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In vitro antimicrobial and antifungal activities of aqueous skin mucus from rainbow trout (*Oncorhynchus mykiss*) on human pathogens.

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ABSTRACT

Extracts and preparations made from the animal origin were used extensively in folk and modern medicine for treating many human diseases. The objective of this study was to determine the antimicrobial and antifungal activities of aqueous skin mucus from rainbow trout. Evaluation of inhibition effect of the aqueous skin mucus against *Staphylococcus aureus*-ATCC 6538, *Escherichia coli*-ATCC 9837, *Citrobacter freundii*-ATCC 8090, *Enterobacter aerogenes*-ATCC 13048, *Neisseria lactamica*-ATCC 23970, *Proteus vulgaris*-ATCC 49990, *Proteus mirabilis*-ATCC 29906, *Pseudomonas aeruginosa*-ATCC 9027, *Pseudomonas fluorescens*-ATCC 13525, *Bacillus cereus*-ATCC 10987, *Klebsiella pneumonia*-ATCC 13883, *Klebsiella oxyttoca*-ATCC 43863, *Streptococcus pneumoniae*-ATCC 49619, *Candida albicans*-ATCC 90028 and *Candida parapsilosis*-ATCC 22019 was tested. The antimicrobial and antifungal effects of mucus on the bacteria were assessed by a disc diffusion assay. The activity was measured in terms of zone of inhibition in mm. The mucus did not show antibacterial and antifungal activities against any of the tested strains of bacteria and fungi yeast.

Introduction

It can be thought fish live in a microbe-rich environment and are vulnerable to invasion by pathogenic or opportunistic microorganisms since water is a perfect medium for bacteria and parasitic microbes (Ellis, 2001). The main function of mucus layer secreted from epidermal and epithelial cells is to compose the biological interface between fish and their aqueous environment (Pickering, 1974; Ellis, 1999). It is also thought to serve as a lubricant in locomotion and osmoregulation (Rosen and Cornford, 1971; Cameron and Endean, 1973), and a defense barrier in the prevention of surface colonization by bacteria and fungi (Alexander and Ingram, 1992). Furthermore, it has a mechanical protective function (Cameron and Endean, 1973), and some function in intra-species chemical communication (Saglio and Blanc, 1989). The skin mucus is produced primarily by epidermal goblet or mucus cells and is composed of biochemically diverse secretions, which contain a number of molecular compounds such as complement, transferrin, lysozyme, C-reactive protein and antimicrobial proteins and peptides (Negus, 1963; Aranishi and Nakane, 1998; Subramanian et al. 2007).

Over the past years, it has also been shown that mucus plays a role in the prevention of colonization by parasites, bacteria and fungi and the antibacterial role of mucus has been known for many years (Austin and McIntosh 1988). Fish mucus was found as a source of antimicrobial products (Hellio et al. 2002). We are not aware of any reports addressed to the antimicrobial and antifungal activities of rainbow trout (*O. mykiss*) skin aqueous mucus on human pathogens. Therefore, we aimed to investigate the *in vitro* antibacterial and antifungal activities of the mucus on human pathogens.

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Material and methods

Animals

One year old 50 freshwater rainbow trout (200±20g) were obtained from the Fisheries Department of Agricultural Faculty at Atatürk University in Erzurum and maintained under standard conditions. Fish treatment protocols were conducted according to Applied Research Ethics National Association (2002).

External mucus collection

Hypothermic stress was used to stimulate mucus production. Live whole fish were firstly cleaned by dipping them into distilled water to remove any apparent dirt. Fish were then placed into an enclosed plastic bag with approximately an equal volume of distilled water at room temperature. The bag was sealed and finally transferred to a freezer at -20°C for 1hr to induce a hypothermic stress condition. At the end of the 1hr, the mucus was collected by wiping the fish clean using the same plastic bag. The mucus extract was centrifuged at 2000 rpm for 5 min. The obtained supernatant was stored at 4°C until analysis.

Preparation of test microorganisms

Gram-positive bacteria (*Staphylococcus aureus*-ATCC 6538, *Bacillus cereus* and *Streptococcus pneumonia*), gram-negative bacteria (*Escherichia coli*-ATCC 9837, *Citrobacter freundii*-ATCC 8090, *Enterobacter aerogenes*-ATCC 13048, *Neisseria lactamica*-ATCC 23970, *Proteus vulgaris*-ATCC 49990, *Proteus mirabilis*-ATCC 29906, *Pseudomonas aeruginosa*-ATCC 9027, *Pseudomonas fluorescens*-ATCC 13525, *Klebsiella pneumonia*-ATCC 13883, *Klebsiella oxyttoca*-ATCC 43863), and fungi yeast (*Candida albicans*-ATCC 90028 and *Candida parapsilosis*-ATCC 22019) were employed for determination of antimicrobial and antifungal activity. Microorganisms that can be pathogenic for humans and animals were used in this study. The bacterial and fungal stock cultures were maintained on Mueller Hinton Agar (Oxoid CM 337, Basingstoke, Hampshire, UK) slants, respectively, which were stored at 4°C. For the purpose of antibacterial evaluation, 13 microorganisms were used. These bacteria were maintained on a Blood Agar Base (Oxoid CM55, Basingstoke, Hampshire, UK). The yeast was maintained on Sabouraud dextrose agar (Oxoid CM41, Basingstoke, Hampshire, UK), which is often used with antifungal for the isolation of pathogenic fungi.

Determination of antimicrobial and antifungal activities

The antibacterial and antifungal activities of the skin mucus were determined by a disc diffusion assay. Agar cultures of the test microorganisms were prepared. For this purpose, 3 to 5 similar colonies were selected and transferred with loops into 5 mL of Tryptone soya broth (Oxoid CM129, Basingstoke, Hampshire, UK). Tryptone soya broth is a highly nutritious and versatile medium, recommended for general laboratory use and used for the cultivation of aerobes and facultative anaerobes, including some fungi. The broth cultures were incubated for 24 h at 37°C. For screening, sterile, 6-mm diameter filter paper discs were impregnated with 45 µL of the mucus extract sterilized by mechanical filtration. 0.85% NaCl solution was used to prepare as the control samples. Positive controls were prepared with the same solution. Then the paper discs were placed onto Mueller Hinton agar (Oxoid CM337, Basingstoke, Hampshire, UK). The inoculums for each organism were prepared from broth cultures. The concentration of the cultures was to 10^8 colony forming units (1×10^8 CFU mL^{-1}). The results were recorded by measuring the zones of growth inhibition surrounding the discs. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All data on antimicrobial activity are the average of triplicate analyses. Ampicillin (10 µg $disc^{-1}$), amoxicillin (25 µg $disc^{-1}$), cefuroxime (30 µg $disc^{-1}$), and antifungal miconazole nitrate (40 µg $disc^{-1}$, DRG International) were used as reference standards to determine the sensitivity of one strain in each tested microbial species, as recommended by the Clinical and Laboratory Standards Institute (46). Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Results

In this study, 13 different bacterial and 2 different Candida species were used to screen the possible antibacterial and antifungal activities of skin mucus extract (Table 1). However, none of the gram-positive and gram-negative bacterial species and the Candida species was inhibited by the skin mucus extract. Ampicillin (10 µg $disc^{-1}$), amoxicillin (25 µg $disc^{-1}$) and cefuroxime (30 µg $disc^{-1}$) were used as positive controls for bacteria. Miconazole nitrate (40 mg $disc^{-1}$) was used as a positive control for antifungal activity.

Discussion

Earlier studies have reported that not only the antimicrobial property of crude epidermal mucus but also a number of antibacterial factors in the mucus of rainbow trout against infectious fish pathogens have been demonstrated in rainbow trout (Austin and McIntosh, 1988; Rainger and Rowley, 1993). However little information is available on the antimicrobial activity of the trout against human pathogens.

The protective role of mucus and its components in various fish species suggests that the epidermal mucus acts as a first line of defense against pathogens and therefore may offer a potential source of various innate antimicrobial agents such as lysozyme, cathepsin B, muramidases and trypsin-like proteases (Fouz et al. 1990; Barnes et al. 2003; Svendsen and Bogwald 1997; Nagashima et al. 2001; Sarmaşik, 2002; Subramanian et al. 2007). These components of crude epidermal mucus exhibit strong pore-forming properties, which were well correlated with antibacterial activity (Ebran et al. 1999). For instance Smith et al. (2000) indicated that the lysozyme from rainbow

Table 1. Antibacterial and antifungal activity of skin mucus extract from rainbow trout against bacteria strains based on disc-diffusion assay. Antimicrobial and antifungal results are the averages of triplicate measurements. AMP: Ampicillin (45 mg $disc^{-1}$); AMC: Amoxicillin (45 mg $disc^{-1}$); CEF: cefuroxime (45 g $disc^{-1}$); MN: Miconazole nitrate (45 mg $disc^{-1}$); ND: Not detected activity at this concentration; S: Sensitivity; R: Resistant.

	Diameter of mucus extract zone (mm)	Antimicrobial agent (mm)						Antifungal activity (mm)	
		AMP		AMC		CEF		Mucus Extract	MN
		R	S	R	S	R	S		
*Staphylococcus aureus*_ATCC 6538	ND	-	12	-	11	-	17	-	-
*Escherichia coli*_ATCC 9837	ND	-	16	-	115	24	-	-	-
*Citrobacter freundii*_ATCC 8090	ND	22	-	21	-	30	-	-	-
*Enterobacter aerogenes*_ATCC 13048	ND	-	ND	-	ND	-	11	-	-
*Neisseria lactamica*_ATCC 23970	ND	24	-	24	-	28	-	-	-
*Proteus vulgaris*_ATCC 49990	ND	-	ND	-	ND	17	-	-	-
*Proteus mirabilis*_ATCC 29906	ND	-	ND	-	ND	17	-	-	-
*Pseudomonas aeruginosa*_ATCC 9027	ND	-	ND	-	ND	-	11		-
*Pseudomonas fluorescens*_ATCC 13525	ND	-	15	-	17	26	-	-	-
*Bacillus cereus*_ATCC 10987	ND	23	-	21	-	35	-	-	-
*Klebsiella pneumonia*_ATCC 13883	ND	-	ND	-	ND	22	-	-	-
*Klebsiella oxyttoca*_ATCC 43863	ND	-	10	-	11	19	-	-	-
*Streptococcus pneumoniae*_ATCC49619	ND	23	-	27	-	37	-	-	-
*Candida albicans*_ATCC 90028	-	-	-	-	-	-	-	ND	21
*Candida parapsilosis*_ATCC22019	-	-	-	-	-	-	-	ND	20

trout mucus showed bacteriolytic activity against *Planococcus citreus*. Besides the lysozyme from yellowtail (*Seriola quinqueradiata*), common carp (*C. carpio*) and rainbow trout (*O. mykiss*) mucus were also shown to have bacteriolytic activity against *Micrococcus lysodeikticus*, *Pasteurella piscicida*, *L. anguillarum* and *Micrococcus luteus* (Takahashi et al. 1987; Itami et al. 1987; Smith et al. 2000). Similarly, proteases such as trypsin and cathepsin B and L from rainbow trout (*O. mykiss*) and eel species (*A. anguilla* and *A. japonica*) mucus, respectively, exhibited bacteriolytic activity against *L. anguillarum*, *Edwardsiella tarda*, and *Flavobacterium columnare* (Hjelmeland et al. 1983; Aranishi et al. 1998; Aranishi, 2000). In addition, two novel skin antibacterial muramidases were purified from rainbow trout and those probably contribute to epithelial defense of the fish against microbes, either alone or in synergism with antibacterial peptides (Svendsen and Bogwald, 1997). An antimicrobial peptide which may play a role in protection against intracellular or extracellular pathogens was also purified by cation exchange and reverse phase chromatography from skin of rainbow trout (Fernandes and Smith, 2002). Pleurocidin isolated from skin mucous secretions of the winter flounder displays a strong antimicrobial activity (Saint et al. 2002). Birkemo et al. (2003) were reported that hipposin in the skin mucus of Atlantic halibut showed strong antimicrobial activity against several bacteria.

In the current study, aqueous mucus extract did not exhibit any antibacterial and antifungal activities on human pathogens. Earlier studies have also reported that, no or little antimicrobial activity had been detected in the aqueous mucus extract of various fish species including Arctic char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), koi carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), haddock (*Melanogrammus aeglefinus*) and hagfish (*Myxine glutinosa*) (Hellio et al. 2002; Subramanian et al. 2008). Similarly, in a different study, the crude, acidic and aqueous extracts were prepared from the epidermal mucus of snakehead fish *Channa striatus* (Wei et al. 2010) and the crude and aqueous extracts showed a detectable level of bactericidal activity against the fish pathogen *Aeromonas hydrophila* but no activity against human pathogens among the three extracts. The absence of antimicrobial and antifungal activities of the aqueous mucus in this study could be due to disuse of different extraction methods and/or the inactivation of the special enzymes such as lysozyme, trypsin, cathepsin B and L in mucus by the incubation temperature and or pH conditions used in the antimicrobial assay.

In conclusion, in this research we investigated the antimicrobial and antifungal activities of rainbow trout (*O. mykiss*) aqueous mucus extract against 15 human pathogens. Aqueous extract inhibited none of the human pathogens. However, further investigations can be focused on the antimicrobial and antifungal activities of the different rainbow trout skin mucus extracts on human and

fish pathogens.

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