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ANTIBIOTIC RESISTANCE OF FECAL COLIFORM BACTERIA INHABITING SEA WATER AND SAND OF MARINE RECREATION BEACH IN THE SOUTHERN BALTIC SEA

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Abstract

The results of the present study showed variability in resistance of FC bacteria isolated from beach sand and sea water against tested antibiotics. Enteric bacteria were the most resistant to clindamycin and penicillin while the most sensitive to ciprofloxacin, gentamycin, neomycin, rifampicin and streptomycin. Fecal coliform bacteria inhabiting sand were more resistant to nearly all tested antibiotics compared to bacteria isolated from sea water. The majority of bacteria inhabiting sea water and sand of the studied beach were resistant to only 1-4 antibiotics out of the 12 antibiotics tested. Fecal coliform bacteria isolated from Ustka beach were the most resistant to β-lactam and lincosamide antibiotics, while the most susceptible to aminoglycosides.

Key words: Baltic Sea, beach, fecal coliform, antibiotic resistance

INTRODUCTION

Numerous studies (Bonilla et al. 2007, Elmir et al. 2007) showed increased contamination by fecal coliforms (FC), of the sand of many recreational beaches. Fecal contamination of beaches can present significant ecological implication, public health hazards, loss of recreation opportunities and costly impact for local economies (Edge and Hill 2007). Main sources of fecal pollution of sandy beaches are municipal wastewater effluents, agricultural runoff, aquaculture, fecal droppings from birds and recreational users (Kümmerer 2009, Ding and He 2010). Hartz et al. (2008) show that bacterial fecal indicators can persist in sand throughout the year without variation and replication of cells because particulate provide microbes with a unique microhabitat affording protection, increased moisture and nutrients.

Beside enteric bacteria, aquatic ecosystems are contaminated with different pharmaceutical substances, mainly antibiotics (Brown et al. 2006). During recent decades, natural and mainly chemically synthesised antibiotics are discharged in various amounts through many pathways into the aquatic environments as a result of indiscriminate use of those organic compounds in medical and veterinary practice for therapeutic and prophylactic purposes and as growth promoters in agriculture and aquaculture (Wose-King et al. 2010). Simultaneous increased concentration of antibiotic in aquatic ecosystems generated new selective pressure on natural bacterial populations (Alpay-Karaoglu et al. 2007). Antibiotics may eliminate sensitive bacteria, which might play an important ecological role of natural microbial communities (Ding and He 2010, Umamaheswari and Anbusaravanan 2010). On the other hand these drugs generate increase of a number of antibiotic-resistant bacteria in aquatic environments (Matyar et al. 2007, Perliński and Mudryk 2009). The antibiotic-resistant bacteria inhabiting water basins may create a serious public hazard due to the possible transformation of resistant factor from non - pathogens to pathogens (Al-Mossawi et al. 1982).

Recently antibiotic resistance of enterobacteria has increased dramatically in marine ecosystems in different parts of the world (Wang et al. 2008, Fernadez-Delgado and Suarez 2009). Fecal coliform bacteria, mainly *Escherichia coli*, have been generally accepted as the predominant vehicle for the dissemination of resistance genes and vectors due to its abundance in water ecosystems (Alpay-Karaoglu et al. 2007). Grabow and Prozesky (1973) showed that about 30% of all fecal coliform bacteria had transferable antibiotic resistance. The rapid increase of antibiotic-resistant fecal coliforms is due, in part, to ability of these bacteria to horizontal transfer of antibiotic resistance genes among the bacterial population by cell to cell contact (Reinthaler et al. 2003). The agent responsible for horizontal transfer of resistance is an extrachromosal genetic element termed an R-factor or R-plasmid. Bacteria containing R-plasmids were isolated from humans and animals at a high frequency of occurrence and this problem is well documented (Alpay-Karaoglu et al. 2007).

Many recent studies were carried out in different water ecosystems, like a coastal lake (Ahmed et al. 2008), bay (Parveen et al. 1997), marine coastal waters (Al-Mossawi et al. 1982), to determine the distribution of fecal coliform bacteria resistant to antibiotics. But to the best of our knowledge nothing is known about antibiotic resistance of those bacteria inhabiting sand of marine beaches. Thus, the aim of the present study was to investigate the incidence of antibiotic resistance among fecal coliform bacteria isolated from sea water and sand of a recreational marine beach.

MATERIAL AND METHODS

1. Study area and sampling

The study was carried out on an exposed sandy beach of Ustka town (54°35`N, 16°51`E; southern Baltic Sea) (Fig. 1). It represents a dissipative beach type with longshore bars and troughs; its width is ca. 75 m. In general, the size of sand grains

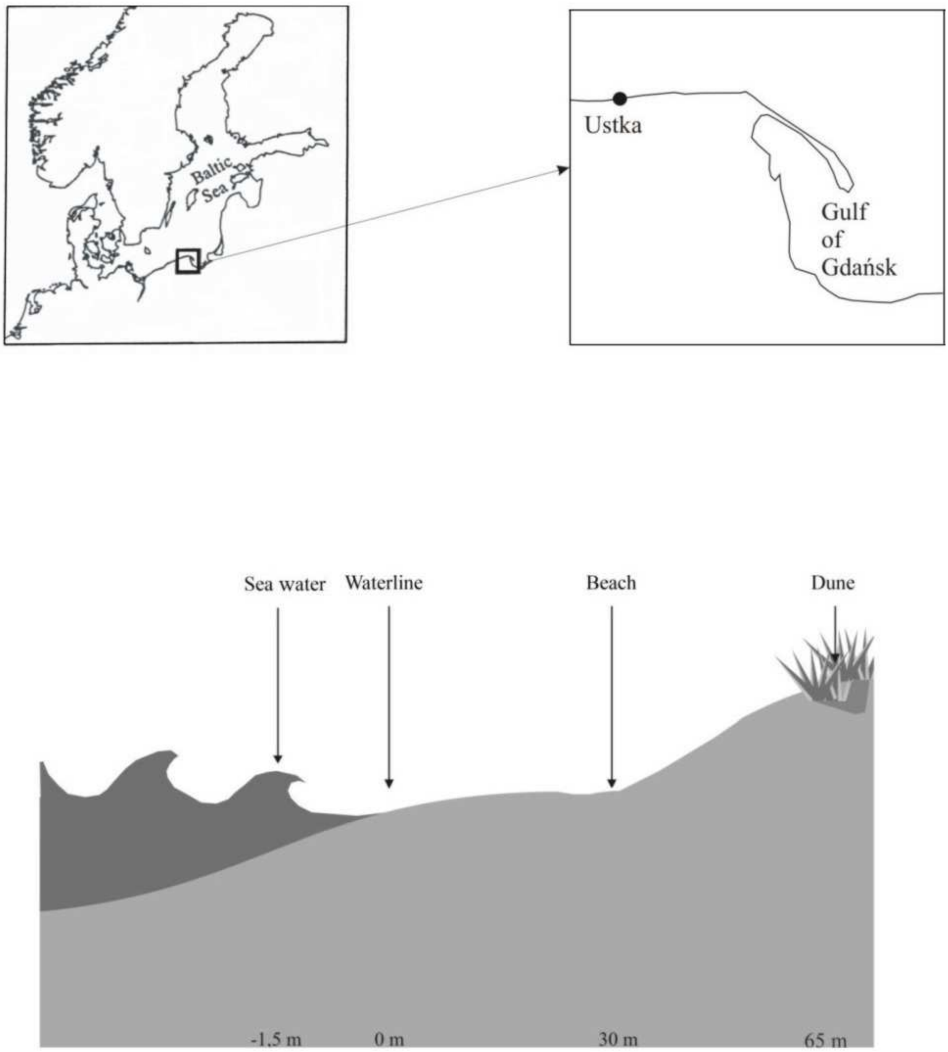

Fig. 1. Location of sampling sites on the sandy beach in Ustka

is between 0.125-0.250 mm (Kramarska et al. 2003). Ustka Beach with its surf zone is one of the most picturesque and very popular bathing beach in Poland. Polish and foreign tourists, and local inhabitants intensively visit this beach and it is usually very crowded, particularly during the summer months. Two local sources of contamination, i.e. pollutants carried by the Słupia River and large shore bird populations have an impact on the studied beach. The Słupia River carries $15.5 m^3/s$ of water into the sea, as well as 200 000 - 300 000 $m^3 \cdot year^{-1}$ of natural and anthropogenic sediments (Zawadzka 1996). There are several colonies of gulls along the studied beach and their population grows rapidly in Ustka town. Moreover large population

of swans breeds in the vicinity and they became increasingly common in the beach areas.
Sand and sea water samples were collected at Ustka Beach during summer 2009 in july. Samples of sand were taken from four sites along a transect perpendicular to the shoreline (Fig. 1). Wet sand was collected from two sites situated at the waterline and approximately 1-1.5 m from the waterline at a depth of about 1 m. Dry sand was also collected from two sites – one located at halfway up the beach at a 30 m distance from the shore and another located on the dune, 60 m away from the shore. Sand core samples were taken with a hand-operated sampler (length – 30 cm, inner diameter – 15 cm). Already in the field, the sampled cores were divided into two sections: 0-5 cm and 10-15 cm and placed in sterile plastic jars. Sea water samples were collected in sterile bottles within a 1.5 m from the waterline at a depth of about 15 cm. Jars and bottles were put into containers with ice and transported to the laboratory. The time between sample collection and bacteriological analyses did not usually exceed 2-3 h.

2. Isolation of fecal coliform bacteria

To isolate fecal coliform (FC) bacteria 45.0 g of sand samples were weighed aseptically and transferred to 180 cm^3 of sterile 8‰ artificial sea water, and then they were shaken vigorously by hand for about 10 min to suspend bacteria. Following 30 min sedimentation, the supernatant and sea water samples were filtered through a 0.45-μm pore size, 47 mm-diameter membrane filter (Whatman ME 25/31 ST). Next the filters with collected bacteria were aseptically transferred to the dish containing 10 cm^3 of ECD MUG Agar (Fluka) and incubated for 48 h at 44°C in a portable incubator. After incubation the colonies on the membrane filter were examined with a Wood's lamp for fluorescence (UV light; 366 nm). Greenish fluorescent colonies indicated cleavage of 4-methylumbelliferyl-ß-D-glucuronide (MUG) by β-D-glucuronidase and release of fluorescent MUG compound, which can be detected under UV light. Fluorescence indicated colonies showing β-D-glucuronidase activity and these were counted as fecal coliform bacteria. A total of 100 fecal coliform strains were isolated from sand (25 from each site) and 25 from sea water samples.

3. Determination of antibiotic resistance

The Bauer-Kriby disc-diffusion method (Arvanitidou et al. 1997) was used to assess antibiotic resistance of fecal coliform bacteria. All the details concerning the methodical determination of antibiotic resistance are presented in the works (Mudryk 2005, Mudryk et al. 2010). We tested fecal coliform bacteria strains with the following twelve antibiotics, which are commonly used in medical and veterinary practice (their concentration is given in parentheses): amoxicillin (AX, 25 μg); ampicillin (AM, 10 μg); ciprofloxacin (CIP, 5 μg); clindamycin (DA, 2 μg); gentamycin (CN, 10 μg); erythromycin (E, 10 μg); neomycin (N, 30 μg); penicillin (P, 10 μg); rifampicin (RA, 5 μg); streptomycin (S, 30 μg); oxytetracycline (T, 30 μg); tetracycline

(TE, 30 μg). The Antibiotic Resistance Index (ARI) of fecal coliform bacteria was calculated according to Webster at al. (2004). All tested antibiotics were divided into groups according to their chemical structure: aminoglycosides (AM) (gentamycin, neomycin, streptomycin), macrolides (MA) (erythromycin), β-lactams (LA) (amoxicillin, ampicillin, penicillin), rifampicins (RIF) (rifampicin), lincosamides (LI) (clindamycin), quinolones (QU) (ciprofloxacin), tetracyclines (TET) (oxytetracycline, tetracycline) (Reinthaler et al. 2003, Mudryk 2005, Mudryk et al. 2010).

RESULT

The incidence of antibiotic resistance of fecal coliform bacteria inhabiting Ustka Beach is given in Table 1. These results showed a great variability in resistance of FC bacteria isolated from sand and sea water against tested antibiotics. The highest percentage (79-96%) of all tested strains isolated from wet and dry sand was resistant to clindamycin and penicillin, while only 3-5% of these strains were resistant to ciprofloxacin, gentamycin, neomycin, rifampicin and streptomycin. Among fecal coliform bacteria isolated from sea water, the highest frequency (92%) of resistance was found against penicillin. All FC bacteria inhabiting sea water were sensitive to gentamycin, neomycin and streptomycin. Data presented in Table 1 showed the difference between fecal coliform bacteria inhabiting sand and sea water in their resis-

Table 1

Resistance to different antibiotic of fecal coliform isolated from sand and sea water of studied beach (percentages derived from the pooled data of two sand layers)

Antibiotics	Stations		
	sea water	wet sand	dry sand
Amoxicillin (AX)	8.0	25.3	20.0
Ampicillin (AM)	20.0	31.3	25.0
Ciprofloxacin (CIP)	4.0	8.1	2.0
Erythromycin (E)	24.0	38.4	29.0
Gentamycin (CN)	0.0	7.1	0.0
Clindamycin (DA)	4.0	96.0	91.0
Neomycyn (N)	0.0	2.0	4.0
Oxytetracycline (T)	24.0	28.3	13.0
Penicillin (P)	92.0	81.8	79.0
Rifampicin (RA)	4.0	3.0	7.0
Streptomycyn (S)	0.0	2.0	4.0
Tetracycline (TE)	28.0	14.1	12.0
ARI	0.17	0.28	0.24

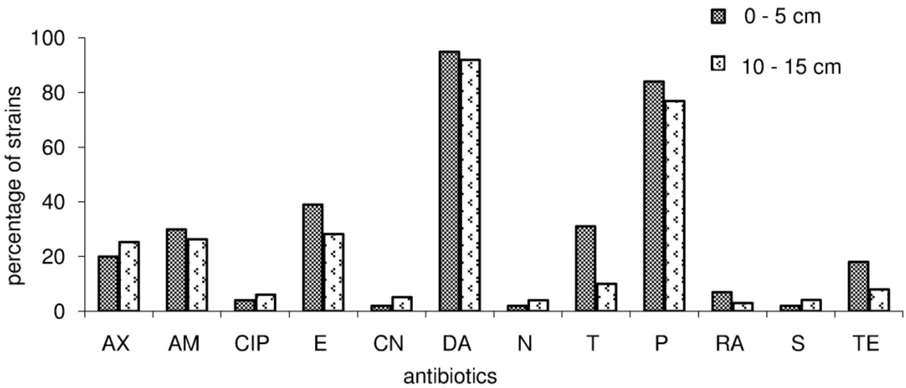

Fig. 2. Percentage of antibiotic resistant bacteria in surface (0-5 cm) and subsurface (10-15 cm) sand layer (percentages derived from the pooled data of all sites)

tance to antibiotics tested in this study. Enteric bacteria isolated from wet sand were more resistant (ARI 0.28) to these antibiotics than bacteria isolated from dry sand (ARI 0.24) and sea water (ARI 0.17).

Data on the antibiotic resistance level of FC strains inhabiting surface (0-5 cm) and subsurface (10-15 cm) sand layers are given in Figure 2. These results showed quite similar proportion of antibiotic-resistant bacteria in both layers. Only in the case of rifampicin, oxytetracycline and tetracycline the resistance level of fecal coliform bacteria isolated from the surface sand layer was higher compared to those isolated from the subsurface layer.

The collection of fecal coliform bacteria isolated from sea water and sand was also analyzed for the multiple antibiotic resistance (MAR) (Table 2). Bacteria resistant against more than one out of the twelve analyzed antibiotics were considered as MAR. Prevalence of the multiple antibiotic resistance varied from 2 to 9 antibiotics in fecal coliform bacteria inhabiting wet sand and from 2 to 8 in those isolated from dry sand. 83-91% of fecal coliform bacteria strains isolated from sand of the studied beach were resistant to at least two tested antibiotics. Most FC strains inhabiting wet

Table 2

Multiple antibiotic resistance fecal coliform strains inhabiting sand and sea water of studied beach (percentages derived from the pooled data of two sand layers)

	Numbers of antibiotics													% multiple resistant
Stations	0R	1R	2R	3R	4R	5R	6R	7R	8R	9R	10R	11R	12R	
Sea water	4.0	40.0	24.0	12.0	16.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	56.0
Wet sand	1.0	8.1	29.3	23.2	15.2	8.1	9.1	4.0	1.0	1.0	0.0	0.0	0.0	90.9
Dry sand	1.0	16.0	36.0	16.0	15.0	9.0	5.0	1.0	1.0	0.0	0.0	0.0	0.0	83.0

(29%) and dry sand (36%) of the studied beach were resistant to 2 out of the 12 tested antibiotics. Triple and quarter resistance against antibiotics was recorded in 15-23% of fecal coliform bacteria inhabiting wet sand and 15-16% in those isolated from dry sand. Only 1-9% of strains showed resistance to 5-8 antibiotics, while no strains isolated from wet and dry sand was resistant to 10-12 antibiotics.

56% of fecal coliform bacteria isolated from sea water showed resistance to at least two antibiotics and most isolated strains (40%) were resistant to one antibiotic (Table 2). Double resistance was observed in 24% of the strains and 12-16% presented triple and quarter resistance. Only 4% of bacteria demonstrated resistance to 5 antibiotics and none of FC bacteria inhabiting sea water was resistant to 6-12 antibiotics. The proportional resistance of enteric bacteria inhabiting sand and sea water to different classes of antibiotics is shown in Figure 3. The results of the present study

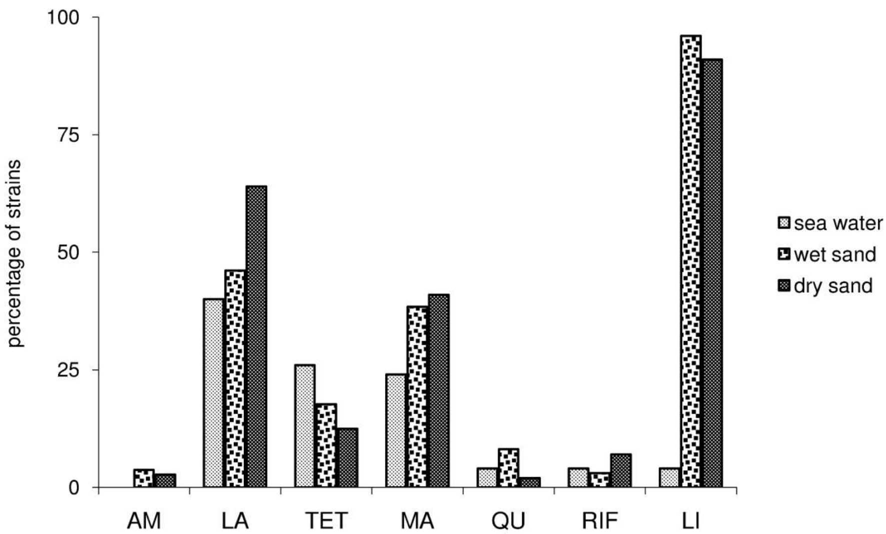

Fig. 3. The resistance of bacteria with respect to their chemical structure (in percentage)

showed that fecal coliform bacteria resistance against antibiotics depended on their chemical structure. FC strains isolated from wet and dry sand of Ustka Beach were the most resistant to lincosamides (91-96%), while they showed the lowest resistance (3-4%) against aminoglycosides.

Fecal coliform bacteria isolated from sea water were the most resistant to β-lactams (40%), while all these bacteria were sensitive to aminoglycoside antibiotics.

DISCUSSION

The results of this study showed occurrence of antibiotic-resistant fecal coliform bacteria in both sea water and sand of the studied recreational Ustka beach. Birds and humans may be the sources of these enteric bacteria. According to Bonilla et al. (2007) and Edge and Hill (2007) the feces of marine birds, mainly gulls, occupying the beaches have been significant non-point source of fecal bacteria in sea water and sand of the beach. Gould and Flechter (1978) estimated that the average wet weight of feces excreted by different gull species ranged from 11.2 to 24.9 g day^{-1} and one gull could produce between 34 and 62 of fecal droppings in a day. This would result in an average daily load of fecal coliform bacteria up to 1.2 x 10^{10} from one gull on the beach. Literak et al. (2010), in the study on the coast of the Baltic Sea in Poland, showed that even if populations of wild birds were not directly influenced by antibiotic practice, still antibiotic-resistant coliform bacteria were found in the feces of these birds.

In Ustka beach recreational users, beside birds, could be significant source of antibiotic-resistant fecal coliform bacteria, particularly in summer, what was already reported from other regions (Whitman and Nevers 2003). Lots of people in summer spend much of their time in sea water and on the sand of beaches which may accumulate fecal bacteria shed from human body (Bonilla et al. 2007). The study by Elmir et al. (2007) showed that bathers shed appreciable amount of bacteria via their bodies into sea water and sand.

Among all enteric bacteria isolated from sea water and sand of Ustka Beach, the highest percentage (79-96%) of strains was resistant to clindamycin and penicillin. Several other studies also showed that resistance to these antibiotics was relatively common amongst FC bacteria isolated from various aquatic ecosystems. Fecal coliform bacteria isolated from sachet-water manufactured in Abakaliki (Nigeria) 100% were resistant to clindamycin (Ngozi et al. 2010). Also Mudryk (2005), Mudryk and Skórczewski (2009) reported that 45-90% of heterotrophic bacteria inhabiting the downtown pond, estuarine lake and marine beach were resistant to clindamycin. Clindamycin is mainly bacteriostatic antibiotic inhibiting protein synthesis by binding to the some of 50s ribosomal subunits and according to Ding and He (2010) is not active against most strains of FC.

Among fecal coliforms isolated from recreational coastal waters of the Caribbean Sea and Aksu River (Turkey) the highest bacterial resistance index was recorded for penicillin, i.e. 100% (Toroglu et al. 2005, Ferndandez-Delgado and Suarez 2009). About 73% of fecal coliform bacteria isolated from the Mhlathuze River (RSA) were resistant to penicillin (Lin et al. 2004). High resistance level of the studied isolates to penicillin could result from cross-resistance, as this antibiotic is inactivated by chromosomal beta-lactamases produced by many species of family *Enterobacteriaceae* (Goni-Urrriza et al. 2000).

According to the results of the present study fecal coliform strains isolated from different parts of the studied beach differed in the level of antibiotic resistance. Bacteria inhabiting wet sand (sea water, shoreline zone) were more antibiotic-resistant than bacteria isolated from dry sand (the middle part of the beach and dune). Also Mudryk et al. (2010) and Oliveira et al. (2010) documented the difference in distribution of bacteria showing varied antibiotic resistance, which inhabited wet and dry sand of marine beaches.

The study carried out by Mudryk (2005) at marine sandy beach in Sopot (southern Baltic Sea) showed that heterotrophic bacteria isolated from the surface sand layers were more resistant to nearly all tested antibiotics compared to bacteria from the subsurface layers. In the present study generally no difference was found in antibiotic resistance of fecal coliform bacteria inhabiting surface and subsurface sand layer. This is in agreement with the results given by Mudryk et al. (2010) for antibiotic-resistant heterotrophic bacteria isolated from a non-recreational beach in Czołpino (southern Baltic Sea).

According to Akinbowale et al. (2006) many bacteria inhabiting water basins show multiple antibiotic resistance coded on plasmids, mutational events or on even smaller and mobile genetic elements called transposons (Meirelles-Pereira et al. 2002). According to Manivasagan et al. (2011) the difference in the level of bacteria resistant to various antibiotics may reflect the history of antibiotics' application and hence serve as an indicator. Skórczewski et al. (2013) reported that the majority of fecal coliform bacteria isolated from a downtown pond and coastal lake were resistant to 6-12 antibiotics out of the 18 tested. In the present study, however, the majority of fecal coliform bacteria inhabiting sea water and sand of the studied beach were resistant only to 2-3 antibiotics out of twelve used in this study. That means that they are not perfectly capable of detoxicating those antimicrobial compounds.

Most classes of antibiotics that vary in molecular structure and physicochemical properties are approved for both human and animal use and are introduced into water ecosystems (Harwood et al. 2000). The present data demonstrated that fecal coliform bacteria isolated from sand of Ustka beach were the most resistant to lincosamide antibiotics. Lincosamides are one of the commonly used antibiotic classes in medical and veterinary practice, which occur in many water ecosystems (Andreozzi et al. 2006). Thus resistance of bacteria isolated from Ustka beach to these antibiotics is not surprising as it corresponds to what has already been found in the studies in other water basins (Calamari et al. 2003, Skórczewski et al. 2013). According to Lüthje and Schwarz (2007) resistance of bacterial strains to these antibiotics can be due to target site modification, active efflux mechanisms, mutations and enzymatic inactivation of the drugs. Our results also indicate that fecal coliform bacteria are able to follow at least one of the mechanisms given above, since such a high percentage of those organisms were resistant to lincosamide antibiotics.

Fecal coliform bacteria isolated from sea water and sand of Ustka beach were the most susceptible to aminoglycoside antibiotics. This means that studied bacteria were not capable of actively detoxifying that class of antibiotics. Goni-Urriza et al. (2000) also reported low resistance of enterobacteria against this antimicrobial group.

In conclusion, the presence of a high number of antibiotic-resistant fecal coliform bacteria in sea water and sand of recreational marine beach may have public health implications. This emphasizes the need for further studies, particularly on occurrence of bacteria in coastal zone as well as the possibility of the returning of resistance genes to the human population through water usage. In our opinion occurrence of antibiotic-resistant fecal coliform bacteria in water basins could possibly be used as an alternative indicator of fecal contamination.

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ANTYBIOTYKOOPORNOŚĆ BAKTERII KAŁOWYCH Z GRUPY COLI ZASIEDLAJĄCYCH WODĘ MORSKĄ I PIASEK REKREACYJNEJ PLAŻY NA OBSZARZE BAŁTYKU POŁUDNIOWEGO

Streszczenie

Badano oporność na antybiotyki bakterii fekalnych wyizolowanych z wody morskiej i piasku rekreacyjnej plaży znajdującej się na południowym wybrzeżu Morza Bałtyckiego. Uzyskane wyniki badań wykazały zmienność w oporności bakterii z grupy coli wyizolowanych z piasku i wody morskiej wobec badanych antybiotyków. Mikroorganizmy te były najbardziej odporne na penicylinę i klindamycynę, a najbardziej wrażliwe na ciprofloksacynę, gentamycynę, neomycynę, ryfampicyny i streptomycyny. Bakterie kałowe z grupy coli zasiedlające piasek były bardziej odporne na prawie wszystkie badane antybiotyki niż te izolowane z wody morskiej. Większość bakterii zasiedlających wodę morską i piasek badanej plaży była odporna na zaledwie 1-4 antybiotyków z 12 badanych. Bakterie kałowe z grupy coli izolowane na plaży w Ustce były najbardziej oporne na antybiotyki beta-laktamowe oraz linkozamidy, a najbardziej podatne na aminoglikozydy.