Original Research Article

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Antibiotic resistance in bacteria isolated from the skin of trout in León Province, Spain

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Abstract

Antibiotic resistance among fish pathogens has become a major concern. Integrons play an important role in dissemination of antibiotics resistance genes among the different species of bacteria. This study was designed to determine the antibiotic susceptibility of 20 bacterial isolates were isolated from the skin of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss) collected from the rivers and fish farms in León Province, Spain and to detect class 1 and 2 integrons in these isolates by PCR to explore the role of these integrons in the antibiotic resistance. The investigated isolates included *Pseudomonas fluorescens (P. fluorescens)* (n=5), *Aeromonas sobria (A. sobria)* (n=3), *Pantoea agglomerans* (*P. agglomerans*) (n=3), *Acinetobacter johnsonii (A. johnsonii)* (n=2), *Serratia fonticola (S. fonticola)* (n=1), *Staphylococcus equorum (S. equorum)* (n=3), *Yersinia kristensenii (Y. kristensenii)* (n=2) and *Xanthomonas retroflexus (X. retroflexus)* (n=1). Antimicrobial susceptibility testing for the isolates revealed that they were sensitive to enrofloxacin (100%) and gentamicin (95%) while resistant to ampicillin (90%) and tetracycline (80%). PCR revealed that all the isolates didn't harbor class 1 or 2 integrons. Therefore, the indiscriminate use of antibiotics must be restricted and more attention should be paid to biosecurity measures in aquaculture. Also, further studies are needed to assess role of integrons and other genetic elements in antimicrobial resistance in aquaculture.

Keywords: Antimicrobial, Integrons, León Province, Resistance, Trout

Introduction

The emergence of antimicrobial resistance among fish pathogens during the past decades, became a major concern in many countries (Schmidt et al., 2001) where it undermines effectiveness of antibiotics in aquaculture and increases the possibilities for passage of these antibiotic-resistant bacteria and their antibiotic resistance determinants to bacteria of terrestrial animals and human, including pathogens (Cabello, 2006). This in addition to that development of new antibiotics by the pharmaceutical industry had essentially stalled due to economic and regulatory obstacles (Bartlett et al., 2013).Antibiotic resistance in bacterial populations can result from the clonal selection under antimicrobial selective pressure or through horizontal genes transfer (Deng et al., 2016). The growth of aquaculture worldwide has been accompanied by a rapid increase in the therapeutic and prophylactic use of antimicrobials including those important in human therapeutics (Cabello et al., 2013). The use of a wide variety of antimicrobials in large amounts ensures their remaining in the aquatic environments for prolonged time periods exerting their selective pressure which has led to emergence of antibiotic-resistant bacteria in aquaculture environments, increase of antibiotic resistance of fish pathogens, and to alteration of bacterial flora in aquaculture environments (Cabello, 2006 and Cabello et al., 2013). Mobile genetic elements as plasmids, transposons, and integrons play a big role in evolution and dissemination of antimicrobial resistance in the aquatic environment through their wide dissemination for antimicrobial resistance genes between bacteria by horizontal gene transfer (Khan et al., 2009). Integrons are specialized genetic elements capable of capturing,

integrating, and mobilizing gene cassettes. They are widely present among bacteria (Xu et al., 2009) and their occurrence in fish-farming environments is well known (Ndi and Barton, 2011). Integrons could be horizontally transferred among bacteria of inter- and intra-species (Lukkana et al., 2012) and they are often situated on conjugative plasmids or transposons which can facilitate and accelerate their lateral transfer between bacteria (Mazel, 2006).Although plasmids are the most important agents for appearance and dissemination of antibiotic resistance (Smith and Romesberg, 2007), integrons constitute an important means of spreading antibiotic resistance genes (Partridge et al., 2009). More than 100 different gene cassettes have been found within integrons, most of them encode for antibiotic resistance (Sarria-Guzmán et al., 2014). Integrons can capture more than one antibiotic-resistant cassette (Sarria-Guzmán et al., 2014) and numerous combinations of gene cassettes have been reported within integrons (Partridge et al., 2009) which may lead to multi-resistance (Fluit and Schmitz, 1999). There are three classes of integrons involved in antimicrobial resistance but class 3 integrons rarely emerges in clinical isolates (Xu et al., 2009).Several recently found genetic elements and resistance determinants for quinolones, tetracyclines, and β-lactamases are shared between aquatic bacteria, fish pathogens, and human pathogens, and appear to have originated in aquatic bacteria (Cabello et al., 2013). Limited data are available about antimicrobial resistance in skin microbiota of trout and role of integrons in this resistance. Therefore, this study was aimed to determine the antimicrobial susceptibility of bacteria isolated from skin of brown and rainbow trout collected from the rivers and fish farms in León Province, Spain and to detect class 1 and 2 integrons in these isolates by PCR to explore role of these integrons in the antimicrobial resistance.

Materials and Methods

1. Bacterial isolates:

This study was performed on 20 different bacterial isolates were isolated and identified by Carbajal-González et al. (2011) from skin of brown (n=18) and rainbow (n=2) trout collected from the rivers (n=13) and fish farms (n=7) in León Province, Spain, these isolates and origin of their isolation were illustrated in Table (1). These bacterial isolates were stored at -80°C in tryptone soya broth (TSB) supplemented with 15% glycerol.

Table 1. The investigated bacterial isolates in this study and origin of their isolation.

Strain code	Bacteria		Origin of isolation	
	Species	Number	Fish species	Source of fish
LE89	*P. fluorescens*	5	brown trout	Porma River
LE98			brown trout	Porma River
LE122			brown trout	fish farm on Porma River
LE141			rainbow trout	fish farm on Duerna River
LE143			rainbow trout	fish farm on Duerna River
LE51	*A. sabra*	3	brown trout	Omaña River
LE74			brown trout	fish farm on Porma River
LE80			brown trout	fish farm on Porma River
LE35	*P. agglomerans*	3	brown trout	Porma River
LE36			brown trout	Porma River
LE37			brown trout	Porma River
LE4	*A. johnsonii*	2	brown trout	Porma River
LE7			brown trout	Porma River
LE52	*S. fonticola*	1	brown trout	Omaña River
LE6	*S. equorum*	3	brown trout	Porma River
LE11			brown trout	fish farm on Porma River
LE13			brown trout	fish farm on Porma River
LE54	*Y. kristensenii*	2	brown trout	Omaña River
LE58			brown trout	Omaña River
LE38	*X. retroflexus*	1	brown trout	Porma River

In this study, the bacterial isolates were defrosted and cultured on TSA (Cultimed, Belgium) and incubated aerobically at 22°C for 24 hours. Subsequently, the isolates identification was confirmed through their morphological characters, Gram staining, oxidase test and catalase test. Furthermore, any doubtful isolate was identified by MALDI-TOF MS (Bizzini and Greub, 2010) and the results were interpreted following the manufacturer's recommendation.

2. Antibiotic susceptibility testing of the isolates:

Antibiotic susceptibility of the isolates was determined by using Kirby-Bauer disk diffusion method against four different antibiotics including ampicillin (10 µg), gentamicin (10µg), enrofloxacin (10µg) and tetracycline (30µg) (Oxoid, Spain). Bacterial suspensions of the isolates were prepared and adjusted to 1.5×108 CFU/mL by using Sensititre™ Nephelometer (Thermo Scientific, USA). Subsequently, an aliquot of each suspension was spread plated onto Mueller-Hinton agar (Oxoid, Spain) then antibiotic disks were dispensed on the inoculated plate and incubated aerobically at 22°C for 18-24 hours. Inhibition zones diameters were measured and interpreted according to CLSI (2014).

3. Molecular detection of class 1 and 2 integron-integrase genes (intI1 and intI2) in the isolates:

3.1. DNA extraction:

Bacterial isolates were cultured on TSA then bacterial colonies were harvested and placed into sterile Eppendorf tubes containing 100 µl of phosphate buffered saline (pH 7.4). Subsequently, the suspensions were heated in water bath at 100º C for 10

min. then centrifuged at 14000 rpm for 5 min. (Holmes and Quigley, 1981). The supernatants were transferred into fresh Eppendorf tubes and used as DNA templates for PCR.

3.2. DNA amplification:

Isolates were screened for presence of class 1 and 2 integrons through amplification of bacterial DNA targeting class 1 and 2 integron-integrase genes (intI1 and intI2) using the oligonucleotide primers illustrated in Table (2) (Shibata et al., 2003). PCR mixture was prepared in 25 µl volumes containing 2.5 µl of 100 mM PCR buffer (Biotools), 1.25 µl of 50 mM MgCl2 (Invitrogen™), 0.8 µl of 4 mM deoxynucleotide triphosphate mix, 0.5 µl of each primer, 2 µl of the extracted DNA, 0.15 µl of 5 U/µl DNA Polymerase (Biotools) and 17.3 µl of sterile milliQ water. PCR was performed in SimpliAmp™ thermal cycler (Thermo Scientific, USA) under the conditions previously described by Dillon et al. (2005); initial denaturation at 95°C for 5 min. followed by 30 cycles of denaturation at 95°C for 30 sec., annealing at 55°C for 30 sec. and extension at 72°C for 90 sec. followed by final extension step at 72°C for 7 min.

3.3. Analysis of PCR products:

PCR products were electrophoresed on 1% agarose gel in 1x Tris-acetate EDTA buffer at 90 V and 400 mA for 40 min. and 100 bp DNA ladder was used for fragments size determination. Thereafter, the gel was photographed by a gel documentation system (Biorad, USA) and data was analyzed.

Table 2. Target genes and oligonucleotide primers used in this study.

Target gene	Primers sequences (5`- 3`)		Product size (bp)
*Int*I1	F	GCATCCTCGGTTTTCTGG	457
	R	GGTGTGGCGGGCTTCGTG	
*Int*I2	F	CACGGATATGCGACAAAAAGGT	789
	R	GTAGCAAACGAGTGACGAAATG	

4. Statistical analysis:

The statistical analysis was performed by using Epi Info™ software (version 7.2.4). The differences in antimicrobial resistance between bacteria isolated from rivers trout and those isolated from farms trout were estimated by Chi-Square and Fisher's exact tests, with an $\alpha = 0.05$ cutoff.

Results

1. Antibiotic susceptibility of the isolates:

Results of antimicrobial susceptibility testing of the bacterial isolates to the tested antibiotics were illustrated in Tables (3) and (4). Statistical analysis revealed that there was no significant difference between the antimicrobial resistance of bacteria isolated from rivers trout and those isolated from farms trout.

Table 3. Results of antibiotic susceptibility of bacterial isolates.

Isolates	Antimicrobial susceptibility result															
	Ampicillin				Gentamicin				Enrofloxacin				Tetracycline			
	Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
P. fluorescens (n=5)	0	0	5	100	5	100	0	0	5	100	0	0	0	0	5	100
A. sobria (n=3)	0	0	3	100	3	100	0	0	3	100	0	0	2	66.7	1 LE51	33.3
P. agglomerans (n=3)	0	0	3	100	3	100	0	0	3	100	0	0	0	0	3	100
A. johnsonii (n=2)	0	0	2	100	2	100	0	0	2	100	0	0	0	0	2	100
S. fonticola (n=1)	0	0	1	100	1	100	0	0	1	100	0	0	0	0	1	100
S. equorum (n=3)	2	66.7	1 LE11	33.3	3	100	0	0	3	100	0	0	2	66.7	1 LE11	33.3
Y. kristensenii (n=2)	0	0	2	100	2	100	0	0	2	100	0	0	0	0	2	100
X. retroflexus (n=1)	0	0	1	100	0	0	1	100	1	100	0	0	0	0	1	100
Total (n=20)	2	10	18	90	19	95	1	5	20	100	0	0	4	20	16	80

Table 4. Results of antibiotic susceptibility of bacterial isolates isolated from rivers trout and farms trout.

Isolates	Antimicrobial susceptibility result															
	Ampicillin				Gentamicin				Enrofloxacin				Tetracycline			
	Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Rivers trout (n=13)	1	7.7	12	92.3	12	92.3	1	7.7	13	100	0	0	1	7.7	12	92.3
Farms trout (n=7)	1	14.3	6	85.7	7	100	0	0	7	100	0	0	3	42.9	4	57.1
Total (n=20)	2	10	18	90	19	95	1	5	20	100	0	0	4	20	16	80

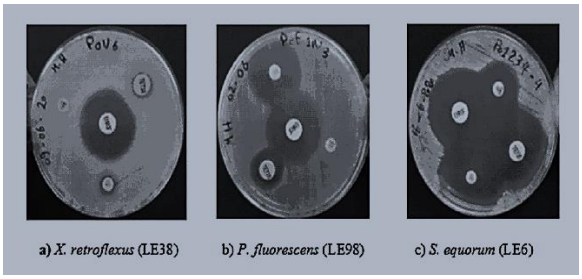

Fig. 1. Antibiotic susceptibility test results of some bacterial isolates.

2. Molecular detection of class 1 and 2 integron-integrase genes (intI1 and intI2):

It was found that all the examined isolates didn't harbor IntI1 or IntI2 genes as illustrated in Fig. (2).

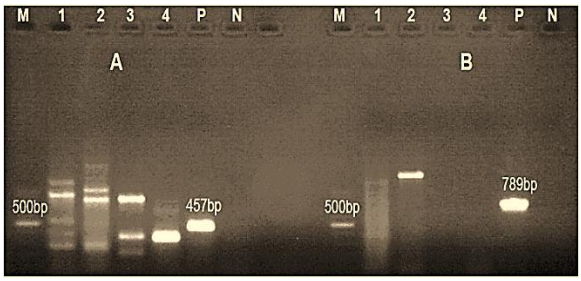

Fig. 2. Agar gel electrophoresis for PCR products using specific primers target IntI1 (Part A) and IntI2 (Part B) genes in the isolates. Lane M: 100 bp molecular weight marker, lane P: positive control, lane N: negative control and lanes 1-4: DNA extracted from LE89, LE141, LE51 and LE80 isolates respectively showing negative results for both genes in all of them.

Discussion

Antimicrobial resistance is an issue of increasing global concern and it is associated mainly with the uncontrolled use of antimicrobials (Barber et al., 2003). There are numerous reports about resistance of fish pathogens to one or several antibiotics (Schmidt et al., 2001). In this study, it was found that (100%) and (95%) of the examined bacterial isolates were sensitive to enrofloxacin and gentamicin respectively while (90%) and (80%) of them were resistant to ampicillin and tetracycline respectively and there was no significant difference between the antibiotic resistance of bacteria isolated from the rivers trout and those isolated from farms trout. These results may be attributed to the prolonged and misuse of ampicillin and tetracycline and presence of regulation framework for use of enrofloxacin and gentamicin in the fish farms and the areas around the rivers from which the samples were collected or may be due to the intrinsic resistome of some bacterial species to ampicillin and tetracycline as the intrinsic resistome of A. sobria to penicillins (Borella et al., 2020 and Dhanapala et al., 2021). Resistance of these different bacterial isolates to ampicillin and tetracycline will result in difficulty of prevention and control of the bacterial diseases of fish, in addition to their epidemiological and public health implications represented in transfer of these resistant bacteria and their resistance genes to human and animals, especially that penicillins and tetracyclines are among the six critically important antimicrobials for human medicine (WHO, 2017). Therefore, antibiotics use in aquaculture and other livestock production sectors must be prudent and supported by antimicrobial susceptibility testing in addition to application of strict hygienic measures in aquaculture.

Our results agreed with findings of Dinçtürk and Tanrıkul (2021) except in the sensitivity to tetracycline where they found that all P. fluorescens isolated from rainbow trout were resistant to ampicillin and sensitive to enrofloxacin and tetracycline. Also, our results agreed with findings of Kozińska et al. (2014) and Borella et al. (2020) who found that all A. johnsonii and A. sobria isolates respectively were resistant to ampicillin and sensitive to gentamicin. While in contrast to our results, Elabd et al. (2020) and Dhanapala et al. (2021) found that all A. johnsonii and A. sobria isolates were resistant gentamycin and enrofloxacin respectively. These differences in the antimicrobial susceptibility may be attributed to some differences including the used antimicrobials, management practices, and time of examination where antimicrobial resistance is increasing over time (Ali et al., 2017).

Integrons constitute an important means for spreading antibiotic resistance (Partridge et al., 2009). Three classes of integrons are involved in the bacterial resistance but class 3 integron rarely emerges in the clinical isolates (Xu et al., 2009). Isolates originating from aquatic sources have been reported to carry integrons and other genes that code for resistance (Ndi and Barton, 2011). In this study, it was found that all the investigated isolates didn't harbor class 1 or 2 integrons as illustrated in Fig. (2). Our results agreed with findings of Ranjbar et al. (2019) who didn't detect class 2 integrons in all investigated Aeromonas species isolated from the diseased freshwater animals and water samples in Iran while disagreed with findings of Dhanapala et al. (2021) who detected class 1 and 2

integrons in (19.9%) of Aeromonas species isolated from freshwater ornamental fish and associated farming environment in Sri Lanka. Class 1 integrons were also detected in A. sobria by Ndi and Barton (2011), Sarria-Guzmán et al. (2014) and Deng et al. (2016).

The genetically determined resistance set up by given bacteria is efficiently transmitted to its clonal expansion and/or other bacteria through the mobile genetic elements as plasmids, transposons, and integrons (Sultan et al., 2018). In this study, it was found that the investigated bacterial isolates were resistant to ampicillin and tetracycline although they didn't harbor class 1 or 2 integrons, resistance of these isolates may be attributed to that genes which conferred such resistance were situated on the other mobile genetic elements as plasmids and transposons which aren't investigated in our study or may be attributed to the intrinsic resistome which is documented in Aeromonas spp. to penicillins (Borella et al., 2020 and Dhanapala et al., 2021). Furthermore, this may be also attributed to the few bacterial isolates number investigated in this study, therefore, further studies are required to explore role of integrons and other genetic elements in the antimicrobial resistance.

Conclusion

The current study revealed that bacteria isolated from skin of brown and rainbow trout collected from the rivers and farms in León Province, Spain were resistant to ampicillin and tetracycline while sensitive to enrofloxacin and gentamicin and didn't harbor class 1 or 2 integrons, representing risk for both human and fishes. Therefore, the indiscriminate use of antibiotics must be restricted and more attention should be paid to biosecurity measures in aquaculture. Furthermore, further studies are required to assess role of integrons and other genetic elements in antimicrobial resistance in aquaculture.

Conflict of interest

The authors declare that they have no competing interest.

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