Antibiotic Resistance of *Escherichia Coli* Isolated from Intestinal Tract of *Cyprinus Carpio*

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Abstract
In the present study, were determined antibiotic resistance of *Escherichia coli* isolated from intestinal tract of fishes (*Cyprinus carpio*), which were intended for human consumption. The 110 samples of *E. coli* were collected from swabs of fish (*Cyprinus carpio).* Cultivation of *Enterobacteriaceae* genera was done at 37 °C during 24 hours. Identification of *E. coli* strains were used MALDI-TOF MS Biotyper. The sensitivity of all *E. coli* strains were tested against: ampicillin 10 μg.disc^{-1}, chloramphenicol 30 μg.disc^{-1}, meropenem 10 μg.disc^{-1}, ceftriaxone 30 μg.disc^{-1}, ofloxacin 5 μg.disc^{-1} and oxytetracycline 30 μg.disc^{-1}. For antibiotic susceptibility testing was used disc diffusion method according by CLSI. In this study was determined that *E. coli* isolated from rectal swab of fish (*Cyprinus carpio*) was resistant to ampicillin (10.90 %) and to chloramphenicol (3.63 %) from 110 *E. coli* isolates. In the cases of meropenem, ceftriaxone and ofloxacin resistance of *E. coli* were not detected. In spite of using oxytetracycline in fish farming was not detected resistance to oxytetracycline. From this work is shown that *Escherichia coli* isolated from freshwater fish (*Cyprinus carpio*) is a resistant to ampicillin and chloramphenicol and it is possible impact of using antibiotics in a last or watercourses pollution of antibiotics is from human activities.

Keywords: Antibiotic resistance, *Cyprinus carpio, Escherichia coli*, fish, intestinal tract

1. Introduction

Monitoring antimicrobial resistance should cover different stages from the entire farm-to-fork chain [1]. Animal feedstuffs are potential vehicles for transmission of resistant bacteria that could colonize the intestinal tract [2]. Several investigations have shown that animal feed, as well as raw feeding materials of vegetable origin, can be contaminated by *Salmonella* spp. [3-5] and other *Enterobacteriaceae* species [2]. Antibiotics are widely used is to protect humans and animals health from pathogenic bacteria by reducing infection.

Antibiotics are released into aquatic environments via the excretion of humans and animals in unaltered forms or metabolites of parent compounds [6]. As a result of the non-hygienic and stressful conditions present in aquaculture facilities, the risk of bacterial infections among aquacultured fish is high. Therefore, heavy amounts of antimicrobials are used in fish feed for preventive and curative purposes in aquaculture facilities worldwide [7]. The heavy use of antimicrobial agents in aquaculture has resulted in the increase of strains resistant to these agents. Potentially these resistant strains can have an impact on the therapy of fish diseases, the therapy

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of human diseases, or the environment of the fish farms [8]. *Escherichia coli* is a common inhabitant of intestinal tract of humans and animals [9], and can be easily disseminated in different ecosystems through the food chain and water. Animal food products are an important source of *E. coli* as faecal contamination of carcasses at the slaughterhouse is frequent. These microorganisms and their possible resistance determinants may be transmitted to humans if these foods are improperly cooked or otherwise mishandled. The level of antibiotic resistance in *E. coli* represents a useful indicator of the resistance dissemination in bacterial populations. There are some reports in which antibiotic susceptibility of *E. coli* isolates from healthy humans [10-12, 18] or animals [13-17] have been studied, but in few cases comparative results have been shown [18, 19] or isolates from foods analyzed. *E. coli* has been shown to exchange genetic material with other bacterial species and it is possible that this organism may pass antibiotic resistance genes to transient bacterial pathogens that cause disease in humans [20]. Keyser *et al*. [21] note that in recent years, accumulating problems with resistant bacteria, leading to predictions that we are back the period before the discovery of antibiotics. One of way around this problem is to introduce new antibacterial preparation which could be isolate from some wild medical plants with antimicrobial effects [22] or it could be operates on a locking mechanism of virulence, more precisely, a type III (T3SS) secretion system. Infections caused by resistant strains of micro organisms causing costly treatment of animals and humans. Such infections prolong the pathological condition and if not treated with the right antibiotics may be increased mortality [23].

In the present study, we determined antibiotic resistance of *Escherichia coli* isolated from intestinal tract of fishes (*Cyprinus carpio*), which were intended for human consumption.

2. Materials and methods

Collection of samples

The 110 samples of *Escherichia coli* were collected from 10 rectal swabs of fish (*Cyprinus carpio*). Eleven samples of *Escherichia coli* were isolated from each rectal swabs of *Cyprinus carpio*. Rectal swabs samples of *Cyprinus carpio* were collected in November 2011 from fish farming in Kolíňany, Slovakia. Fishes used in this experiment were 5 months old and Rupin special (brand name of antibiotic - oxytetracycline) has been used on fish farm for treatment. Pond where the fishes raised was associated with wastewater treatment plant. Samples were collected by rectal swab kit containing (Copan Inovation, Brescia) and transported in medium to laboratory of Department of microbiology, Faculty of biotechnology and food sciences in SUA in Nitra.

Cultivation and isolation of E. coli

Bacterial samples were spread on the surface of agar by rectal swab directly. For cultivation of bacterial strains MacConkey agar (Biomark, Pune) was used. Cultivation of *Enterobacteriaceae* genera was done at 37 °C during 24 hours. After the first incubation was need recultivation to obtain pure culture of *E. coli* in the same conditions. For recultivation and probably identification of *E. coli* strains Chromogenic coliform agar (Oxoid, UK) was used. For obtaining the pure culture of *E. coli* four-ways streak plate method was used. Every these steps of recultivation was done in the same conditions.

Identification of E. coli strains

Initial identification of *E. coli* strains were done on Chromogenic coliform agar (Oxoid, UK) and Triple sugar iron agar (Biolife, Italy). Biochemical identification of *E. coli* was done by ENTERO test 24 (Erba Lachema, Brno). Working procedure for biochemical testing is described into the manufacturer manual. Evaluation of biochemical test was done by identifying computer program TNW Lite 7.0 software (Erba Lachema, Brno). For better identification of *E. coli* strains were used MALDI-TOF MS Biotyper (Brucker Daltonics GmBH, Germany) and method for prepare of samples to identification was done by Kmeť and Drugdová, [24].

Antibiotic susceptibility testing

The pure inoculum of *E. coli* strains were prepared by suspending of colonies into the physiological solution from agar plates and every suspensions were adjusted to equal a 0.5 McFarland standard. The sensitivity of all *E. coli* strains were tested against: ampicillin (AMP 10) 10 $\mu g.disc^{-1}$, chloramphenicol (C 30) 30 $\mu g.disc^{-1}$, meropenem (MEM 10) 10 $\mu g.disc^{-1}$, ceftriaxone (CRO 30) 30 $\mu g.disc^{-1}$, ofloxacin (OFX 5) 5 $\mu g.disc^{-1}$ and

oxytetracycline (OT 30) 30 µg.disc^{-1}. For antibiotic susceptibility testing was used disc diffusion method according by EUCAST [25]. (The European Committee on Antimicrobial Susceptibility Testing). Incubation of *E. coli* strains were done at 35 ± 2 °C on Mueller-Hinton agar (Biomark, Pune). Interpretation of inhibition zones around the disc was according by EUCAST [25] (Breakpoint tables for interpretation of MICs and zone diameters. Version 3.1, valid from 2013/2/11). The inhibition zones were controlled with the references sensitivity *Escherichia coli* CCM 3988.

Statistical evaluation

From obtained data were calculated basic variation-statistical values by using statistical program Statgraphic. In this study were calculated values like: average, standard deviation, minimum, maximum, coefficient of variation and frequency of size of inhibition zones.

3. Results and discussion

In our experiment was studied antibiotic resistance in 110 *E. coli* strains isolated from rectal swabs of *Cyprinus carpio*. In this study was determined that *E. coli* isolated from rectal swab of fish (*Cyprinus carpio*) was resistant to ampicillin (10.90 %) and to chloramphenicol (3.63 %) from 110 *E. coli* isolates. *E. coli* was sensitive to meropenem, ceftriaxone and to ofloxacine. Results of antibiotic resistance of *E. coli* are described into the figure 1. Sarter et al., [26] studied antibiotic resistance in Gram-negative bacteria isolated from farmed catfish and these authors isolated 92 bacterial species from 3 different catfish farms. In contrast with our study, they determined that Gram-negative bacteria isolated from catfish were resistant (60 – 90 %) to ampicillin and 20 – 50 % to chloramphenicol. Similarly, Subramanian Kumaran et al., [27] monitored antibiotic resistance of *E. coli* isolated from see fish and they determined 56.25 % resistance to ampicillin and 2.5 % resistance to chloramphnicol.

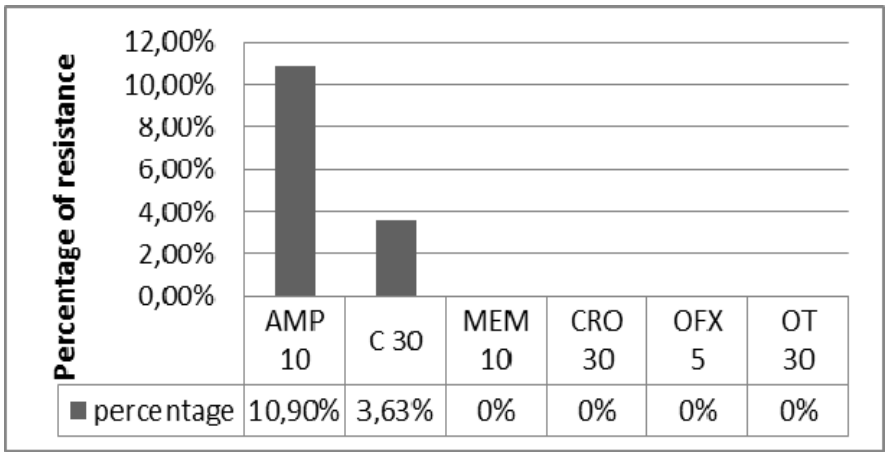

Figure 1. Percentage of antibiotic resistance of *Escherichia coli* isolated from rectal swabs of *Cyprinus carpio* (AMP 10 – ampicillin, C 30 – chloramphenicol, MEM 10 – meropenem, CRO 30 – ceftriaxone, OFX 5 – ofloxacin, OX 30 – oxytetracycline)

Numbers of resistant and sensitive samples of *E. coli* isolated from rectal swabs of *Cyprinus carpio* are described into the table 1. In experiment was determined that 12 *E. coli* strains were resistant and 98 samples were sensitive to ampicillin. Also, 4 strains of *E. coli* were resistant and 106 samples were sensitive to chloramphenicol. In the cases of meropenem, ceftriaxone and ofloxacin resistance of *E. coli* were not detected. Also, intermediate susceptibility was not detected. Likewise, multiresistant strains of *E. coli* were not detected. Conversely, Sarter et al., [26] determined 8 strains of *E. coli* which were multiresistant to several antibiotics. In their study, they isolated *E. coli* from intestinal microcenose of freshwater catfish. Also, Ryu et al., [28] isolated 179 *E. coli* from commercial fish and sea food which were resistant to ampicillin (12 strains) and to chloramphenicol (21 strains). However, these authors found resistant strains to ceftriaxone in 3 isolates of *E. coli*. In our study resistance to ceftriaxone we not detected. Also, Ryu et al., [28] found

multiresistant *E. coli* isolated from commercial fish and sea food. Also, Miranda and Zemelman, [29] found about 65 strains of bacteria resistant to ampicillin and about 68 strains resistant to chloramphnicol. These strains were isolated form intestinal tract of fish from fish farming. Hleba et al., [30] isolated *Enterobactericaceae* species (including *E. coli*) from intestinal tract of duck, which come from the same environment like researched fish. They used tetracycline (TE30), sulfonamid (S10), chloramphenicol (C30) and ampicilin (AMP10) in this research and they determined that *Enterobacteriaceae* genera were resistant to all observed antibiotics. The most resistance was to tetracycline, than sulfonamid and ampicillin and chloramphenicol. Equally, they determined that *E. coli* was resistant to tetracycline (37.5%), streptomycin (12.5%), amipicillin (6.25%) and to chloramphenicol resistance was not detected.

Table 1. Antibiotic resistance profile of *E. coli* isolated from rectal swabs of *Cyprinus carpio*

Antibiotics	No. of *E. coli* isolates		
	Resistant	Intermediate	Sensitive
AMP 10	12	22	76
C 30	4	ND	106
MEM 10	ND	ND	110
CRO 30	ND	ND	110
OFX 5	ND	ND	110
OT 30	ND	ND	110

Legend: ND – not detected, AMP 10 – ampicillin, C 30 – chloramphenicol, MEM 10 – meropenem, CRO 30 – ceftriaxone, OFX 5 – ofloxacine, OT 30 - oxyttracycline

Statistical evaluation of inhibition zones determined that the greatest variability was in the samples of *E. coli* against to ampicillin (22.97 %). Minimum and maximum of inhibition zones ranged from 12 to 34 mm and average of inhibition zones was 22.97 mm. Conversely, the lowest variability of inhibition zones of *E. coli* samples was determined into meropenem (10.07%). Minimum and maximum values of inhibition zones ranged from 26 to 36 mm and average of inhibition zones was 31.80 mm. Others variation-statistical values are described into the table 2.

Table 2 The basic variation-statistical values of inhibition zones of *E. coli* isolated from rectal swabs of *Cyprinus carpio* in mm

Antibiotics	The basic variation-statistical values						
	Count	Average	Standard deviation	Coeff. of variation	Minimum	Maximum	Range
AMP 10	110	22.97	6.36	27.69%	12	34	22
C 30	110	26.32	3.67	13.95%	12	30	18
CRO 30	110	30.10	3.63	12.08%	24	34	10
MEM 10	110	31.80	3.20	10.07%	26	36	10
OFX 5	110	32.30	3.92	12.13%	28	40	12
OT 30	110	22.18	3.79	17.10%	20	30	10

Legend: AMP 10 – ampicillin, C 30 – chloramphenicol, MEM 10 – meropenem, CRO 30 – ceftriaxone, OFX 5 – ofloxacin, OT 30 – oxytetracycline

For determining and better showing frequency of the size of inhibition zones around the discs in *E. coli* isolates isolated from rectal swabs of *Cyprinus carpio* we prepared histograms. Boundaries between resistant, intermediate and sensitive *E. coli* isolates isolated from rectal swabs of *Cyprinus carpio* are shown in these histograms with red line and red number values (R – resistant part, I – intermediate part, S – sensitive part). The evaluation of inhibition zones around the discs for ampicillin showed that the highest frequency was in the sensitive range from 21.1 to 25 mm and mass was about 30 %. Conversely, the lowest frequency was in the sensitive range from 28.8 to

32.5 mm and mass was about 2 %. Frequency of the size of inhibition zones in the resistant range was from 10 to 14 mm and mass was about 12 %. From the frequency histogram for ampicillin we can see that about 88 % *E. coli* isolates had sizes of inhibition zones in the sensitive range. About 12 % was in the resistant range only. Also we can see that about 20 % of *E. coli* isolates was the boundary between resistance and sensitivity. More detailed results are shown in the figure 2.

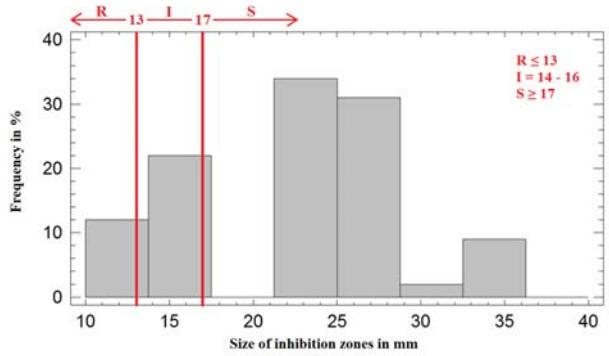

Figure 2 Histogram for frequency of the sizes of inhibition zones in *E. coli* against ampicillin.

Evaluation of inhibition zones around the discs for chloramphenicol showed that the highest frequency was in the sensitive range from 23.5 to 26 mm and mass was about 40 %. Conversely, the lowest frequency was in the resistant and intermediate range from 11 to 13.5 mm and mass was about 4 %. From the chloramphenicol frequency histogram we can see that more like 96 % *E. coli* isolates had sizes of inhibition zones in the sensitive range and about 4 % *E. coli* isolates was in the resistant and intermediate range. Also from this histogram we can see sharp boundary between resistant and sensitive *E. coli* isolates. More detailed results are shown in the figure 3.

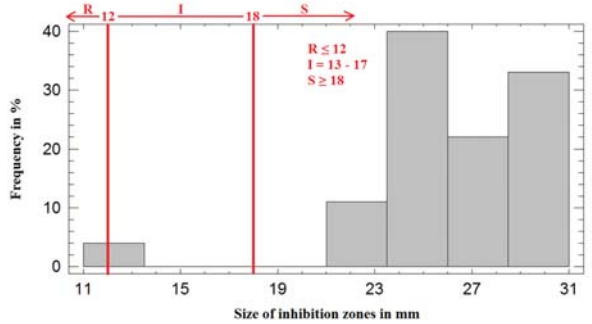

Figure 3 Histogram for frequency of the sizes of inhibition zones in *E. coli* against chloramphenicol.

Evaluation of inhibition zones around the discs for meropenem showed that the highest frequency was in the sensitive range from 29.5 to 31 mm, from 32.5 to 35.5 mm and mass was about 20 % for all three. Conversely, the lowest frequency was in the sensitive range from 25 to 28 mm and mass was about 10% for both. From frequency histogram for meropenem we can see that every *E. coli* isolates was in the sensitive range. More detailed results are shown in the figure 4.

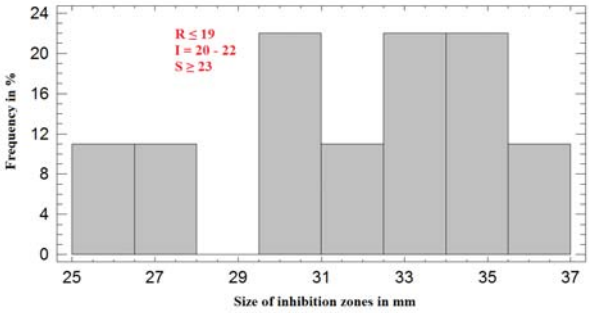

Figure 4 Histogram for frequency of the sizes of inhibition zones in *E. coli* against meropenem.

Evaluation of inhibition zones around the discs for ceftriaxone showed that the highest frequency was in the sensitive range from 30.5 to 32 mm and mass was about 32 %. Conversely, the lowest frequency was in the sensitive range from 26 to 27.5 from 29 to 30.5 mm and from 32 to 33.5 mm and mass was about 10 % for all three. From ceftriaxone frequency histogram we can see that every *E. coli* isolates was in the sensitive range, but about 20 % *E. coli* isolates were the boundary between intermediate and sensitivity. More detailed results are shown in the figure 5.

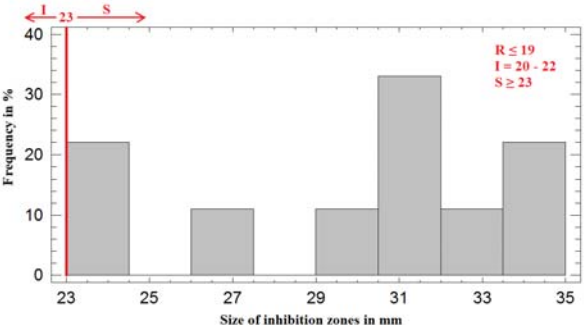

Figure 5 Histogram for frequency of the sizes of inhibition zones in *E. coli* against ceftriaxone.

Evaluation of inhibition zones around the discs for ofloxacin showed that the highest frequency was in the sensitive range from 30.8 to 32.7 mm and mass was about 44 %. Conversely, the lowest

frequency was in the sensitive range from 28.9 to 30.8 and from 32.6 to 34.5 mm and mass was about 10 % for both. From ofloxacin frequency histogram we can see that every *E. coli* isolates was in the sensitive range and no *E. coli* isolates were the boundary between resistance and sensitivity. More detailed results are shown in the figure 6.

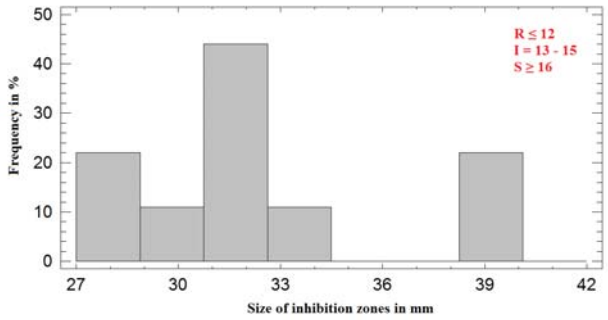

Figure 6 Histogram for frequency of the sizes of inhibition zones in *E. coli* against ofloxacin

Evaluation of inhibition zones around the discs for ofloxacin showed that the highest frequency was in the sensitive range from 19 to 20.5 mm and mass was about 62 %. Conversely, the lowest frequency was in the sensitive range from 22 to 23.5 and mass was about 1 %. From oxytetracycline frequency histogram we can see that every *E. coli* isolates was in the sensitive range and about 62% *E. coli* isolates were the boundary between intermediate and sensitivity. More detailed results are shown in the figure 7.

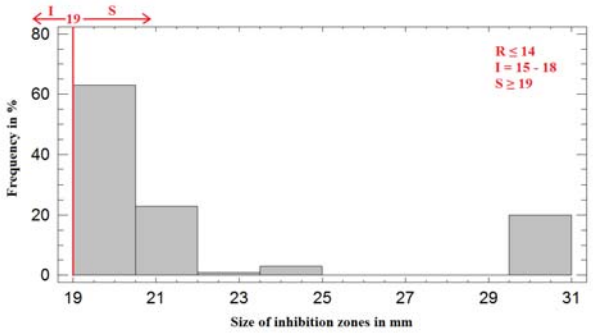

Figure 7 Histogram for frequency of the size of inhibition zones in *E. coli* against oxytetracycline.

4. Conclusions

Antibiotic resistance is a global problem not only for human health in this time. Antibiotic resistance can be transfer from animal to environment and conversely. Also all the ways to human body and it can to lead to problems in the treatment of bacterial diseases. Bacteria from faeces of fish can to survive in the water condition very well and it can to spread their resistant genes to another animals or humans. Antibiotics create selective pressure for creation of antibiotic resistance. Therefore, role of monitoring of resistance is very important. From this work is shown that *Escherichia coli* isolated from freshwater fish (*Cyprinus carpio*) is a resistant to ampicillin and chloramphenicol and it is possible impact of using antibiotics in a last or watercourses pollution of antibiotics is from human activities. In spite of using oxytetracycline in fish farming was not detected resistance to oxytetracycline. Probably, time which was used for treatment of fish is not sufficient to create a resistance.

Acknowledgements

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