EFICÁCIA *IN VITRO* DE FLORFENICOL E BICICLOMICINA PARA BACTÉRIAS PATOGÊNICAS DE PEIXES DE ÁGUA DOCE

DANIELA TUPY DE GODOY

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do curso de Mestrado em Ciências Veterinárias, para a obtenção do título de Mestre.

Orientador
Prof. Dr. Henrique César Pereira Figueiredo

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DEDICATÓRIA

Ao meu pai, a minha mãe e a minha irmã, dedico.

Esta conquista é nossa!

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SUMÁRIO

RESUMO

Godoy, Daniela Tupy de. **Eficácia *in vitro* de florfenicol e biciclomicina para bactérias patogênicas de peixes de água doce.** Lavras: UFLA, 2006. 47p. (Dissertação de Mestrado em Ciências Veterinárias)*

Dentre os patógenos que acometem as pisciculturas no Brasil, destacam-se as aeromonas móveis e o *Streptococcus agalactiae*. Responsáveis por surtos de septicemia e encefalite, respectivamente, levam a altas taxas de mortalidade e a antibioticoterapia é a principal forma de controle. O objetivo deste trabalho foi determinar a concentração inibitória mínima (MIC) de florfenicol (FLO) e biciclomicina (BCM) para aeromonas móveis isoladas de peixes e água de cultivo, e *S. agalactiae* isolados de peixes, oriundos de diferentes estados brasileiros. Foram utilizadas 118 amostras de aeromonas móveis e 27 de *S. agalactiae.* A metodologia utilizada para determinação do MIC foi a microdiluição em caldo, de acordo com "Method for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals", estabelecido pelo Clinical and Laboratory Standards Institute (CLSI, 2006). Os testes foram incubados a 28ºC e foram utilizadas diluições seriadas de base dois para os antibióticos florfenicol variando de 0,06 a 64μg mL^{-1} e biciclomicina variando de 0,195 a a 200μg mL^{-1}. As amostras foram classificadas como susceptíveis ou resistentes, de acordo com o padrão de distribuição modal dos valores de MIC. Para *S. agalactiae* os valores de MIC para florfenicol e biciclomicina variaram de 1μg mL^{-1} a 16 μg mL^{-1} e 3,12 μg mL^{-1} a 12,5 μg mL^{-1}, respectivamente. Uma distribuição unimodal dos resultados de MIC foi observada em ambos os casos e todas as amostras foram classificadas como susceptíveis a BCM e ao FLO. Para aeromonas móveis, 100% das amostras foram susceptíveis ao florfenicol ($0,5 \leq MIC \geq 16$ μg mL^{-1}) e à biciclomicina (0,78-100 μg mL^{-1}). Com relação à fonte das amostras, caso clínico ou água de cultivo, apenas as amostras de água de cultivo apresentaram padrão bimodal de distribuição dos resultados para biciclomicina, caracterizando a existência de uma população sensível ($MIC \leq 6,25$ μg mL^{-1}) ao antibiótico e outra resistente ($MIC \geq 50$μg mL^{-1}). O florfenicol e a biciclomicina apresentaram alta eficácia *in vitro* contra aeromonas móveis e *S. agalactiae.*

* Orientador: Prof. Henrique César Pereira Figueiredo.

ABSTRACT

Godoy, Daniela Tupy de.***In vitro*** **efficacy of florfenicol e bicyclomycin to pathogenic bacteria from freshwater fish.** Lavras: UFLA, 2006. 47p. (Master Dissertation in Veterinary Science)*

The *Streptococcus agalactiae* and the motile aeromonads are major pathogens for several tropical fish species, causing encephalitis and septicemia outbreaks, respectively, with high mortality in intensive culture systems. The use of antibiotic is the main control measure. The objective of this work was to determine the minimum inhibitory concentration (MIC) of florfenicol (FLO) and bicyclomycin (BCM) for motile aeromonads isolated of diseased fish and pond water, and *S. agalactiae* isolated from fish. One hundred eighteen strains of motile aeromonads and 27 strains of *S. agalactiae* were selected from different Brazilian states. The methodology used for determination of the MIC was the microdilution in broth, in accordance with "Method for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals", established for the Clinical and Laboratory Standards Institute (CLSI, 2006). All test were carried out with an incubation temperature of 28ºC and serial two-fold dilution of florfenicol (0,06 – 64 μg mL^{-1}) and bicyclomycin (0,195 – 200μg mL^{-1}) were used. All strains were classified as susceptible or resistant according to the profile of data distribution (continuous or modal). To *S. agalactiae*, the MIC values to FLO ranged from 1 μg mL^{-1} to 16 μg mL^{-1} and to BCM from 3.12 μg mL^{-1} to 12.5 μg mL^{-1}. In both cases it was observed the distribution of values in one cluster and all strains analyzed were classified as susceptible to FLO and BCM. To motile aeromonads, the MIC values to FLO ranged from 0.5 μg mL^{-1} to 16 μg mL^{-1} and to BCM from 0.78 μg mL^{-1} to 100 μg mL^{-1}. To the both antibiotics the MIC values presented a continuous distribution. In the motile aeromonads isolated from pond water it was observed a bimodal profile for MIC values of BCM, characterizing a susceptible population (MIC $\leq$ 6.25 μg mL^{-1}) and other resistant (MIC $\geq$ 50 μg mL^{-1}) to the antibiotic. Both antibiotics presented a high effectivity *in vitro* against motile aeromonads and *S. agalactiae* strains.

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INTRODUÇÃO

No Brasil, a piscicultura representa um novo ramo do agronegócio, com previsão de crescimento do consumo de pescado, tanto no mercado interno como para exportação. Apesar da diversidade de peixes nativos e das excelentes características zootécnicas de algumas destas espécies, a tilápia do Nilo (*Oreochromis niloticus*), que é uma espécie de peixe exótica, destaca-se dentre as cultivadas no Brasil. Com o aumento expressivo de novos empreendimentos financeiros nessa área, inclusive com linhas de crédito governamentais, observam-se grandes entraves técnicos à produção, notadamente nos aspectos de sanidade e doenças infecciosas que acometem os peixes.

Segundo dados da FAO (2004), a contribuição da aqüicultura no fornecimento global de peixes, crustáceos e moluscos continua crescendo, de 3,9% da produção, em 1970 para 29,9%, em 2002. A aqüicultura cresce mais rápido em relação a outros setores que produzem alimento de origem animal. Mundialmente, este setor cresceu a uma taxa de 8,9% ao ano, desde 1970, enquanto a pesca por captura cresceu 1,2% e os sistemas terrestres de produção de carne 2,8%, no mesmo período. O Brasil ocupa o quarto lugar no ranking dos dez maiores produtores, com relação ao crescimento. De 2000 a 2002, a taxa de crescimento foi de 18,1%.

Dentre os patógenos freqüentes na piscicultura podem-se destacar o *Streptococcus agalactiae* e as aeromonas móveis.

O *Streptococcus agalactiae* é capaz de causar doença em uma vasta gama de hospedeiros. Ele foi primeiramente isolado no homem, causando meningite neonatal e em bovinos, causando mastite. Ocasionalmente, também pode causar infecções em ratos, gatos, cães, hamsters, camelos e sapos. Nos peixes, causa surtos de encefalite e é responsável por grandes perdas econômicas, alcançando

90% de mortalidade, podendo ocorrer em peixes de ambientes estuarínos, marinhos ou de água doce. Dentre os peixes de água doce cultiváveis, o maior impacto econômico é observado em criações de tilápia do Nilo.

Dentre os patógenos de peixes de água doce, as aeromonas móveis têm assumido, nos últimos anos, uma posição importante, devido a sua ocorrência como agente primário causador de lesões e também por causar doenças em seres humanos, tanto pela ingestão de água como de alimentos contaminados, além de estarem envolvidas na difusão de genes de resistência a antibióticos entre bactérias. Considerada de ocorrência mundial é, provavelmente, a doença bacteriana mais comum em peixes de água doce. A infecção por aeromonas móveis está associada a fatores predisponentes, como o excesso de matéria orgânica na água, oxigênio dissolvido abaixo das concentrações adequadas e alta densidade animal.

O número de antibióticos usados na piscicultura é limitado devido ao custo das drogas para o tratamento de grandes populações, bem como por sua forma de administração. A via oral é a mais comumente utilizada, estando o antibiótico incorporado à ração, o qual não deve alterar a palatabilidade do alimento. No Brasil, encontra-se em tramitação, no Ministério da Agricultura, Pecuária e Abastecimento (MAPA), a aprovação dos antibióticos florfenicol e biciclomicina para uso oral na aqüicultura. Quando aprovados, serão os primeiros antibióticos licenciados no país para este uso.

O perfil de resistência bacteriana aos antibióticos deve ser foco de atenção, devido ao grande uso destas drogas, tanto na medicina humana, quanto em medicina veterinária. A resistência bacteriana aos antibióticos possui origem genética. Parte destes genes são móveis e podem ser transferidos entre populações de microrganismos. O fenômeno da transferência de genes de resistência entre bactérias pode resultar em aumento da severidade e duração das

doenças, aumento das taxas de mortalidade, elevação do custo com tratamentos e o ressurgimento de doenças já conhecidas, que se tornam não tratáveis.

O monitoramento da resistência aos antibióticos é determinante para o uso racional dessas drogas e a literatura apresenta dados escassos sobre o perfil de resistência aos antibióticos de bactérias isoladas de peixes. Dessa forma, o objetivo deste trabalho foi determinar a concentração inibitória mínima de florfenicol e biciclomicina para *Streptococcus agalactiae* e espécies de aeromonas móveis isoladas de peixes oriundos de diferentes estados brasileiros. Os resultados são apresentados em dois artigos científicos, a seguir.

FLORFENICOL AND BICYCLOMYCIN RESISTANCE PATTERNS TO BRAZILIAN STRAINS OF MOTILE AEROMONADS**.**

(Preparado de acordo com as normas da revista “Aquaculture”).

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Key words*:* florfenicol; bicyclomycin; motile aeromonads; minimal inhibitory concentration; fish.

Abstract

The minimum inhibitory concentration (MIC) of florfenicol (FLO) and bicyclomycin (BCM) to 118 motile aeromonads strains (*Aeromonas hydrophila, A. caviae e A. sobria*) isolated from diseased fish and pond water in Brazil was determined according to the new guideline of Clinical and Laboratory Standards Institute for MIC determination in bacteria isolated from aquatic animals (CLSI, 2006). Considering the three bacterial species together the MIC values to FLO ranged from 0.5 μg mL^{-1} to 16 μg mL^{-1} and to BCM from 0.78 μg mL^{-1} to 100 μg mL^{-1}. To the both antibiotics the MIC values presented a continuous distribution. In the motile aeromonads isolated from pond water it was observed a bimodal profile for MIC values of BCM, characterizing a susceptible population (MIC $\leq$ 6.25 μg mL^{-1}) and other resistant (MIC $\geq$ 50 μg mL^{-1}) to the antibiotic. Both antibiotics presented a high effectivity *in vitro* against motile aeromonads strains.

1. Introduction

The genus *Aeromonas* comprises a group of ubiquitous aquatic bacteria having a wide distribution in the freshwater and marine environment (Schimidt et al., 2000; Hatha et al., 2005). Aeromonads are linked with a variety of diseases in different fish species and motile aeromonads are often involved in disease outbreak in ponds. Among the motile aeromonads, *Aeromonas hydrophila*, *A. sobria* and *A. caviae* are most commonly associated with fish diseases (Wahli et al., 2005). It has been found to cause skin lesions and ulcerations, hemorrhages in the body surface, necrosis in the liver and kidney and septicemia, with high mortality rates (Popovíc et al., 2000, Wahli et al., 2005). There is no vaccine commercially available yet and the use of antibiotics is the major alternative to control the outbreaks. As farmed fish are often treated with antibiotic, this has resulted in the development of resistant bacterial strains. The emergence of antibiotic resistance in fish pathogens has been reported from temperate and tropical aquaculture systems (Hatha et al., 2005).

The use of antibiotics like florfenicol (FLO) and bicyclomycin (BCM) in aquaculture has been increased in last years. FLO is an

antibiotic of large spectrum, derived from chloramphenicol, which impairs the bacterial protein synthesis by direct linkage to the ribosome (Cannon et al., 1990; Schwarz et al., 2004). BCM is a novel antibiotic isolated from *Streptomyces sapporonensis* and *S. aizumenses.* Its structure is unrelated to other antibiotic classes and presents large spectrum against Gram-positive and Gram-negative bacteria (Magyar et al., 1999; Kohn & Widger, 2005). The BCM targets the rho, a transcription terminator that regulates bacterial gene expression. Without rho there is a loss of cell viability (Moyse et al., 2001; Kohn & Widger, 2005; Skordalakes et al., 2005). BCM is poorly absorbed in most animal species, with major action at the gastrointestinal tract (Hornick, 2003). The pharmacokinetics of BCM in tropical fish is unknown.

Several methods for minimum inhibitory concentration (MIC) determination have been proposed for bacteria isolated from aquatic animals (Martinsen et al., 1992; Alderman & Smith, 2001; Samuelsen et al., 2003 Coyne et al., 2004a). In 2006, based on a previous inter-laboratorial research (Miller et al, 2005), the CLSI (Clinical and Laboratory Standard Institute) established a harmonized protocol with

accurate quality controls to determine the MIC of antibiotics to bacteria isolated from aquatic animals.

The aim of this work was to determine minimum inhibitory concentration of florfenicol and bicyclomycin to motile aeromonads strains isolated from diseased fish and pond water in different Brazilian states, using the guideline of CLSI.

2. Material and methods

A total of 118 motile aeromonads strains were selected, comprising 92 *Aeromonas hydrophila,* 16 *A. caviae* and 10 *A. sobria*. The strains were isolated from *Oreochromis niloticus* (Linnaeus, 1758), *Brycon orbignyanus* (Vallenciennes, 1849), *Pseudoplatistoma corruscans* (Spix & Agassiz, 1829) and *Rhamdia quelen* (Quoy & Gaimard, 1824), in the period 2002–2006, and originated from eleven farms in the following Brazilian states: Mato Grosso do Sul, Minas Gerais , Rio de Janeiro e Rio Grande do Sul (Table 1). 74 strains were derived from diseased fish (hemorrhagic septicemia) and 44 strains from pond water of healthy fish

farms. All strains were previously identified by biochemical tests (Janda & Abott, 1998).

The MICs were determined in accordance with "Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline", (CLSI, 2006). Briefly, the bacterial strains, stocked in freezer –70^{o}C, were inoculated in cation-adjusted Mueller-Hinton broth (CAMHB, Difco, USA) and incubated at 28ºC for 24 hours. Bacterial suspension was prepared in saline solution (0.85%), adjusted to 0.5 of MacFarland standard (BioMérieux, France) and then diluted 10 times in CAHMB. The minimum inhibitory concentration tests of FLO were performed in sterile dry-form microplates (Sensititre, Trek Diagnostic system, U.K.) with the antibiotic concentration ranging from 0.06 μg mL^{-1} to 64 μg mL^{-1}. The reconstitution was made adding 100 μL of CAHMB in each well. An inoculum of approximately 5.0 x 10^{5} CFU mL^{-1} of the bacterial suspension was inoculated per well. To BCM MIC a stock solution (400 μgmL^{-1}) of BCM (Searchem corp., Japan) was prepared in each day of test. In sterile microplates (Kartell, Italy) 2-fold serial dilutions of BCM in 100 μL of CAHMB were made ranging from

0.195 μg mL^{-1} to 200 μg mL^{-1}. An inoculum of approximately 5.0 x 10^5 CFU mL^{-1} of the bacterial suspension was inoculated per well.

The microplates of FLO and BCM were sealed and incubated at 28ºC for 24 hours and then the results were read. *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 were used as quality control of plates and procedures. All strains were tested in duplicate. The MIC was defined as the lowest concentration of antibiotic that prevented a visible bacterial growth. The strains were classified as susceptible or resistant according to the modal profile of MIC values distribution showing two or more distinct populations. If the MIC distribution of an antimicrobial was bimodal the breakpoint was set as the midpoint between the peaks of each MIC distribution (Brunn et al., 2000).

3. Results

The distribution for MIC values of florfenicol and bicyclomycin to the 118 strains of motile aeromonads are shown on Figure 1. The MIC values to florfenicol ranged from 0.5 μg mL^{-1} to 16 μg mL^{-1}, and to

bicyclomycin from 0.78 μg mL^{-1} to 100 μg mL^{-1}. To the both antibiotics were observed a continuous distribution of MIC values.

Analysing the results for each bacterial species tested, the distribution of FLO MIC values to *A. hydrophila* was bimodal, with 99% of strains presenting MIC $\leq$ 4 μg mL^{-1} and 1% of strains with MIC $\geq$ 16 μg mL^{-1}. The same distribution pattern was obtained to BCM, with 99% of strains presenting MIC $\leq$ 6.25 μg mL^{-1} and 1% of strains with MIC $\geq$ 100 μg mL^{-1} (Figure 2). *A. caviae* strains showed a continuous distribution of MIC values to both antibiotics (Figure 3). *A. sobria* presented a bimodal profile to FLO (90% of strains with MIC $\leq$ 1 μg mL^{-1} and 10% with MIC $\geq$ 4 μg mL^{-1}) and a continuous distribution to BCM (Figure 4).

The motile aeromonads strains analysed were isolated from two different sources, diseased fish and pond water. The distribution of MIC values to FLO was continuous to the both sources, ranging from 0.5 μg mL^{-1} to 2 μg mL^{-1} for bacterial strains of diseased fish and from 0.5 μg mL^{-1} to 16 μg mL^{-1} for bacterial strains of pond water. To BCM the strains from diseased fish showed a continuous distribution (0.78 μg mL^{-1}

to 25 μg mL^{-1}) and the strains from pond water presented a bimodal profile (MIC ≤ 6.25 μg mL^{-1} and MIC ≥ 50 μg mL^{-1}) (Figure 5).

4. Discussion

At the present in Brazil there are no licensed antibiotics for use in pisciculture. However, the Brazilian Ministry of Agriculture are analysing the approval of oral formulations of FLO and BCM to use in aquaculture. Florfenicol have been shown effective to control several bacterial diseases in fish and is approved to use in European Union, United States, Canada, Japan and South Korea (Michel, et al., 2003; Lewbart et al., 2005; Yanong & Curtis, 2005; Kawanishi et al., 2006) and the bicyclomycin is approved to pseudotuberculosis treatment in fish in Japan (Kawanishi et al., 2006).

Considering the results obtained from the motile aeromonads with no bacterial species distinction all strains seems to be susceptible to FLO and BCM. The classification criteria of MIC values by modal distribution is applied generally when there are no established breakpoints based on

pharmacokinetics data or there are few reports on the behavior of a single bacterial species in relation to a given antibiotic (Bruun et al., 2000).

Although the MIC values to BCM had presented a continuous distribution, the broad range observed (Figure 1) suggests the occurrence of a resistant subpopulation, with high MIC values. This was evident in the data obtained from *A. hydrophila* (Figure 2B) and from motile aeromonads strains originated from pond water (Figure 5B), where resistant subpopulations were clearly observed. As BCM is a drug of low absorption, with main action at GI tract, the efficacy against strains with high MIC values could be proved by experimental infection and treatment under controlled conditions. In a study conducted in Japan with 74 strains of *Photobacterium damselae ssp. piscicida* all strains were susceptible to FLO and BCM, with MIC values ranging from 0.25 to 0.5 μg mL^{-1} and from 2 to 4 μg mL^{-1}, respectively (Kawanishi et al., 2006). In the present study 91% of motile aeromonads strains showed MIC to BCM ranging from 0.78 to 3.12 μg mL^{-1}. However, this comparison is limited by intrinsic differences among bacterial species in relation to antibiotic resistance.

The MIC values obtained to motile aeromonads strains were similar to previous reports. In France, 49 strains of motile aeromonads presented a continuous distribution of MIC values, ranging from 0.25 μg mL^{-1} to 32 μg mL^{-1} (Michel et al., 2003). In Australia, of 22 strains of *Aeromonas* sp. analyzed, only one was resistant to FLO with MIC ≥ 128 μg mL^{-1} and the susceptible strains with MIC ≤ 16 μg mL^{-1} (Akinbowale et al., 2006). From 313 danish strains of *Aeromonas* sp. only one strain was resistant (MIC = 32 μg mL^{-1}), and the other strains showed MIC ≤ 2 μg mL^{-1} (Schimidt et al., 2000). In Taiwan, 41 strains of *Aeromonas hydrophila* isolated from aquatic animals were tested, with MIC_{90} (MIC_{90}- the minimal concentration that could inhibit 90% of tested isolates) to FLO of 12.5 μg mL^{-1} (Ho et al., 2000). The MIC values to FLO in motile aeromonads have been similar, in spite of geographical origin of strains and distinct methodologies applied.

The strains of *Aeromonas hydrophila* presented a bimodal distribution of FLO and BCM MICs. However, for both antibiotics, the resistant population was composed only by one strain, named AE 262-03, which showed MIC to FLO and BCM of 16 μg mL^{-1} and 100 μg mL^{-1}, respectively. The FLO MIC value to AE 262-03 was close to the MIC

values of susceptible population, with no clear definition of a true resistant strain. To BCM an extensive gap was observed between susceptible population and the strain AE 262-03 (Figure 2) characterizing a resistant strain. A similar pattern was observed in FLO MIC values to *A. sobria* (Figure 4A).

The molecular basis of BCM resistance is poorly understood. The resistance seems to be plasmid-mediated through a gene (*bcr*) that encodes a transmembrane efflux protein. This protein also induces cross-resistance to oxytetracycline and some other antibiotics (Bentley et al., 1993). Mutation of gene encoding rho protein is also a source of BCM resistance (Yanofsky & Horn., 1995; Moyse et al., 2001). The molecular determinants of BCM resistance were not evaluated in this study.

Considering the distribuition of MIC values to motile aeromonads, a clear breakpoint could not be established. Another way to determine breakpoint is the use of pharmacokinetic data, based on the Cmax (maximum plasma concentration) of a drug. There is no available mathematical model to establish breakpoints for fish and some methods have been adapted (BSAC, 1991; Coyne et al., 2004b). However, for the

Brazilian tropical fish species, the Cmax values for FLO and BCM are unknown.

The outbreaks of septicemia by motile aeromonads are commonly associated to the occurrence of two or more *Aeromonas* species in the same farm. In consequence, the analysis of MIC values taking together different motile aeromonads species generate reliable results to apply in field conditions. Furthermore, the low number of *A. sobria* and *A. caviae* strains used in this study limits the determination of the populational behavior of that species against FLO and BCM.

The strains of motile aeromonads from diseased fish and pond water showed a similar pattern of MIC values distribution, to both antibiotics tested. There are no extensive reports comparing these two environmental sources for motile aeromonads and the data suggest that the analyzed sources do not exert a selective pressure for antibiotic resistance.

The differences in methods applied to determine the MIC for aquatic bacteria limit a complete comparison of generated data (Ho et al., 2000; Schimidt et al., 2000; Alderman & Smith, 2001; Michel et al., 2003; Akinbowale et al., 2006). The new protocol established by the

Clinical and Laboratory Standards Institute (CLSI, 2006) will allow in the future a full comparison of antibiotic resistance patterns in different regions and countries and a better management of antibiotic efficacy in aquaculture worldwide.

5. Conclusion

The high frequency of susceptible strains to FLO and BCM indicates the potential of these drugs to control motile aeromonads infections. Studies on the pharmacokinetics of these antimicrobials in tropical fish are desirable to the future use of FLO and BCM in field conditions.

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Table 1 –Brazilian strains of motile aeromonads used in this study.

Species	**Host/ Source**	**Geographic origin (State)**	**Farm**	**Strains (n)**
A. hydrophila	*P. corruscans*	Mato Grosso do Sul	A	4
A. sobria				2
A. caviae	*O. niloticus*	Minas Gerais	B	2
A. hydrophila				3
A. sobria				3
A. hydrophila	Pond Water			4
A. caviae	*O. niloticus*	Minas Gerais	C	5
A. hydrophila				12
A. caviae	*B. orbignyanus*	Minas Gerais	D	4
A. hydrophila				5
A. sobria				4
A. hydrophila	*O. niloticus*			7
A. caviae	Pond Water	Minas Gerais	E	5
A. hydrophila				1
A. hydrophila	Pond Water	Minas Gerais	F	5
A. hydrophila	Pond Water	Minas Gerais	G	4
A. hydrophila	Pond Water	Minas Gerais	H	4
A. hydrophila	Pond Water	Minas Gerais	I	3
A. sobria				1
A. hydrophila	Pond Water	Minas Gerais	J	17
A. hydrophila	*R. quelen*	Rio Grande do Sul	L	15
A. hydrophila	*O. niloticus*	Rio de Janeiro	M	8
Total				118

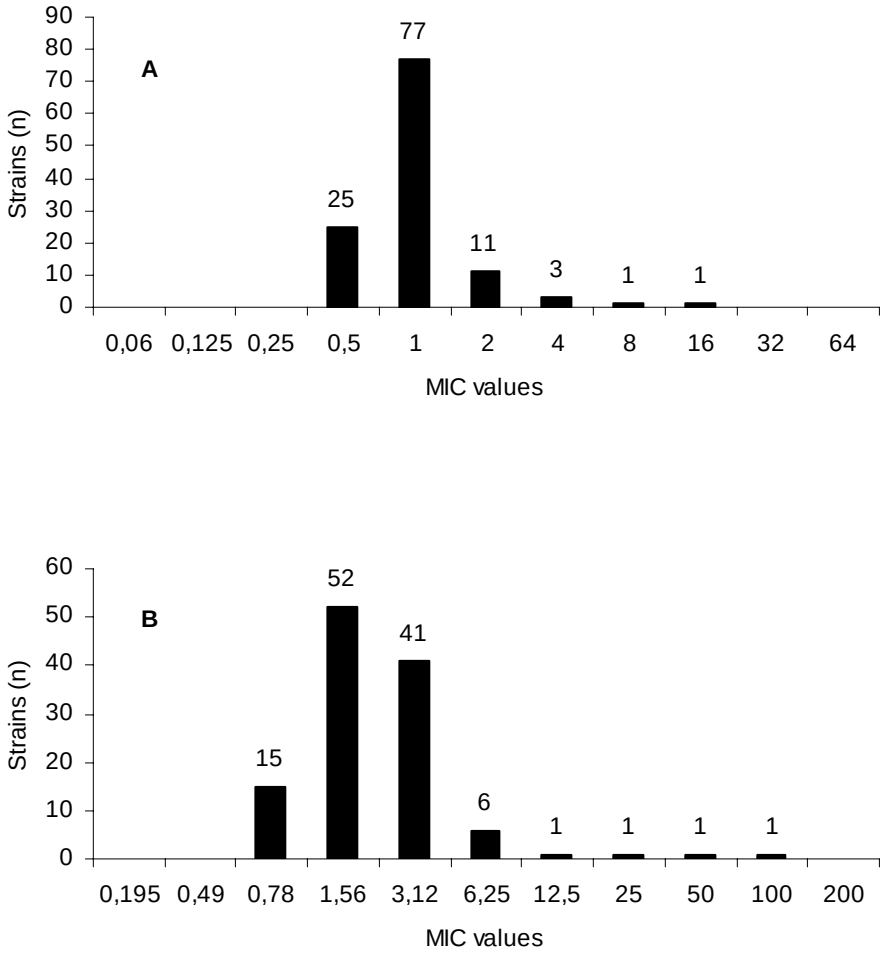

Figure 1 - Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B) to motile aeromonads.

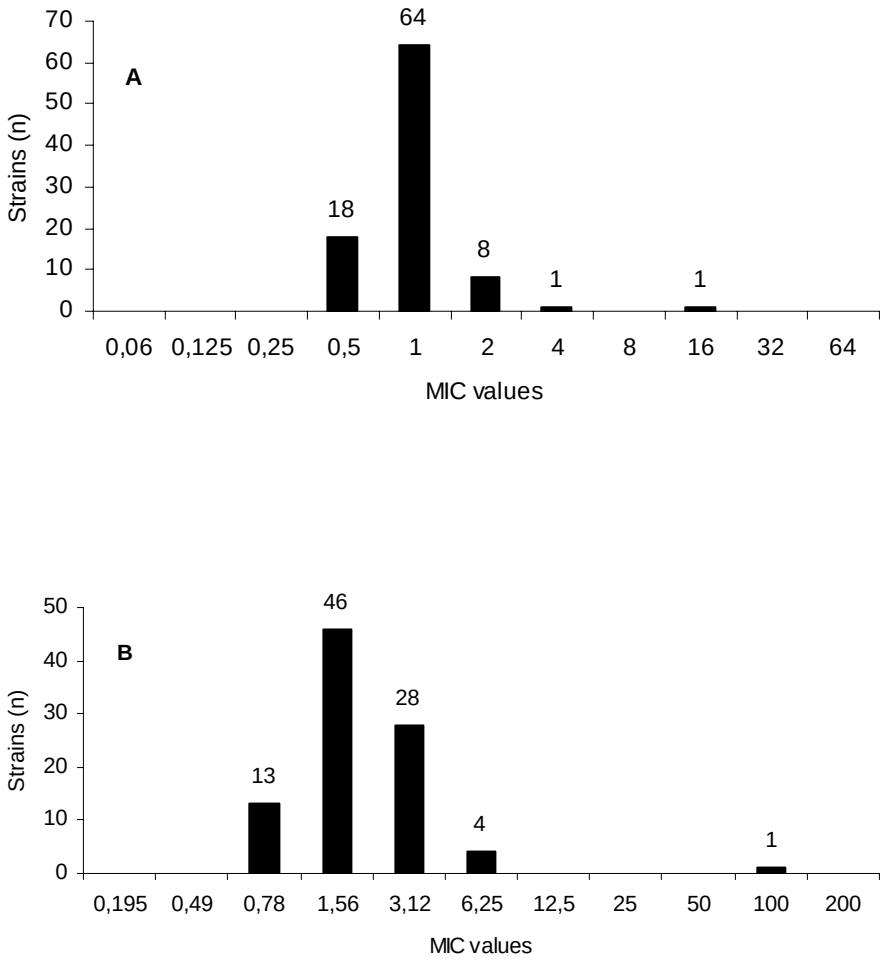

Figure 2 - Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B) to *Aeromonas hydrophila.*

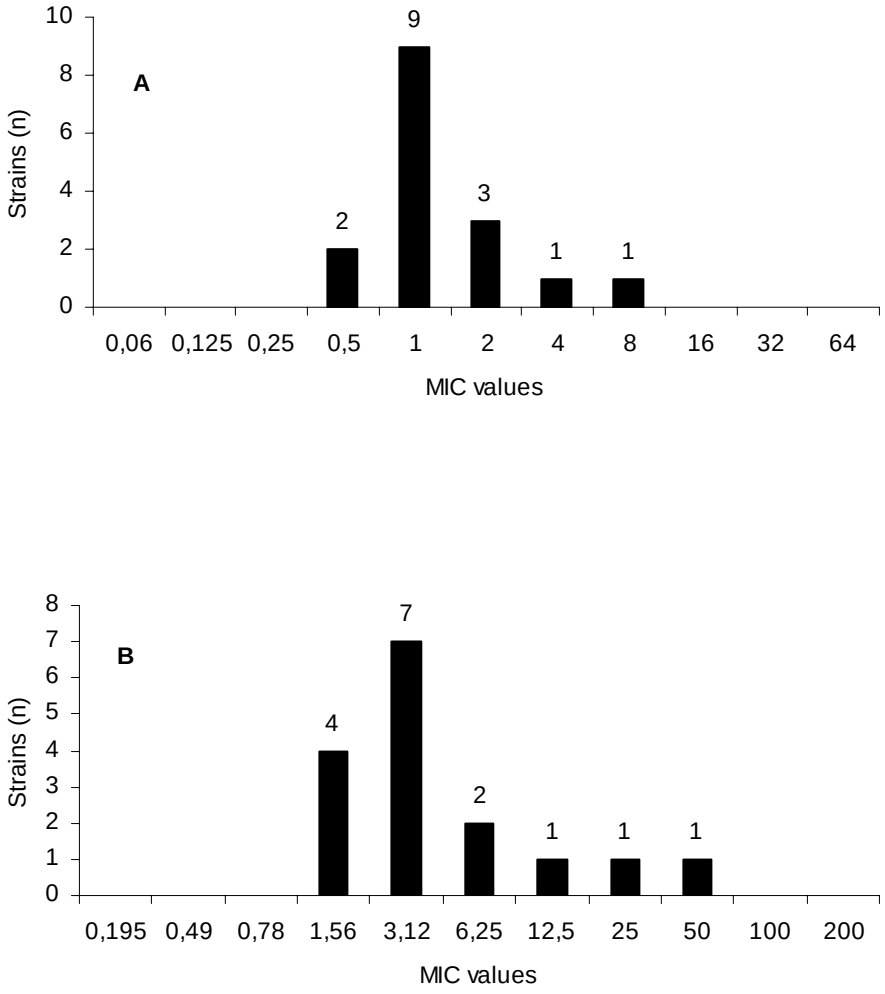

Figure 3 - Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B) to *Aeromonas caviae.*

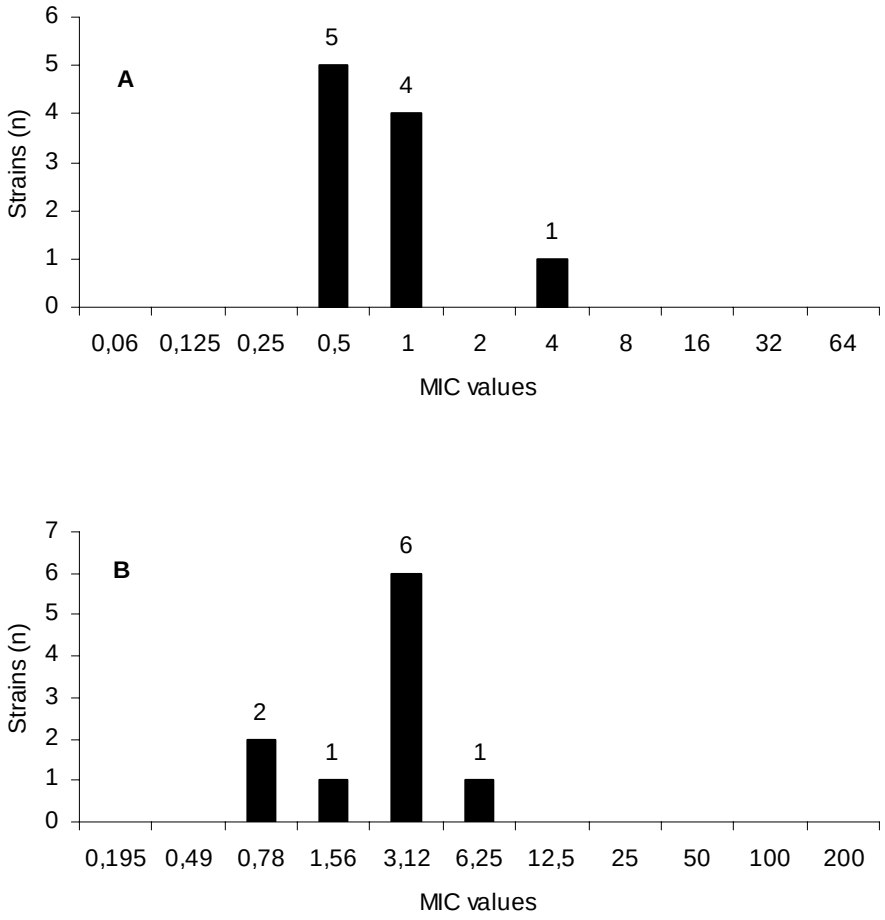

Figure 4 - Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B) to *Aeromonas sobria.*

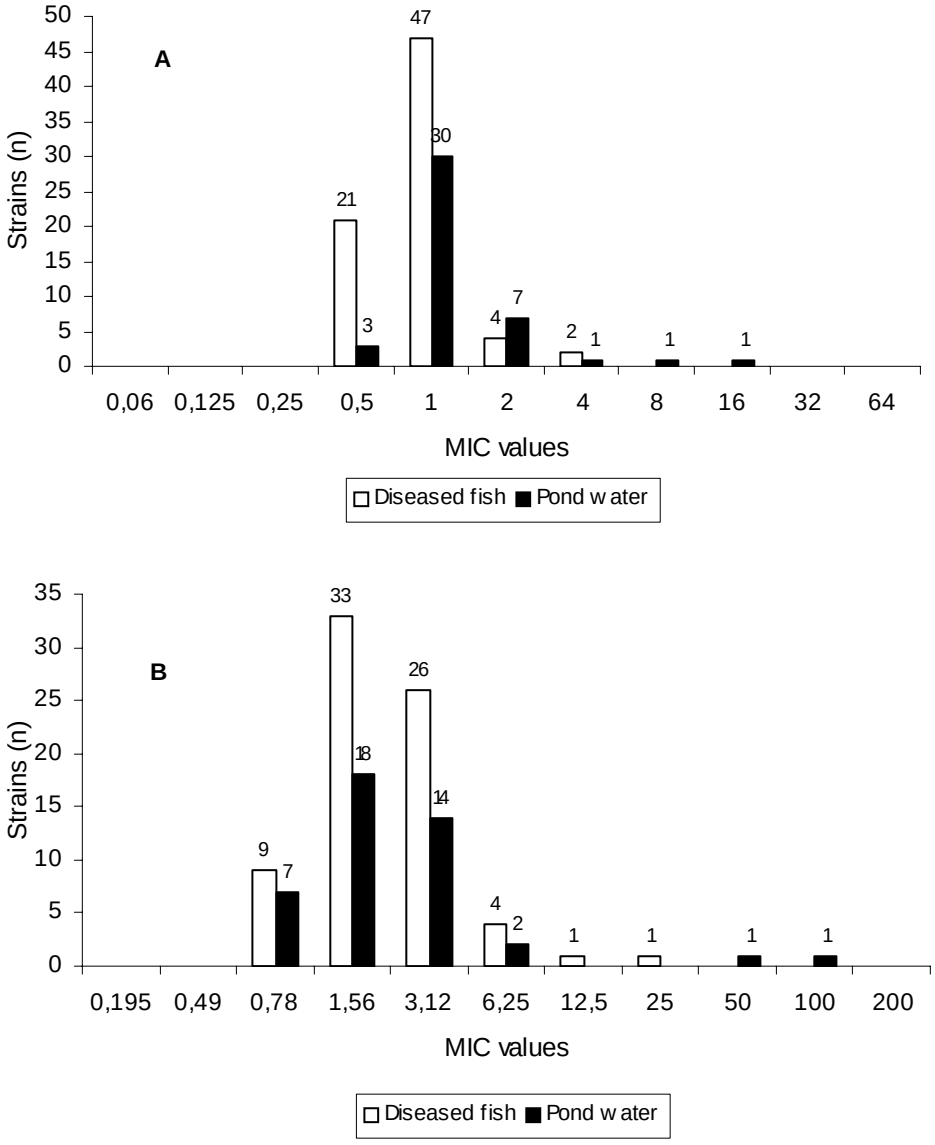

Figure 5 - Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B) to motile aeromonads isolated from diseased fish and pond water.

MINIMUM INHIBITORY CONCENTRATION OF FLORFENICOL AND BICYCLOMYCIN TO *Streptococcus agalactiae* STRAINS FROM NILE TILAPIA *Oreochromis niloticus.*

(Preparado de acordo com as normas da revista " Diseases of Aquatic Organisms")

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Running head: Antimicrobial MIC's for *S. agalactiae.*

Key words: MIC, Bicyclomycin, Florfenicol, *Streptococcus agalactiae*, fish.

Abstract

The aim of this study was to determine the minimum inhibitory concentration (MIC) of florfenicol (FLO) and bicyclomycin (BCM) for Brazilian strains of *Streptococcus agalactiae* isolated from Nile tilapia. The MICs were determined according to the new guideline of Clinical and Laboratory Standards Institute for MIC determination in bacteria isolated from aquatic animals (CLSI, 2006). The MIC values to FLO ranged from 1 μg mL^{-1} to 16 μg mL^{-1} and to BCM from 3.12 μg mL^{-1} to 12.5 μg mL^{-1}. In both cases it was observed the distribution of values in one cluster. All strains analyzed were classified as susceptible to FLO and BCM.

Text

Streptococcus agalactiae is an emerging pathogen in the pisciculture, associated to encephalitis and septicemia outbreaks, with high mortality rates. Several fish species from estuarine, marine and

freshwater environments are susceptible to the *S. agalactiae*, including the Nile tilapia, *Oreochromis niloticus* (L.) (Evans et al, 2002; Pasnik et al., 2005). The main predisposing factors to the infection seem to be the elevated temperature in the pond water and high animal density. There is no vaccine commercially available yet and the use of antibiotics is the major alternative to control the outbreaks.

The use of antibiotics like florfenicol (FLO) and bicyclomycin (BCM) in aquaculture has been increasing in last years. The FLO is a fluorinated chloramphenicol derivative and has large spectrum against Gram-positive and Gram-negative bacteria. It also has been effective in the control of *Aeromonas salmonicida, Vibrio salmonicida and Edwardsiella ictaluri* (Ho et al, 2000; Schwarz et al, 2004; Lewbart et al, 2005). The BCM is an antibiotic isolated from *Streptomyces sapporonensis e S. aizumenses*. Its structure is different from the other known antibiotics and has large spectrum against Gram-positive and Gram-negative bacteria (Kohn & Widger, 2005). The resistance patterns of fish strains of *S. agalactiae* against FLO and BCM are not determined.

In 2006, the CLSI (Clinical and Laboratory Standard Institute) established a harmonized protocol to determine the minimum inhibitory

concentration (MIC) of antibiotics to bacteria isolated from aquatics animals, based on a previous inter-laboratorial research (Miller et al, 2005); That will allow the comparison of data generated in distinct geographic origins. Due to the scarce data about the *S. agalactiae* resistance to FLO and BCM and the clinical meaning of this pathogen to the Brazilian pisciculture, the aim of this study was to determine the minimum inhibitory concentration of FLO and BCM to *Streptococcus agalactiae* strains isolated from Nile tilapia in different Brazilian states.

A total of 27 *Streptococcus agalactiae* strains were selected. The strains were isolated from diseased Nile tilapia (*Oreochromis niloticus*), in the period 2003-2006, and originated from eight different farms in the following Brazilian states: São Paulo, Minas Gerais, Espírito Santo, Paraná and Bahia (Table 1). All strains were previously identified by standard biochemical and serological tests (API 20 Strep and Slidex Strepto kit, BioMérieux, France).

The MICs were determined in accordance with "Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline" (CLSI, 2006). Briefly, the bacterial strains, stocked in freezer –70^{o}C, were streaked in blood agar plates and

incubated at 28°C for 24 hours. Bacterial suspensions were prepared in saline solution (0.85%), adjusted to 0.5 of MacFarland standard (BioMérieux, France) and then diluted 10 times in cation-adjusted Mueller-Hinton broth (CAMHB, Difco, USA) supplemented with 2.5% of lysed horse blood (LHB). The MIC tests of FLO were performed in sterile dry-form microplates (Sensititre, Trek Diagnostic system, U.K.) with the antibiotic concentration ranging from 0.06 μg mL^{-1} to 64 μg mL^{-1}. The reconstitution was made adding 100 μL of CAMHB supplemented with 2.5% de LHB in each well. An inoculum of approximately 5.0 x 10^5 CFU mL^{-1} of the bacterial suspension was inoculated per well. To BCM MIC a stock solution (400 μg mL^{-1}) of BCM (Searchem corp., Japan) was prepared in each day of test. In sterile microplates (Kartell, Italy) 2-fold serial dilutions of BCM in 100 μl of CAHMB supplemented with 2.5% of LHB were made ranging from 0.195 μg mL^{-1} to 200 μg mL^{-1}. An inoculum of approximately 5.0 x 10^5 CFU mL^{-1} of bacterial suspension was inoculated per well. The microplates of FLO and BCM were sealed and incubated at 28°C for 24 hours and then the results were read. *Escherichia coli* ATCC 25922 in CAMHB was used as quality control of plates and procedures. All strains were tested in duplicate. The MIC was

defined as the lowest concentration of antibiotic that prevented a visible bacterial growth. The strains were classified as susceptible or resistant according to the modal profile of MIC values distribution (Brunn et al., 2000).

The minimum inhibitory concentration results for FLO and BCM to *S. agalactiae* are shown in table 1. The MIC values to FLO ranged from 1 μg mL^{-1} to 16 μg mL^{-1} and to BCM from 3.12 μg mL^{-1} to 12.5 μg mL^{-1}. The distribution of MIC values for both antibiotics are shown in figure 1. The distribution of values in one cluster, without gaps, indicates that all tested strains were susceptible to FLO and BCM.

At the present in Brazil there are no licensed drugs for pisciculture use. However, the Brazilian Ministry of Agriculture are analysing the approval of oral formulations of FLO and BCM to use in aquaculture.

Florfenicol have been approved to use in aquaculture in Europe, Norway, United States, Canada, Japan and South Korea (Michel, et al., 2003; Lewbart et al., 2005; Yanong & Curtis, 2005; Kawanishi et al., 2006) and the bicyclomycin is approved to pseudotuberculosis treatment in fish since 1992 in Japan (Kawanishi et al., 2006).

Breakpoint values to FLO and BCM applied to aquatic animals bacteria are not available yet (CLSI, 2006). There is no data about pharmacokinetics of these drugs in tropical fish species, such as Nile tilapia, essential to establish accurate breakpoints.

Reports on the antibiotic resistance in *S. agalactiae* strains isolated from fish are rare and limited to the method of disk diffusion. Generally are described only resistance to gentamicin and streptomycin and occasionally to bacitracin. (Evans et al, 2002; Plumb et al, 1974). In our knowledge this is the first report about MIC values to FLO and BCM to *S. agalactiae* isolated from fish. FLO and BCM are reasonable choices to control *S. agalactiae* infection outbreaks in Nile tilapia culture.

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Table 1 – *Streptococcus agalactiae.* Minimum inhibitory concentration values to florfenicol and bicyclomycin according to geographic origin.

Strain*	**Species**	**Geographic origin (State)**	**Farm**	**MIC (μg ml-1)**	
				Bicyclomycin	**Florfenicol**
ST 07-05	*S. agalactiae*	Bahia	A	3,12	2
ST 08-05				3,12	8
ST 05-04		Espírito Santo	B	3,12	2
ST 06-04				3,12	4
ST 09-05			C	3,12	2
ST 11-05				3,12	2
ST 13-05				3,12	4
ST 01-03		Minas Gerais	D	3,12	2
ST 02-03				3,12	1
ST 03-03				3,12	2
ST 04-03				3,12	2
ST 17-06		Paraná	E	6,25	2
ST 26-06				3,12	2
ST 20-06			F	6,25	2
ST 15-06		São Paulo	G	6,25	16
ST 16-06				3,12	2
ST 18-06				6,25	2
ST 19-06				6,25	2
ST 30-06				12,5	2
ST 31-06				12,5	2
ST 32-06				12,5	2
ST 33-06			H	3,12	2
ST 34-06				3,12	4
ST 36-06				3,12	4
ST 37-06				3,12	2
ST 38-06				3,12	2
ST 39-06				3,12	2

*All strains were isolated from Nile tilapia (*Oreochromis niloticus*)

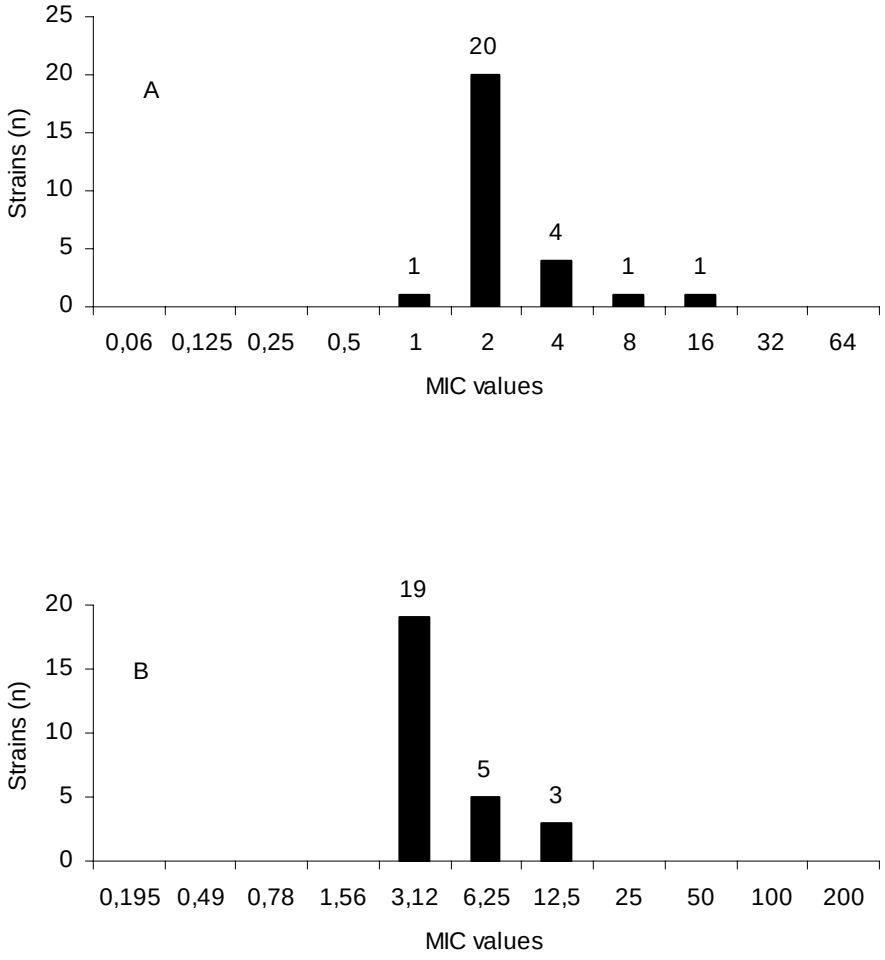

Figure 1 – *Streptococcus agalactiae.* Distribution of minimum inhibitory concentration values (μg mL^{-1}) of florfenicol (A) and bicyclomycin (B).

CONCLUSÕES

As concentrações inibitórias mínimas das amostras de *Streptococcus agalactiae* para florfenicol variaram de 1 μg mL^{-1} a 16 μg mL^{-1} e, para biciclomicina, de 3,12 μg mL^{-1} a 12,5 μg mL^{-1}. A distribuição dos resultados foi contínua e 100% das amostras foram classificadas como susceptíveis ao florfenicol e à biciclomicina. As amostras foram classificadas como resistentes ou susceptíveis, de acordo com a distribuição modal dos valores de MIC. Os isolados de aeromonas móveis apresentaram valores de MIC, para florfenicol, variando de 0,5 μg mL^{-1} a 16 μg mL^{-1} e, para biciclomicina de 0,78 μg mL^{-1} a 100 μg mL^{-1}. O florfenicol e a biciclomicina apresentaram alta eficácia contra as aeromonas móveis.

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