ANTIMICROBIAL RESISTANCE IN *Streptococcus agalactiae* IN TILAPIA (*Oreochromis* sp.) FARMING IN NORTHERN VIETNAM

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ABSTRACT

Antimicrobial resistance (AMR) is a threat to animal and human health globally. Antimicrobials are used in aquaculture to control different bacterial diseases. However, inappropriate use of antimicrobials can accelerate the emergence of AMR. This study was conducted to determine antimicrobial resistance in *Streptococcus agalactiae*, which affects and causes diseases with high mortality in tilapia. A total of 26 *S. agalactiae* isolates were streaked from kidney/brain/liver samples of diseased farmed tilapia from 2017 to 2021 at Hai Duong, Bac Ninh, Hoa Binh, Yen Bai provinces. The sensitivity of the isolates to 7 antimicrobials was established by disc diffusion (Oxiod). The results showed that levels of resistance vary from *S. agalactiae* to DOX (34.4%), RIF (64.1%), TET (45.0%), ERY (64.8%), TMP-SMX (70%), FLO (35.3%), and AMP (75%). The resistance rate to DOX was significantly different ($p<0.05$) compared to ERY. *S. agalactiae* has the highest resistance to 2 types of antibiotics at 19.2%, followed by resistance to 3 types (15.4%), and resistance to 6 and 7 types of antibiotics at a rate of 11.5%. The resistance in 1 type, 4 types, and 5 types of antibiotics was observed lowest at 7.7%. Vietnamese tilapia farmers need guidelines for effective antimicrobial treatment and wwider take up of vaccine or sustainable farming technology to prevent infections caused by *S. agalactiae* and reduce emergence of AMR.

Keywords: Tilapia*; Streptococcus agalactiae*; antimicrobial; resistance.

1. Introduction

Antimicrobial resistance (AMR) remains a public health threat globally and south-east Asian countries have a high antimicrobial usage (AMU) and AMR [1]. Vietnam's rates of AMR are among the highest in Asia, with multi-drug-resistant infections causing thousands of deaths annually [2]. Vietnam has the highest rates of penicillin resistance (71.4%) and erythromycin resistance (92.1%) in Asia [3]. AMR is generally driven by the irrational use of antimicrobials at all levels of the health care system. However, in aquaculture and livestock production, AMU

may also contribute to AMR. Overuse of antimicrobials in livestock, use of substandard drugs, inadequate AMR surveillance, low public awareness, and inadequate regulation have all been associated with increasing AMR rates in Vietnam [4].

Fish farming is one of the fastest-growing sectors in Vietnam and plays an important role in the national economy. In 2021, the value of seafood export reached 8.9 billion US dollars; the total fishery production was 8.73 million tons, of which aquaculture production contributed 4.8 million tons [5]. The main aquaculture species cultured in Vietnam are Pangasius (catfish), brackish water shrimp and more recently tilapia. Oriented plan upto 2030, tilapia will become the main product farmed species after brackish water shrimp and pangasius, which will focus on developing and producing main 3 species of tilapia including: striped tilapia (*Oreochromis niloticus*); tilapia Cross-species hybrid between striped tilapia (*O. niloticus*) and green tilapia (*O. aureus*); and red tilapia (*Oreochromis* spp.) [6]. In 2020, the tilapia farming area in Vietnam reached 37,697 hectares in earth pond, over 1.2 million cubic meters (m^3) in cage, strongly developed in the northern provinces such as Hai Duong, Bac Giang, Bac Ninh, Phu Tho, Hoa Binh, Hanoi, Yen Bai; Quang Ninh and some provinces in the Mekong Delta such as An Giang, Dong Thap, Hau Giang [7]. Vietnam has a tilapia production of about 270,000 tons, ranking 7th in the world after China, Indonesia; Egypt; Brazil; Philippines and Bangladesh [8], and ranked 6th in the world in terms of export volume [9].

Although the aquaculture sector, including tilapia farming, is quite established in Vietnam, it faces several challenges including diseases. A report on tilapia disease and anomalous symptoms in northern provinces (2017-2021) demonstrated that reduced feeding, scattered death, haemorrhage, cloudy/explosion eyes infected with bacteria were common and some samples also experienced parasitic disease [7]. Bacterial infections, in particular *S. agalactiae* has caused the highest mortality in Vietnamese tilapia culture across provinces [10-12]. Applying probiotics, chemicals and antimicrobials is the common practice of farmers to prevent and control disease during the grow-out phase. Approximately 70% of drugs used for animals and aquatic animals in Vietnam are antimicrobials [13]; monitoring of both livestock and aquaculture has shown that many antimicrobials critical for human health are used by farms [14]. Interviews conducted in 2022 with tilapia farmers in three northern provinces showed that amoxicillin (28.6%) and doxycycline (21.4%) were commonly used as were antimicrobials sold in pharmacies for human use (14.3%). In Bac Giang province, amoxicillin was commonly used (77.8%), whereas doxycycline/flophenicol were often used in Bac Ninh province (55.6%) [15]. However, data related to bacterial AMR in aquaculture systems are still limited, especially for tilapia pathogenic bacteria. Therefore, the determination of antibiotic resistance of bacteria isolated from infected tilapia is the aim of this research.

2. Materia and methods

2.1. Materials

Diseased tilapia samples showed clinical signs such as hemorrhage, abdominal distension, protruding eyes, and fluid in the abdominal cavity. The mortality rate of cultured fish in the pond was also recorded. Fish samples were collected from various

provinces including Hoa Binh, Hai Duong, Bac Ninh, and Yen Bai. A total of 26 strains were subjected to antibiotic analysis. The standard bacterium E. coli ATCC®25922™ was used as a positive control for each antibiotic tested.

The bacterial isolation method used in this study followed the protocol outlined by Buller in 2004 [16]. Liver, kidney, brain, and spleen samples from tilapia were cultured on TSA (Trypticase soy agar) nutrient medium and incubated at 28-30°C for 24-48 hours. After incubation, the bacterial cultures were subjected to proliferation culture, grams staining, and biochemical testing using API20Strep. The results of the biochemical reactions were analyzed using the Apiweb TM-API20Strep software, which identified bacterial species that matched 99% similarity with *S. agalactiae*.

The commercial discs antibiotics used in this study included doxycycline (DOX) at a concentration of 30 µg/disc, rifamycin (RIF) at 30 µl/disc, tetracycline (TET) at 30 µg/disc, erythromycin (ERY) at 15 µg/disc, trimethoprim/sulfamethoxazole (TMP-SMX) at a ratio of 1.25/23.75 µg/disc, florfenicol (FLO) at 30 µg/disc, and ampicillin (AMP) at 10 µg/disc.

2.2. Methods

The sensitivity of antibiotics was performed according to the Kirby-Bauer agar diffusion method [17]. The criteria for assessing the resistance of bacteria are based on the standards table of the Clinical and Laboratory Standards Institute (CLSI) updated in 2012.

The diameter of the zone inhibition (mm) measured using a graduated ruler after incubation at 28-30^{o}C after 24 hours. The obtain results of resistance, sensitivity or average resistance to the tested antibiotic based on according to CLSI guidelines (Table 1).

Table 1: *CLSI guidelines for the determination of antibiotic resistance in bacteria*

Antibiotic paper circle	**Susceptible (S)**	**Intermediate (I)**	**Resistant (R)**
	Measure unit (mm)		
Doxycycline (DOX 30)	≥14	11-13	≤10
Rifamycin (RIF 30)	≥20	17-19	≤16
Tetracycline (TET 30)	≥15	12-14	≤11
Erythromycine (ERY 15)	≥23	14-22	≤13
Trimethoprim/sulfamethoxazole (TMP-SMX 1.25/23.75)	≥17	13-16	≤12
Florfenicol (FLO 30)	≥20	17-19	≤16
Ampiciline (AMP 10)	≥17	14-16	≤13

Excel 2010 software is used for descriptive statistical analysis and the differences between formulas were investigated using LSD on SPSS 20 software.

3. Results and discussion

3.1 Current status of resistance to some antibiotics of Streptococcus agalactiae

The analysis results indicated that *S. agalactiae* strains isolated from diseased tilapia samples exhibited varying rates of antibiotic resistance, ranging from 34.4% to

75% for the seven antibiotics investigated (Table 2). It is worth noting that the resistance rate for the antibiotic doxycycline (DOX) was 34.4%, which was significantly different ($p<0.05$) from the resistance rate observed for Erythromycin (ERY). These findings align with previous studies conducted by Dang Thi Lua and Truong Thi My Hanh in 2019, which demonstrated that *Streptococcus* sp. isolated from tilapia and climbing perch cultivated in Phu Tho and Hai Duong exhibited resistance to AMP and TMP-SMX [18]. Additionally, another study conducted by Phuoc et al. in 2015 found that *Streptococcus* sp. causing disease in tilapia cultivated in the Mekong Delta showed resistance to various antibiotics, including ofloxacin, nitrofuran, tetracycline, oxacillin, bacitracin, ampicillin, amoxicillin, and streptomycin [19]. Thereby, the antibiotic resistance of *Streptococcus* sp. isolated from cultured tilapia has been clearly identified. However, there are differences in the types of antibiotics used in different research areas. This is because each region of aquaculture uses different types of medications.

Some investigation results on antibiotic use in diffirent aquaculture areas demonstrated: In An Giang province, sulfonamide and trimethoprim were mostly used (95.0%), followed by amoxicillin (55.0%), florfenicol (50.0%). In Tien Giang province, there were 8 antibiotics, among those the most used were sulfonamide and trimethoprim (87.0%), followed by amoxicillin (56.5%). In Dong Thap province, there were 11 antibiotics included amoxicillin (86.4%), sulfonamide and trimethoprim (81.8%), ciprofloxacin (63.6%), oxytetracycline (59.1%), florfenicol (59.1%) and enrofloxacin (50.0%). The tilapia farmers in Vinh Long province have used 6 types of antibiotics in the treatment of fish diseases. The most used products are still sulfonamide and trimethoprim (81.0%), amoxicillin (76.2%), florfenicol (76.2%), doxycycline (71.4%) and other antibiotics such as ampicillin, colistin, gentamycin, florfenicol and doxycycline, erythromycin, tobramycin, etc. with low usage rates [12]. In Hai Duong province, the usage rate of amoxicillin is highest (28.6%), followed by doxycline (21.4%), especially sometimes using human medicine for fish (14.3%). Similar to Hai Duong, 77.8% of households in Bac Giang use amoxicillin, then trimethoprim-sulfamethoxazole (66,7%), doxycycline (44,4%.), while tilapia farmers in Bac Ninh commonly use a combination of two antibiotics, doxycycline and flophenicol (55,6%) [15].

In terms of sensitivity, the sensitivity rates of the 7 selected antibiotics for research on *S. agalactiae* ranged from 12.5% to 61.2%. The highest sensitivity rate of DOX at 61.2% demonstrated a significant difference ($p<0.05$) compared to AMP (12.5%), while the other 5 antibiotics did not show any significant difference ($p>0.05$) among themselves as well as compared to other experimental antibiotics (Table 2). Meanwhile, the intermediate results of 7 drugs tested for *S. agalactiae* ranged from 4.4-29.5% and there was no significant difference between them (Table 2). The TET and DOX antibiotics of the tetracycline group have high sensitivity to *Streptococcus* sp. has also been report in tilapia farming areas in Phu Tho and Hai Duong [18].

Table 2: *Rate of antimicrobial resistance of S. agalactiae to some antibiotics*

No.	Antibiotic paper circle	Resistant (R) (%)	Intermediate (I) (%)	Susceptible (S) (%)
1	Doxycycline (DOX 30)	34.4 ± 10.2^{a}	4.4 ± 4.4^{a}	61.2 ± 15.8^{b}

No.	Antibiotic paper circle	Resistant (R) (%)	Intermediate (I) (%)	Susceptible (S) (%)
2	Rifamycin (RIF 30)	61.4±18.6^{ab}	5.7±5.7^{a}	32.9±19.1^{ab}
3	Tetracycline (TET 30)	45.0±17.8^{ab}	25.0±13.1^{a}	30.0±17.9^{ab}
4	Erythromycine (ERY 15)	64.8±17.0^{b}	25.2±8.0^{a}	30.0±20.0^{ab}
5	Trimethoprim/sulfamethoxazole (TMP-SMX 1.25/23.75)	70.0±20^{ab}	10.0±10.0^{a}	20.0±20.0^{ab}
6	Florfenicol (FLO 30)	35.3±19.4^{ab}	29.5±18.7^{a}	35.2±18.4^{ab}
7	Ampiciline (AMP 10)	75.0±14.4^{ab}	12.5±12.5^{a}	12.5±12.5^{a}

Note: *The data in the table are the Mean ± Standard error. The data in the same column containing different letters are statistically significant ($p<0.05$)*

Furthermore, the analysis of the inhibition diameter of *S. agalactiae* samples collected from 2017 to 2021 for the same antibiotic revealed a gradual decrease in the sterile ring diameters over time, as indicated in Figure 2. This observation further supports the existence of antibiotic resistance in *S. agalactiae*. In 2017, the average inhibited ring diameter for the tested antibiotics ranged from 15-30 mm. However, in subsequent years, there was a sharp decline in the average inhibited ring diameter, particularly for TMP-SMX, where the average inhibited ring diameter was only 2.5mm in 2021. The average inhibited ring diameter of S. agalactiae for DOX, RIF, TET, and ERY also exhibited a gradual decrease over time from 2017 to 2021, with significant reductions observed during the periods of 2017-2018 and 2020-2021, resulting in a sterile diameter average reduction of 10-15 mm (Figure 1).

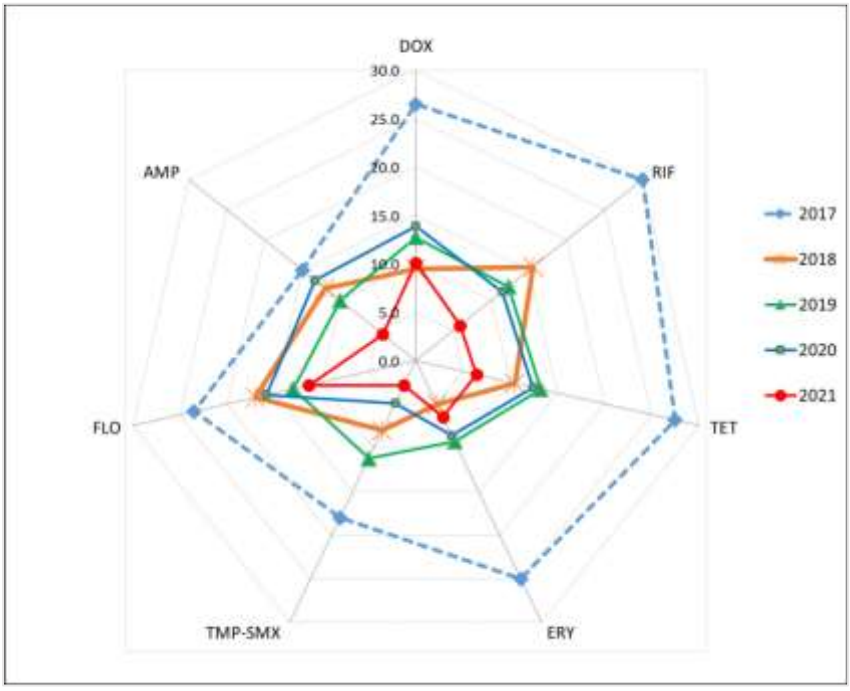

Figure 1: *The average inhibition diameter of S. agalactiae with some antibiotics*

3.2 Multi-antibiotic resistance of Streptococcus agalactiae

The study results indicate that *S. agalactiae* is both mono and multi-antibiotic resistant. *S. agalactiae* has the highest rate of resistance to 2 types of antibiotics at 19.2%, followed by resistance to 3 types of antibiotics (15.4%), and finally resistance to 6 and 7 types of antibiotics at a rate of 11.5%. The lowest resistance was seen in 1 type, 4 types, and 5 types of antibiotics at 7.7% (Figure 2). Multi-drug resistance in pathogenic bacteria in aquaculture has been studied and identified in various different farming subjects. *Streptococcus* sp. isolated from samples of carp and catfish farming in Phu Tho and Hai Duong provinces showed resistance to at least 2 antibiotics and up to 6 antibiotics [18]. *Vibrio* sp. obtained from shrimp in Long An, Ben Tre, Bac Lieu, and Ninh Thuan provinces exhibited 100% strain resistance to 1 antibiotic, 95% strain resistance to 4 antibiotics, with over 50% of bacterial strains showing resistance to more than 10 types of antibiotics, including one strain resistant to 21 types of investigated antibiotics [20]. *V. parahaemolyticus*, the causative agent of AHPND in farmed shrimp in Quynh Luu district, Nghe An province, demonstrated the highest rate of resistance to 4 types of antibiotics (33.3%), followed by resistance to 6 and 3 types of drugs (22.2%), and finally resistance to 2 and 5 types of drugs (11.1%) [21]. *Edwardsiella ictaluri*, which causes disease in farmed Tra catfish in the Mekong Delta, was found to have up to 86% of strains showing resistance to 3 or more drugs, and 70% of strains resistant from 6 to 9 types of drugs [22]. *Listeria seeligeri* and *L. welshimeri* bacteria showed resistance to 2 types of antibiotics at a rate of 10.9% [23]. Therefore, the phenomenon of multi-drug resistance in bacteria has been identified in aquaculture, posing a potential risk of transferring multi-drug resistance genes from pathogenic bacteria in aquatic animals (such as shrimp) to bacteria causing diseases in humans and livestocks.

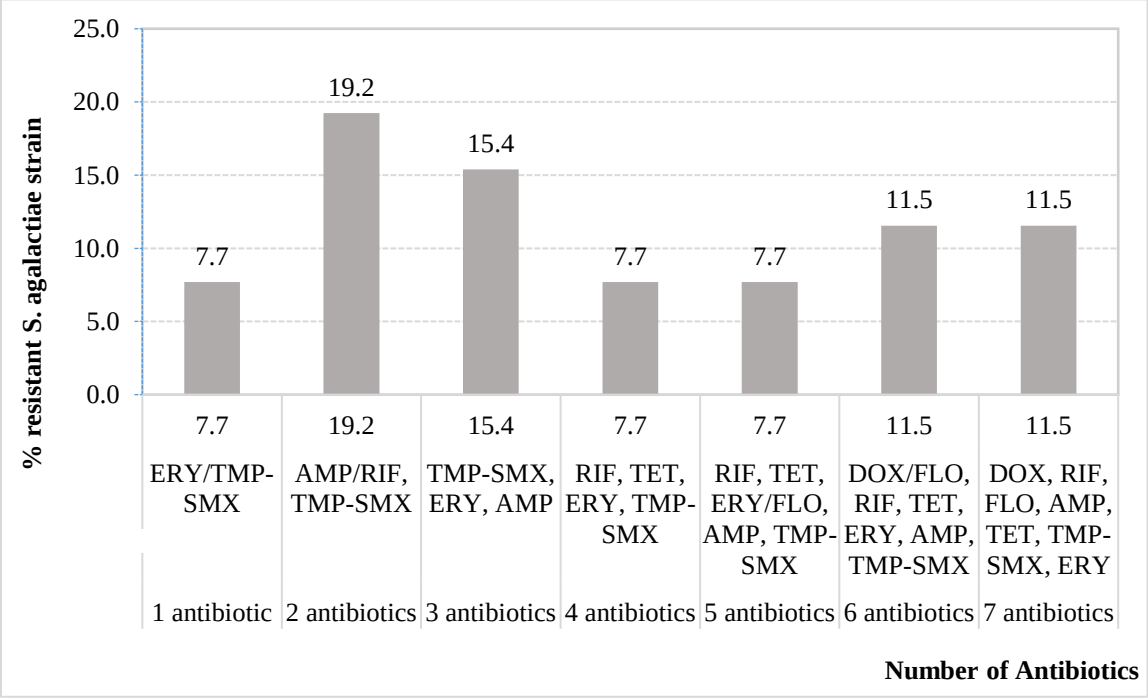

Figure 2: *Mono and multi-antibiotic resistance of S. agalactiae*

4. Conclusion

S. agalactiae, isolated from diseased tilapia samples in Hai Duong, Bac Ninh, Hoa Binh, and Yen Bai provinces, exhibited varying resistance rates to different antibiotics. The resistance rates were as follows: DOX (34.4%), RIF (64.1%), TET (45.0%), ERY (64.8%), TMP-SMX (70%), FLO (35.3%), and AMP (75%). The resistance rate to DOX was significantly different ($p<0.05$) compared to ERY.

Among the resistant strains of *S. agalactiae*, the highest rate was observed for resistance to 2 types of antibiotics (19.2%). This was followed by resistance to 3 types of antibiotics (15.4%), then resistance to both 6 and 7 antibiotics at a rate of 11.5%. On the other hand, the lowest resistance rates were seen in strains resistant to only 1 antibiotic, 4 antibiotics, and 5 antibiotics, at 7.7%.

5. Proposal

In tilapia farming areas, including Hai Duong, Bac Ninh, Hoa Binh, and Yen Bai, it has been indicated that *S. agalactiae* exhibits resistance to certain antibiotics. Therefore, before choosing for antibiotic treatment for fish diseases, it is essential to collect samples and assess the disc antibiotic diffusion test.

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TÓM TẮT

KHÁNG KHÁNG SINH CỦA *Streptococcus agalactiae* GÂY BỆNH TRÊN CÁ RÔ PHI (*Oreochromis* sp.) Ở MIỀN BẮC VIỆT NAM

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Ngày nhận bài 04/12/2023, ngày nhận đăng 05/3/2024

Kháng kháng sinh là mối đe dọa đối với sức khỏe động vật và con người trên toàn cầu. Thuốc kháng sinh được sử dụng trong nuôi trồng thủy sản để trị các bệnh do vi khuẩn. Nhưng việc sử dụng thuốc kháng sinh không phù hợp có thể dẫn đến kháng kháng sinh. Nghiên cứu này được thực hiện nhằm xác định tình trạng kháng kháng sinh của vi khuẩn *Streptococcus agalactiae*. Với tổng số 26 *S. agalactiae* được phân lập từ các mẫu thận/não/gan của cá rô phi nuôi bị bệnh từ năm 2017 đến 2021 tại các tỉnh Hải Dương, Bắc Ninh, Hòa Bình, Yên Bái. Độ nhạy của vi khuẩn phân lập với 7 loại kháng sinh được xác định bằng phương pháp khuếch tán đĩa (Oxiod). Kết quả cho thấy mức độ kháng khác nhau, với DOX (34,4%), RIF (64,1%), TET (45,0%), ERY (64,8%), TMP-SMX (70%), FLO (35,3%) và AMP (75%). Tỷ lệ *S. agalactiae* kháng với DOX khác biệt rõ rệt ($p<0,05$) với ERY ($p<0,05$). *S. agalactiae* cho kết quả kháng cao nhất với 2 loại kháng sinh (19,2%), tiếp đến kháng với 3 loại kháng sinh (15,4%) và kháng với 6 và 7 loại kháng sinh chiếm 11,5%. Tỷ lệ kháng với 1, 4 và 5 loại kháng sinh là thấp nhất chiếm tỷ lệ 7,7%. Người nuôi cá rô phi Việt Nam cần có hướng dẫn về điều trị kháng sinh hiệu quả và sử dụng vắc-xin hoặc công nghệ nuôi bền vững rộng rãi hơn để ngăn ngừa nhiễm khuẩn do *S. agalactiae* gây ra và giảm sự xuất hiện của kháng kháng sinh.

Từ khóa: Cá rô phi; vi khuẩn *Streptococcus agalactiae*; kháng sinh; kháng kháng sinh.