and even the induction of *in vitro* resistance by repeated passages on culture media containing subinhibitory doses of antimicrobials (El Behiry *et al.*, 2012).

Unicellular algae of the genus *Prototheca* have become increasingly involved in the production of human and animal diseases, in both warm-blooded and cold-blooded animals. The disease, called protothecosis is manifested by the formation of granulomatous lesions with chronic evolution,

localized in different tissues and organs, with systemic forms described the cows (Taniyama *et al.*, 1994) and dogs (Souza *et al.*, 2009). Illness occurs more frequently in patients showing immunodeficiencies or other chronic diseases that are subject to abusive treatment with antibiotics. The greatest risk is observed in dairy cows with mastitis that can evolve as an epidemic outbreak. Following the multiplication and development of granulomas, normal tissue architecture and function is impaired, even affecting vital organs.

As a result of increasing frequency of cases and the antibiotic inefficiency, papers that have described the effect of iodine and iodine based products on *Prototheca* were published, being underlined their algacide effect. An *in vitro* study on 27 *P. zopfii* strains isolated from cow's milk

Fig. 4. *Prototheca zopfii* (strain 22)

Fig. 5. *Prototheca wickerhamii* (strain 16529 ATCC)

shows that iodine preparations showed algaecide effect at low concentrations (0.15652-0.625%) and recommends its use for routine hygiene (as pre- and post-dipping) in cattle mastitis control (Salerno *et al.*, 2010). The algaecide effect of povidone iodine was also highlighted on 20 *P. zopfii* strains, both biotype 1 (10 strains) and biotype 2 (10 strains) (Sobukawa *et al.*, 2011). In another study on 15 *P. zopfii* strains tested for sensitivity to iodine and chlorhexidine using dilution method on Sabouraud dextrose broth, was demonstrated that both drugs have algaecide effect, with minimum microbicide concentration (MMC): 0.3125%-1.25% for iodine and 0.0048 - 0.0195% for chlorhexidine (Krukowski *et al.*, 2013).

Iodine and iodine based products (inorganic and organic), having a high antimicrobial potential and broad spectrum, may find use in the treatment of many diseases, including those caused by unicellular algae of the genus *Prototheca*.

CONCLUSIONS

By agar diffusimetrical technique it was established that all tested *Prototheca* strains were sensitive to iodine products, the induced effect being algaecide.

Testing on different inoculum densities, allowed to conclude that the inhibitory effect occurred regardless of density, with small differences in the size of the inhibition zones.

Fig. 6. *Prototheca wickerhamii* (strain 16529 ATCC)
algaecide effect for different contact times (no colonies developed)

Fig. 7. *Prototheca zopfii* (strain 22)
algaecide effect for different contact times (no colonies developed)

Algaecide effect was also demonstrated by dilution method in liquid culture medium (dilution rate $\times 10$), stating that it occurred only at 10^{-1} dilution, and never produced at higher dilutions, respectively 10^{-2} to 10^{-5} .

In relation to the contact time, set for iodine tincture and betadine, it can be shown that the algaecide effect is fast, within 5 min of contact.

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