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Short communication

Patterns of resistance to florfenicol and bicyclomycin in Brazilian strains of motile aeromonads

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ABSTRACT

The minimum inhibitory concentration (MIC) and diameter of zone of inhibition of florfenicol (FLO) and the MIC of bicyclomycin (BCM) were determined for 118 motile aeromonad strains isolated from diseased fish and pond water in Brazil. Tests were performed according to Clinical and Laboratory Standards Institute guidelines. FLO MIC values ranged from 0.5 to 16 μg/mL and BCM MIC values from 0.78 to 100 μg/mL. According to MIC frequency distributions, the strains were classified as wild-type (WT) and non-wild-type (NWT) and provisional epidemiological cutoff values for FLO (WT ≤4 μg/mL) and BCM (WT ≤6.25 μg/mL) were estimated. Disk susceptibility tests revealed a provisional epidemiological cutoff of WT ≥23 mm for FLO. The lack of groups of bacterial strains with NWT behavior to FLO and BCM suggests that the estimated cutoffs should be revised in the future. Both antibiotics exhibited high efficacy *in vitro* against motile aeromonad strains.

1. Introduction

The genus *Aeromonas* comprises a group of ubiquitous aquatic bacteria with a wide distribution in freshwater and marine environments (Schmidt et al., 2000). Motile aeromonads are linked to a variety of diseases in different fish species and are often involved in disease outbreaks in ponds. Among motile aeromonads, *Aeromonas hydrophila*, *A. sobria* and *A. caviae* species are most commonly associated with fish diseases (Wahli et al., 2005). At present there is no commercially available vaccine and antibiotics are the primary means to control outbreaks. The routine use of antibiotics to treat farmed fish has resulted in the development of resistant bacterial strains, with the emergence of antibiotic resistance in fish pathogens reported for both temperate and tropical aquaculture systems.

Antibiotics such as florfenicol (FLO) and bicyclomycin (BCM) have become increasingly utilized in aquaculture in the last few years. FLO is a broad-spectrum antibiotic derived from chloramphenicol that inhibits bacterial protein synthesis by direct binding 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 it exhibits broad-spectrum activity against Gram-positive and Gram-negative bacteria (Magyar et al., 1998; Kohn and Widger, 2005). BCM targets rho, a transcription terminator that regulates bacterial gene expression. Without rho there is a loss of cell viability (Moyse et al., 2001; Kohn and Widger, 2005; Skordalakes et al., 2005). BCM is poorly absorbed in most animal species, limiting most bioactivity to the gastrointestinal tract (Hornick, 2003). BCM has been used for the treatment of non-specific diarrhea in humans, bacterial diarrhea in calves and pigs, and pseudotuberculosis in fish (Kohn and Widger, 2005). The pharmacokinetics of BCM in tropical fish is, to date, unknown.

Table 1
Brazilian strains of motile aeromonads used in this study

Geographic origin (State)	Farm	Host/source	Motile aeromonads strains (*n*)
Mato Grosso do Sul	A	*P. coruscans*	6
Minas Gerais	B	*O. niloticus*	8
		Pond water	4
Minas Gerais	C	*O. niloticus*	17
Minas Gerais	D	*B. orbignyanus*	13
		O. niloticus	7
Minas Gerais	E	Pond water	6
Minas Gerais	F	Pond water	5
Minas Gerais	G	Pond water	4
Minas Gerais	H	Pond water	4
Minas Gerais	I	Pond water	4
Minas Gerais	J	Pond water	17
Rio Grande do Sul	K	*R. quelen*	15
Rio de Janeiro	L	*O. niloticus*	8
Total			118

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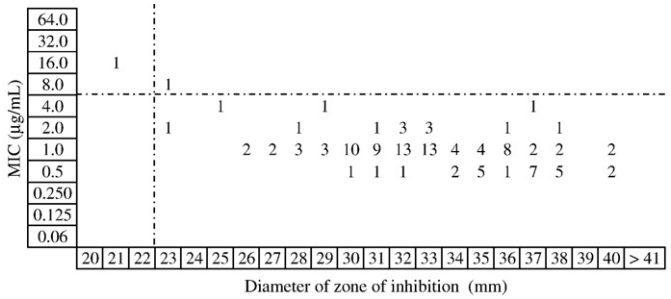

Fig. 1. Frequency distribution of MIC values and diameter of inhibition zones for isolates of motile aeromonads tested against florfenicol (30 µg). Epidemiologic cutoff values are indicated for MIC (horizontal dashed line) and diameter of the zone of inhibition (vertical dashed line).

FLO is effective in controlling several bacterial diseases in fish and is approved for use in the European Union, United States, Canada, Japan and South Korea (Michel et al., 2003; Lewbart et al., 2005; Yanong and Curtis, 2005; Kawanishi et al., 2006) and BCM is approved for treating fish in Japan (Kawanishi et al., 2006).

At present, no antibiotics are licensed for use in aquaculture in Brazil and the Brazilian Ministry of Agriculture is currently analyzing the approval of oral formulations of FLO and BCM.

Several methods for determining the minimum inhibitory concentration (MIC) of an antibiotic for bacteria isolated from aquatic animals have been proposed (Martinsen et al., 1992; Alderman and Smith, 2001; Samuelsen et al., 2003). In 2006, based on previous interlaboratory research (Miller et al., 2005), the Clinical and Laboratory Standards Institute (CLSI) established a harmonized protocol with precise quality controls to determine the MIC of antibiotics against bacteria isolated from aquatic animals (CLSI, 2006a).

The aim of the present work was to determine FLO and BCM MIC values for motile aeromonad strains isolated from diseased fish and pond water and then establish provisional epidemiological cutoffs for these antibiotics.

2. Materials and methods

A total of 118 motile aeromonad strains were selected. The strains were isolated in the period 2002–2006 from *Oreochromis niloticus*, *Brycon orbignyanus*, *Pseudoplatystoma coruscans* and *Rhamdia quelen* from 11 farms in the following Brazilian states: Mato Grosso do Sul, Minas Gerais, Rio de Janeiro, and Rio Grande do Sul (Table 1). A total of 74 strains were derived from diseased fish (hemorrhagic septicemia) and 44 strains from pond water were taken from healthy fish farms. All strains were identified by biochemical tests according to Abbott et al. (2003).

MIC values were determined in accordance with the CLSI (2006a) guideline. Briefly, bacterial strains stored at −70 °C were inoculated into Mueller–Hinton broth (Difco, USA) previously supplemented with divalent cations (20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}; CAMHB) and incubated at 28 °C for 24 h. Bacterial suspensions were prepared in saline solution (0.85%), adjusted to McFarland standard 0.5 (BioMérieux, France) and then diluted 10-fold in CAHMB. FLO MIC tests were performed in sterile dry-form microplates (Sensititre, Trek Diagnostic Systems, UK) using antibiotic concentrations ranging from 0.06 to 64 µg/mL. For reconstitution, 100 µL of CAHMB was added to each well. Then an inoculum of approximately 5.0×10^5 CFU/mL of the bacterial suspension was also added to each well. For BCM tests, a stock solution (400 µg/mL, Searchem Corp., Japan) was prepared fresh each day. In sterile microplates (Kartell, Italy) serial dilutions of BCM in 100 µL of CAHMB were made to yield a range from 0.195 to 200 µg/mL. A sample of approximately 5.0×10^5 CFU/mL of the bacterial suspension was inoculated into each well.

Microplates for FLO and BCM test were sealed and incubated at 28 °C for 24 h and then the results were read. *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 were used for quality control of plates and procedures in each day of MIC assay. All strains were tested in duplicate. The MIC was defined as the lowest concentration of antibiotic that prevented visible bacterial growth.

Disk diffusion tests for FLO were carried out in accordance with the CLSI (2006b) guideline. *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were again used for quality control of plates and procedures. All tests were conducted on Mueller–Hinton agar (Difco, USA), with incubation at 28 °C for 24–28 h. Disks containing

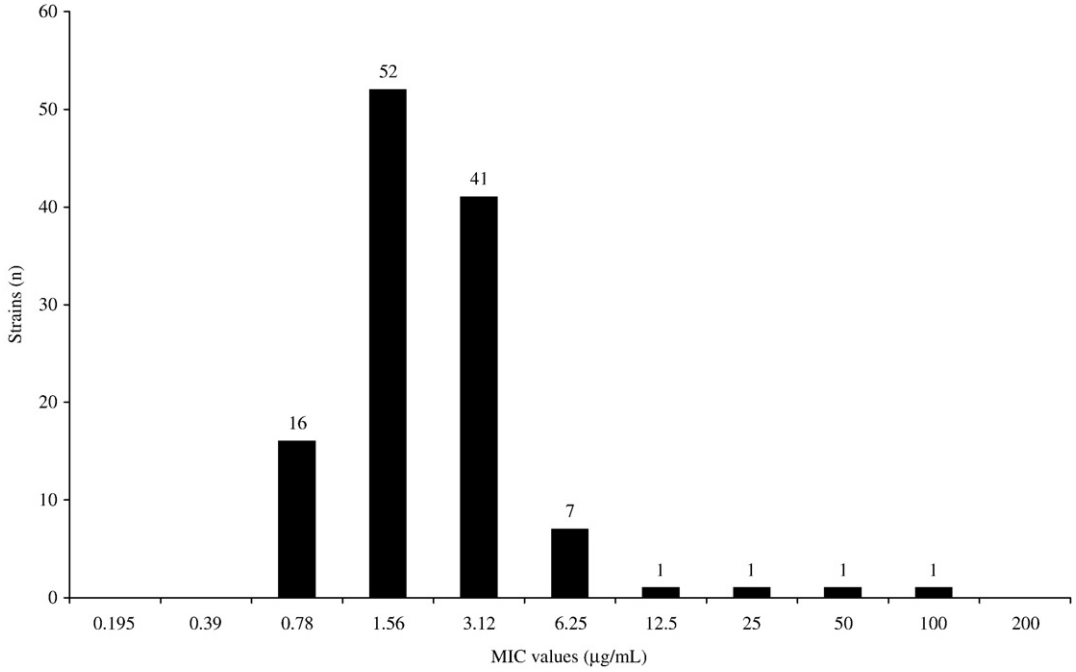

Fig. 2. Distribution of minimum inhibitory concentrations of bicyclomycin for motile aeromonads.