

The Situation of Antibiotic Resistance of Bacteria Isolated from Fresh Water Fish in Hai Duong Province

Nguyen Thi Giang, Pham Duc Ngoc, Tran My Hanh, Bui Thi Viet Ha*

Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam

Received 15 July 2016

Revised 25 August 2016; Accepted 09 September 2016

Abstract: In aquaculture, antibiotics have been used mainly for therapeutic purposes and as prophylactic agents. The abuse of antibiotics in aquaculture farming is a big problem. To achieve high yields and profits, many farmers are now applying intensive farming methods. The improper use of antibiotics causes the phenomenon of drug-resistant bacteria and residue accumulation of antibiotics in aquatic meat. Another reason causing antibiotics-resistant bacteria is the use of antibiotics to small quantities of aquatic feed as a growth stimulant. So that in this study, the initial evaluation of the antibiotic resistance of bacteria isolated from freshwater fish was determined. The experimental results showed that 33/36 strains isolated from fish samples were resistant to 8 types of antibiotics reached 91%. Most of them are multi-drug resistant strains. They are capable of resisting two or more antibiotics. Especially, C23 strain, belong to *Pseudomonas monteilii* species, was capable of resisting to all 8 antibiotics. This strain shows the ability to withstand the temperature and pH range and can use a wide variety of different nutrients.

Keywords: Antibiotic resistance, *Pseudomonas monteilii*, freshwater fish.

1. Introduction

Fish aquaculture constitutes a rapidly growing industry in worldwide. Infectious diseases are always a hazard and may cause significantly stock losses and problems with animal welfare. Intensive fish farming has promoted the growth of several bacterial diseases, which has led to an increase in the use of antimicrobials [1] (Defoirdt et al., 2011). Current levels of antimicrobial use in aquaculture worldwide are not easy to determine because different countries have

different distribution and registration systems. Nevertheless, Burrige et al. (2010) reported that the amount of antibiotics and other compounds used in aquaculture differed significantly between countries [2]. Defoirdt et al., (2011) previously estimated that approximately 500–600 metric tons of antibiotics were used in shrimp farm production in Thailand in 1994; the large variation between different countries, with antibiotic use ranging from 1 g per metric ton of production in Norway to 700 g per metric ton in Vietnam [1, 3]. But the most dangerous is that, a large proportion of the world's antimicrobial industrial production is used as prophylactics and as growth promoters that far outweigh their

*Corresponding author. Tel.: 84-4-38588856
Email: habtv@vnu.edu.vn

use as therapeutics (Bush et al., 2011 and Cabello et al., 2013) [4, 5]. Antibiotics are one of the most common groups used as feed additives in the frame of growth promoter. Several antibiotics have been in use as growth promoters in fish farms ever since. In order to know about the antibiotics resistance of bacteria in fish, some results have been illustrated in the result part [6, 7].

2. Materials and methods

Collecting samples and isolating bacteria:

Seven fish samples were taken directly from freshwater fish ponds in the two fish farm areas of Cam Giang and Gia Loc in Hai Duong province, from January to June 2015. After that, all the samples were immediately incubated in the ice box, and then taken to the laboratory to isolate bacteria on the agar medium. The bacteria strains were preserved at 4⁰C in the cabinet to implement the follow-up experiment.

Antibiotic tests (diffusion susceptibility test): To evaluate antibiotic resistance and sensitivity, antibiograms for strains were obtained using the radial diffusion method, according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 1997), known as disk diffusion or Kirby-Bauer testing [8]. Inhibition zones were measured for 9 antibiotics including amoxicillin, ciprofloxacin, bacitracin, norfloxacin, penicillin, vancomycin, erythromycin, nitro furantoin, tetracycline [4].

Determining the morphology, physiology, biochemistry including: shape and size of cell, colony morphology, ability to form extracellular enzymes, to resist to salt, to assimilate sugar, portable ability.

16S rDNA sequencing and phylogenetic analysis: The sequences of 16S rDNA was determined after amplifying the DNA using PCR and directly analysed from the PCR product (Takashima and Nakase, 1999). Generated sequences were aligned with related species using CLUSTALR® ver.1.83 software (Thompson et al., 1994) [9]. Reference sequences used for the phylogenetic study were obtained from GenBank database. The phylogenetic tree was constructed from the evolutionary distance data according to Kimura (1990) using the neighbor-joining method (Saitou and Nei, 1987). Sites where gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were performed from 1000 random resamplings. All of phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1993) [9].

3. Results and discussion

Total 36 strains of bacteria were isolated from freshwater fish (data not show). These bacteria were tested with 8 types of antibiotics. The antibiotic testing results were divided into 3 parts: resistant, intermediate and susceptible with antibiotics. The results are shown in Table 1.

Table 1. Antibiotic testing results of bacteria calculated in terms of percentage

Antibiotic	Concentration of each paper (µg)	Number of strains	Inhibition zone (D-d, mm)			Percentage (%)		
			R	I	S	R	I	S
Amoxicillin (AMC)	20	36	≤13	14-17	≥18	41,67	2,78	55,55
Ciprofloxacin (CIP)	5	36	≤ 15	16-20	≥ 21	11,11	8,33	80,56
Bacitracin (B)	10	36	≤ 11	12-15	≥ 16	63,89	8,33	27,78
Norfloxacin (NOR)	5	36	≤12	13-16	≥17	30,56	13,89	55,55

Penicillin (P)	6	36	≤3	4-9	≥10	61,11	8,33	30,56
Vancomycin (VA)	30	36	≤9	10-11	≥12	36,11	8,33	55,56
Erythromycin (E)	15	36	≤13	14-22	≥23	63,89	22,22	13,89
Nitrofurantoin (Ft)	300	36	≤16	17-22	≥23	33,33	13,89	52,78

*Antibiotic-impregnated disk (6mm) with amount.

+ Diameter of inhibition from three individual experiments. S. sensitive; I. intermediate; R. resistant.

All of 8 antibiotics were resisted by those bacteria. In that, the bacitracin and erythromycin have the highest resistance with the same percentage is 63.89%, while ciprofloxacin has the lowest resistant rate 11.11%.

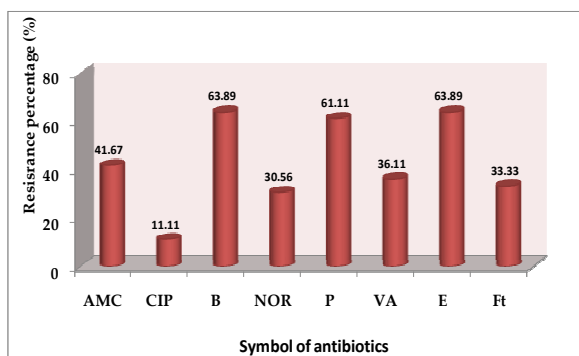


Fig 1. The percentage of antibiotics resistant bacteria.

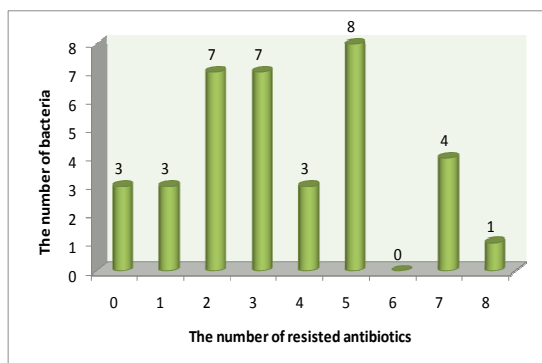


Fig 2. The multidrug resistance of bacteria.

Antibiotics used for treatment of aquaculture diseases in Hai Duong are ineffective because of the drug-resistant and multi-drug resistant bacteria which are populated. The result illustrates that almost all bacteria resist at least 2 kinds of antibiotics. There are only 3 bacteria strains vulnerable to antibiotics and 3 bacteria strains that resist 1 kind of antibiotics, and the rest, 30 strains, resist at least 2 kinds of antibiotics. This result proves that the percentage of antibiotic-resistant bacteria is very high due to the resistance gene being ubiquitous in the aquatic environment. As such, antibiotics are not effective. Out of 8 kinds of antibiotics, only ciprofloxacin has a low resistant rate, 11.11%, meaning that it has the highest efficiency against these bacteria. In contrast, the remaining

faces with high resistant rates of over 30%, especially bacitracin and erythromycin, both at 63.89%, demonstrating their low efficiency against bacteria living in freshwater, as well as in water in Hai Duong.

Overall, the high rate of multiple drug resistance shows the possibility of drug resistance genes being transmitted among bacterial species. Resistance genes can also spread to humans through water. Hence, there will be more risks as drug-resistant bacteria will spread indirectly through gene conversion information and directly in the aquatic environment. The frequency of infection and ineffective treatment will therefore increase. That antibiotics are no longer effective in both aquatic and human disease treatment leads to the difficulty in curing bacteria-caused

infection, and possibly to the outbreak of an epidemic which is caused by drug-resistant bacteria. This results in a declination in antibiotics efficiency, and exacerbates the treatment of diseases that require the companion of antibiotics in aquaculture, seafood, livestock and human.

Diagram shows that most of the bacteria are multidrug-resistant. Among 36 strains of bacteria, only 3 ones are not able to resist to certain antibiotics and the other 33 strains cannot be killed by at least one antibiotic, accounting for 91.67%. In which, 30 strains can resist two or more antibiotic sample. This represents that the majority of the bacteria are capable of acquiring multiple genes associated with antibiotic resistance. Especially, C23 strain resists to all 8 antibiotics. Proved that, they carry genes coding for antibiotic resistance. So their abilities to transmit one or more genes associated with antibiotic resistance to other microorganisms are common.

The mechanisms by which antimicrobial resistant bacteria, initially derived from food-producing animals, contribute to the emergent and increasing threat of antibiotic resistance in

people are complex and varied. The main routes bacteria can take to move from animals to humans include via food or other animal product contamination, occupational exposure for farm workers and fish keepers, veterinary surgeons and health workers. Bacteria can also transmit through environmental contamination like manure containing resistant bacteria, resistance genes, and antibiotic residues, along with recreational pursuits like swimming and fishing. The prevention of build up of resistant bacteria in waterways as a result of fish farming practices, terrestrial agriculture run-off or sewage outflow surrounding fish farms is a major concern for the aquaculture industry.

Morphological, physiological, biochemical and molecular characteristics of C23 strain

Colony morphology: small colonies, round, convex, yellow

Results dyeing unit: rod cell, grown separately or in cloud

Gram staining results: bacterial cells arrested in pink (Gram negative).

Cell size: length is 2.05 μm, width is 1.08 μm.

Indicators	Result
The ability to form extracellular enzyme (<i>D-d, mm</i>)	
Amylase	32
Cellulase	24
Protease	12
Salt-tolerant capability (%)	5
Portable capability	+
Capability to assimilate sugar	
Glucose	+
Manitol	±
Sacarose	+
Lactose	+
Fructose	+

Physio-biochemical characteristics of C23 strain

The result of the comparison of morphological characteristics, physiological,

biochemical and molecular characteristics of C23 strain and *Pseudomonas monteilii*, *Pseudomonas plecoglossicida* demonstrates that C23 belongs to *Pseudomonas monteilii*.

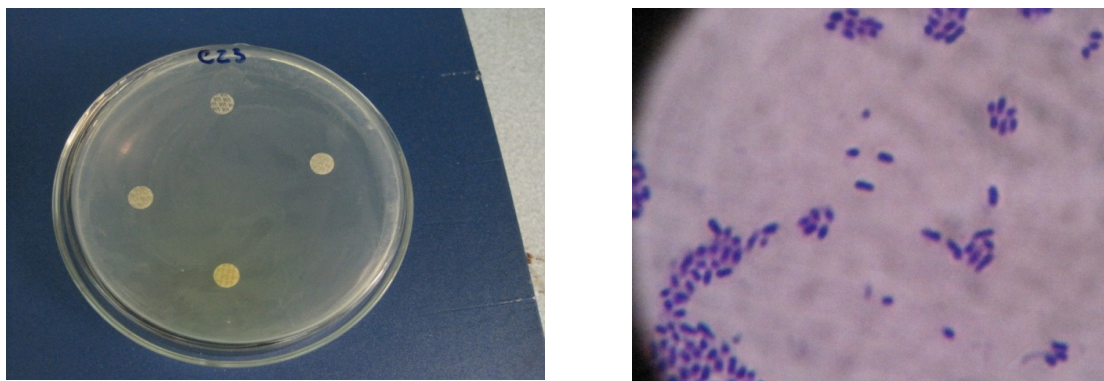


Figure 5. Phylogenetic relationship between the C23 strain and other closely related species. Neighbour-joining phylogenetic tree based on the 16S rDNA sequences showing the relationship between C23 strain and representatives of the genus *Pseudomonas*. Numbers at nodes are bootstrap values based on 1000 resamplings; only values greater than 50% are shown. Bar, 0.01 substitutions per nucleotide position. The 16S rDNA sequence of the bacterium *Burkholderia*, an outgroup of the *Pseudomonas* genus, was used as the root of the phylogenetic tree.

Compared to the bacterial 16S rRNA of *Pseudomonas monteilii*, 16S rDNA sequence of strain C23 is 98.6% similar (1429/1450). This species was first discovered by Elomari M & et al in 1997 from clinical specimens [10]. The clinical significance of *P. monteilii* is not known. This strain should be further investigated to determine their role in nosocomial infections. Their hypothesis was that *P. monteilii* is a rare opportunistic pathogen or colonizer [11].

So based on the morphological and physio-biochemical and molecular characteristics of C23, it is suggested that C23 is belong to *Pseudomonas monteilii* species and signated as *Pseudomonas monteilii* C23.

This strain C23 exists in many fish and environments, causing high risk of transferring antibiotic resistant gene among the bacteria in different ecological environments. If C23 is a pathogenic strain of a fish or people or things, the possibility that it can be treated with antibiotic will be very low.

4. Conclusion

In summary, this study reveals that drug resistance is very common in the environment,

and the efficiency of antibiotics in the treatment of infection is very low. Results have illustrated that 8 antibiotic samples were resisted at rate of 91% (33/36 strains). Remarkably, most of them are multi-drug resistant, i.e. they can resist two or more antibiotic, with one strains capable of resisting all 8 kinds of antibiotics. Which is isolated, marked as C23 strain belongs to *Pseudomonas monteilii*. It is important to note that excessive abuse of antibiotics is happening every day, increasing the risk of antibiotics resistance in bacteria.

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Tình trạng kháng kháng sinh của một số vi khuẩn phân lập từ cá nước ngọt ở tỉnh Hải Dương

Nguyễn Thị Giang, Phạm Đức Ngọc, Trần Mỹ Hạnh, Bùi Thị Việt Hà

Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam

Tóm tắt: Việc lạm dụng thuốc kháng sinh trong nuôi trồng thủy hải sản đang là vấn đề đáng lo ngại. Để đạt được sản lượng và lợi nhuận cao nhất, nhiều ngư dân hiện đang áp dụng các phương thức nuôi thâm canh. Nhưng các vật nuôi lại bị ảnh hưởng nhiều hơn bởi những áp lực và bệnh tật dẫn đến những vụ dịch bệnh gây chết hàng loạt. Trong số các bệnh của thủy sản thì nguyên nhân chủ yếu là do vi khuẩn gây ra với những vụ dịch bệnh có qui mô lớn. Thông thường, người ta sử dụng thuốc kháng sinh để kiểm soát các vi khuẩn gây bệnh. Do việc sử dụng không đúng cách và quá nhiều các loại thuốc kháng sinh nên đã gây ra hiện tượng vi khuẩn kháng thuốc và tích tụ dư lượng thuốc kháng sinh trong thịt thủy sản. Một nguyên nhân khác gây ra hiện tượng vi khuẩn kháng thuốc là việc sử dụng các loại kháng sinh với hàm lượng nhỏ trong thức ăn của thủy sản như một chất kích thích sinh trưởng. Trong nghiên cứu này đã bước đầu đánh giá tình hình kháng chất kháng sinh của các chủng vi khuẩn và thực trạng lạm dụng chất kháng sinh trong nuôi trồng thủy sản. Đánh giá hiệu quả của kháng sinh trong sử dụng hiện nay và đưa ra cảnh báo về tình trạng lây truyền của các gen kháng thuốc trong các vi sinh vật tồn tại trong thủy hải sản đặc biệt là các vi khuẩn nguy hiểm cho vật nuôi và con người. Kết quả thực nghiệm cho thấy 33/36 chủng vi khuẩn phân lập từ các mẫu cá của đã kháng lại 8 loại kháng sinh với ở tỷ lệ 91%. Hầu hết trong số họ là đa kháng thuốc, do chúng có thể chống lại hai hoặc nhiều loại kháng sinh. Đặc biệt, chủng C23 có khả năng chống lại tất cả 8 kháng sinh, được xác định thuộc về loài *Pseudomonas monteilii*. Loài này có khả năng chịu được dải nhiệt độ cũng như pH rộng và có sử dụng đa dạng nguồn dinh dưỡng khác nhau.

Từ khóa: Kháng kháng sinh, *Pseudomonas monteilii*, cá nước ngọt.