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Efficacy of fosfomycin in controlling streptococcosis in Nile tilapia (Oreochromis niloticus)

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A study was conducted to investigate the efficacy of fosfomycin in controlling streptococcosis in Nile tilapia (*Oreochromis niloticus*). The minimum inhibitory concentrations (MICs) of fosfomycin against multiple *S. iniae* isolates showed a sensitivity range of 12.5- 25 µg/mL. The fosfomycin dose levels tested were 40, 60, and 80 mg of active ingredient per kilogram fish per day. Administration of medicated feed started one day after infection by immersion exposure to *S. iniae* and continued for eight days. Survival rates of fosfomycin treated groups were 90, 100 and 96.6% with an average survival of 95. 53 %. On contrary, survival rates of infected non treated groups were 3.3%. All survivors and negative control groups showed no clinical signs, no gross pathology together with negative *S. iniae* re-isolation.

Streptococcosis of fish is a generic term used to designate similar, but different, diseases in which any member (s) of at least three different genera of Gram-positive cocci, (GPC) including streptococci, lactococci, and vagococci are involved (Muzquiz *et al*., 1999). Such infections associated with those bacterial pathogens have been reported in many countries and in different marine, brackish as well as freshwater fish species and became as one of the most important risk factors in the aquaculture.

As a major etiological agent of streptococcosis, *Streptococcus iniae* affects more than 30 species of farmed and wild finfish, including trout, yellowtail, tilapia, barramundi, and hybrid striped bass (Ferguson *et al.*, 2000; Shoemaker *et al.*, 2001; Agnew and Barnes, 2007; Cheng *et al.*, 2010). Estimated economic impact from infections caused by *S. iniae* on aquaculture industry is approximately more than US $ 100 million globally (Hussein and Hatai, 2006; Shoemaker *et al*., 2010). In fish, streptococcosis generally results in meningitis and panophthalmitis, ultimately producing high levels of morbidity and mortality (Bromage and Owens 2009). Disease progression in fish is somewhat variable and dependent on such factors as virulence of the pathogen, route of infection, fish age, and environmental rearing conditions (Agnew and Barnes 2007). Clinical signs typically include skin darkening, lethargy, erratic and spiral swimming, ulcers, and exophthalmia (Plumb 1999; Agnew and Barnes 2007; Buchanan *et al*. 2008).

In Egypt, aquaculture of tilapias, particularly, Nile tilapia (*Oreochromis niloticus*) and their related species are of great importance because of its high economic value among local consumers. *Streptococcus iniae* has been frequently reported associated with the mortality of tilapia species in different localities (Al-Harbi, 1994; Eyngor *et al.,* 2008). Consequently, health management goals for cultured fish usually include preventing disease and minimizing adverse effects of disease outbreaks when they occur. Maintaining healthy rearing conditions, providing proper nutrition, routinely monitoring fish health and vaccines can help to prevent disease outbreaks (Alderman, 1988; Plumb, 1999). However, timely treatment with therapeutic drugs may be useful for controlling mortality and preventing epizootics when disease outbreaks occur (Darwish, 2007; Bowker *et al*., 2010). Relatively few laboratory or field studies have been conducted to evaluate the effectiveness of chemotherapeutants (e.g., enrofloxacin: Stoffregen *et al*., 1996; amoxicillin: Darwish and Ismaiel, 2003; florfenicol: Darwish, 2007; Bowker *et al*., 2010) in controlling mortality associated with *S. iniae.*

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In an era of extensive bacterial drug resistance, especially among pathogenic Gram-positive and Gram-negative species, emphasis should be given not only to the development of new drugs but also to the re-evaluation of older and 'forgotten' drugs (Falagas *et al*., 2008). Fosfomycin is a drug representing the latter category, discovered almost 40 years ago. Fosfomycin is a unique antibiotic that is chemically unrelated to any other known antibacterial agents. It inhibits bacterial cell wall biosynthesis in both Gram-positive and Gram-negative bacteria by inactivating the UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme that catalyses the first step in bacterial cell wall synthesis (Falagas *et al*., 2010). This broad-spectrum antibiotic has principally been used in the treatment of uncomplicated urinary tract infections (UTIs), however, recent data suggest that it may be considered as an alternative in the treatment of Gram-negative and Gram-positive infections other than of UTIs. Adwifos® (active ingredient: fosfomycin 25% (calcium salt), vitamin E 6% and fructose-1-6-phosphate 18%) is an antibiotic, approved by Egyptian Government, used for treatment and control of bacterial diseases caused by both Gram-positive and Gram-negative pathogens in poultry farms.

Up to date, there is currently no published information about the use or efficacy of fosfomycin in controlling streptococcosis in fishes, particularly, in tilapias. Therefore, this study was conducted to determine the *in vitro* sensitivity of *S. iniae* to Adwifos® (25% fosfomycin) together with the drug's efficacy in controlling streptococcosis in Nile tilapia.

Materials and Methods

Antimicrobial agent. Adwifos® (fosfomycin, 25%), was obtained from Adwia company, El-Oboor City, Cairo, Egypt. A stock solution of fosfomycin was made by dissolving 1 g Adwifos® (250 mg fosfomycin) in 100 mL distilled water. The stock solution was filter-sterilized by passing through syringe filter with a poor size of 0.45 µm (Advantec, Cat No. 25CS045AS, Tokyo, Japan). A fosfomycin solution of 200µg/ml was prepared by adding 1 mL of stock solution to 12.45 mL of Todd Hewitt broth (THA; Sigma).

Bacterial strains. Four strains of *S. iniae* previously isolated from outbreaks of streptococcosis were obtained from different locations as shown in (Table 1). The selected *S. iniae* isolates were isolated from outbreaks of streptococcosis at different years, purified, morphologically, biochemically and polymerase chain reaction (PCR) identified (Hussein and Hatai, 2006; 2007), then kept for further experiments in micro-bank tubes under (–80°C) and/or in semisolid agar at 4°C with regular subculture every three months at Department of Fish, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. All tested *S. iniae* isolates were exposed to pathogenicity test before being used in this study. For determination of minimal inhibitory concentrations (MIC) assays, all selected *S. iniae* isolates were subjected to the assays, while only *S. iniae* BNS 0014 was used for infection and treatment trials.

Table 1. *Streptococcus iniae* isolates used for minimal inhibitory concentration (MIC) assays of fosfomycin.

No.	Isolate	Year of isolation	Host or source	Location
1	BNS 0014	2009	Nile tilapia	Beni-Suef, Egypt
2	B0035	2003	Tilapia species	Beni-suef, Egypt
3	JFM 0005	2006	Red tilapia	Bali, Indonesia
4	NJB 02-011	2002	Rainbow trout	Nagano, Japan

Fish. All experiments were performed at an aquatic Laboratory Unit, Fish Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt. Sub-adult Nile tilapia with an average weight of 40±2 g and 14±1 cm length of both sexes were obtained from a private tilapia hatchery at Beni-Suef Governorate, Egypt. The fish were maintained in 500L fiberglass tank, the water quality and temperature were monitored daily and kept within the acceptable range for tilapia. The fish were fed commercial pellet feed (Geo-trad, El-Asher men Ramadan, Cairo, Egypt) twice daily at a ratio of 2% of their body weight and kept under observation for 2 weeks prior to the experiment. During the observation period, the fish were randomly sampled and their kidneys and livers aseptically streaked on TH and/or brain heart infusion agar plates (BHI; Oxoid) to determine whether the fish were free of bacterial infection.

All fish experiments were conducted in 50 L aquaria at 25 ± 2º C. Ten tilapia individuals weighting 40 ±2 g were placed in each aquarium 24 h prior to the experiments.

Determination of minimal inhibitory concentrations (MIC). The MIC value of fosfomycin for each selected *S. iniae* isolates was determined by using the micro-titer broth micro-dilution method described by (Qaiyumi, 2007) and modified by (Hussein and Hassan, 2010). Briefly, Adwifos® solution was initially adjusted to 200 µg/ml TH broth and then subjected to a doubling dilution series on a U-bottom wells micro-titer plate containing TH broth till column number 10 to obtain final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3. 125, 1.563, 0.781 and 0.391 µg/ml. Then, a suspension of each tested *S. iniae* strain in phosphate buffered saline (PBS, pH 7.4) was prepared and adjusted to 1.5 X 10^8 CFU/mL, according to McFarland tube No. 0.5. Then, 20 µL of each tested bacterial isolates was inculcated into one wells row of the micro-plate. Columns number 11 and 12 were designated as controls with the former containing TH broth with PBS and the later contained only TH broth. After incubating at 25ºC, the bacterial growth was determined after 24 h. The viability of the tested bacterial strains was confirmed by plating 20 µL from each well onto TH and/or BHI agar plates and observed over a period of 24 and 48 hours. MIC of fosfomycin was taken as the lowest concentration causing no growth of the tested *S. iniae* strains.

Preparation of fosfomycin diets. Adwifos® was incorporated into a commercial feed ration to provide 40, 60, 80 and 0.0 (control) mg of fosfomycin/kg of fish per day when fish are fed 2% of their body weight per day. The four diets contained 8, 12, 16 and 0.0 mg of Adwifos® per gram of feed representing 2, 3, 4 and 0.0 mg of fosfomycin/g feed, respectively.

The commercial feed was pulverized in V-mixer (Krups 50, model 202, Sony trading, Japan) before adding the required antibiotic, then the mixture was thoroughly blended again for 3 min. Each diet mixture was mixed with distilled water (0.5 ml/g) and fish oil at a rate of 0.5% w/v until a homogenous mixture was obtained. The mixture was passed through a minced-meat processing machine (National, MK-8710N, Osaka, Japan), producing extruded strings, which were air dried at 25ºC for 24 h, manually broken down to about 2-mm-long pellets and then frozen in screwed containers at (–18°C) until used. Small quantities of each diet were thawed and refrigerated at 4°C until used.

Experimental design.

Inoculum preparation and challenge protocol. *Streptococcus iniae* BNS 0014 (Table 1) was passed two times in Nile tilapia to maximize its virulence, and then re-isolated, purified, biochemically identified and the stock was kept frozen in TH broth with 15% glycerol at (–80°C) until used. For challenge trial, 100 mL of TH broth was inoculated with 100 µL of the frozen isolate. The inoculated broth was incubated at 25°C for 24 h on a rotatory shaker (SK-L330-Pro, SCI-Logex, Rhsin Land, USA), and then centrifuged at 5000 rpm for 20 min at 10°C. The bacterial pellet was washed three times with PBS (pH 7.2). In the challenge treatment, the fish from each aquarium were placed in 10 L of aerated water containing *S. iniae* at a concentration of 3×10^7 CFU/ml (Hussein and Hatai, 2006) for 15 min and then returned to their home aquarium. The positive control groups were treated at the same manner, while those of negative control not exposed to *S. iniae*. Fish were observed daily for their behavioral changes and clinical signs. Dead and moribund fish were removed, necrotized, and bacterial isolation attempted from kidney, brain and eye on TH agar. At the end of the experimental period, all survivors were subjected to bacteriological examination. Cultured bacteria were biochemically identified (Hussein and Hatai, 2007).

Efficacy trials. One hundred and fifty Nile tilapia (*Oreochromis niloticus*) of both sexes were randomly distributed into fifteen 50-L aquaria, with 10 individuals in each. Aquaria containing fish were divided into 5 groups, namely A, B, C, D and E. Prior to exposure; fish in each aquarium were collectively weighted to calculate the amount of diet (2% of their body weight) to be administered daily. Control diet

was fed to all experimental groups for 5 days to acclimate them to experimental conditions.

After acclimatization elapsed (5 days), feed was withheld for 24 h and the fish in each aquarium were collectively weighted. Five treatments were assigned to aquaria (A, B, C, D and E), each treatment group consisting of three replicate aquaria. The five treatments consisted of three groups of fish (A, B and C) that experimentally infected with *S. iniae* and then fed fosfomycin medicated feed at rate of 40, 60, or 80 mg/kg, respectively. One group (D) that were experimentally infected with *S. iniae* and then fed non-medicated feed (positive control), and one group (E) that was not infected and fed non medicated feed (negative control). At all levels tested, feeding of appropriate medicated or non medicated diets was started 24 h after *S. iniae* infections and continued for 8 days. All groups of fish were then fed the control diet for additional 10 days.

Results

Determination of MIC of fosfomycin. The MIC assays of fosfomycin against the four *S. iniae* were varied considerably. For example, the MIC values for B0035, JFM 0005, and NJB 02-011 isolates were found to be 12.5µg/mL. However, the MIC for BNS 0014 isolate was 25µg/mL. Furthermore, those MIC values (12.5 and 25µg/mL) of fosfomycin could kill the four *S. iniae* isolates tested and that was evident when no growth occurred after a 20µL from those concentrations wells were plated onto TH and BHI agar plates and observed over a period of 24 and 48 hours.

Efficacy trial. Fosfomycin was highly effective in reducing mortalities in Nile tilapia infected with *S. iniae* BNS 0014 (Table 2, Figure 1). There was a significant difference between the survival rates of fosfomycin medicated treatments and the infected non-medicated treatment (Table 2). Average survival in the three medicated treatments was 95.53 with no statistical differences between any of them and the uninfected negative control groups, which had survival rates of 100%. In contrast, survival rates of fosfomycin medicated treatment infected groups A, B and C were 90, 100 and 96.6%, respectively; while those of non-medicated infected groups (D) and non-medicated uninfected groups (E) were 3.3% and 100%, respectively. On the other hand, severe infection was achieved among fish in (D) groups as the mortalities were reached to 96.7% with positive bacterial re-isolation from moribund and dead fish. Mortalities started 48-72 hours and continued for 18 day post infection. After the end of the experiment (24 day post infection), all fish individuals remained from challenged treatments and negative control groups showed no clinical signs, no gross pathology together with negative *S. iniae* re-isolation.

Table (2): The survival percent of Nile tilapia experimentally infected with *S. iniae* (BNS 0014) and administered different fosfomycin-medicated diets for 8 days.

Fish Group	Fosfomycin dose (mg/Kg fish/day)	Number of dead fish/number of tested fish			Survival %
		Replicate			
		I	II	III	
A	40	2/10	1/10	0/10	90
B	60	0/10	0/10	0/10	100
C	80	0/10	0/10	1/10	96.6
D*	0.0	10/10	10/10	9/10	3.3
E**	0.0	0/10	0/10	0/10	100

*positive control,
** negative control

Figure (1): Cumulative mortality of Nile tilapia experimentally infected with *S. iniae* (BNS 0014) and administered different fosfomycin-medicated diet for 8 days.

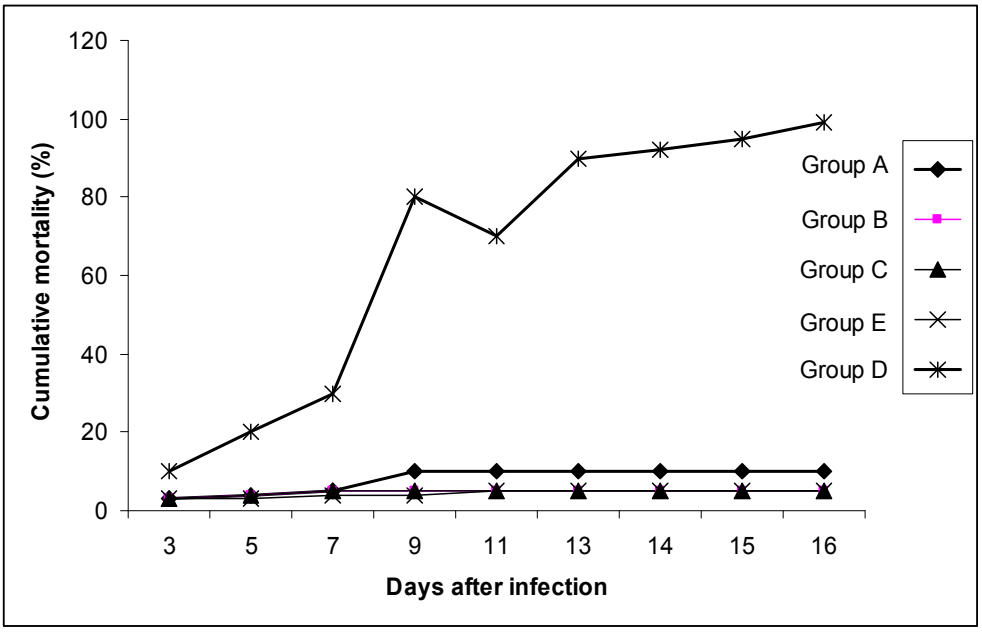
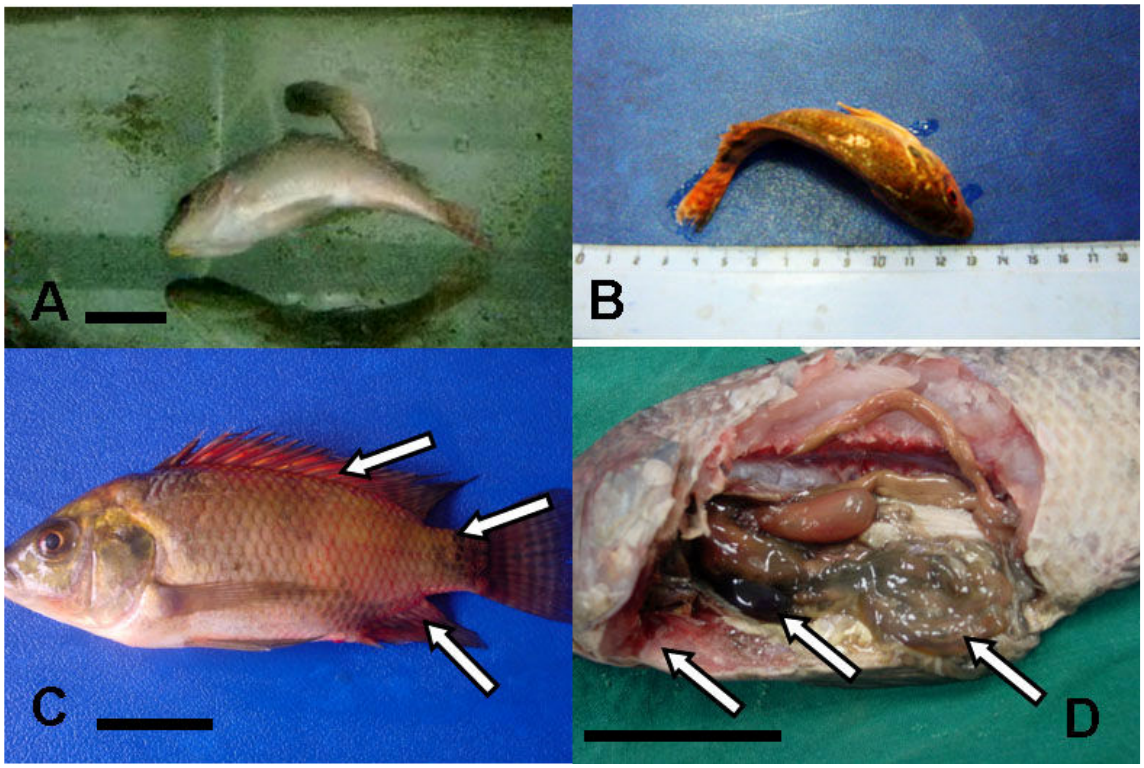


Figure 2. Clinical signs of streptococcosis in Nile tilapia experimentally infected with *S. iniae* by immersion exposure to 3 × 10^7 CFU/ml. (A and B) Moribund fish lied on their side in "C"-shaped posturing. **(C)** Massive hemorrhages at the base of the fins and peduncle region (arrows). **(D)** Severe congestion and enlargement of the liver and spleen accompanied with empty intestine and hemorrhages in the musculature (arrows). Scale bar = 5 Cm.



Clinical signs and gross pathology

Clinical signs and/or gross pathology characteristic to streptococcosis were basically observed in (D) groups and to a little extend in A and C groups. Moribund fish were lethargic and anorexic at 48-72 hours post infection with erratic swimming at random directions, whirling, and lies in "C"-shaped posturing (Figure 2 A, B). Externally, hemorrhages were scattered on the body surface, particularly, at the base of lower jaw, base of the dorsal fin, and massively at the peduncle region and tail fin (Figure 2 C). Internally, congestion of the liver together with distended gall bladder. Spleen was enlarged and dark, while alimentary tract was devoid of food (Figure 2 D). At the end of the experiment, all treated fish showed neither clinical signs, internal lesions, nor abnormal behaviors. Similarly, fish in control negative groups showed no clinical signs and/or abnormal behaviors.

Discussion

Fosfomycin is a bactericidal broad-spectrum antibiotic that is not structurally related to other classes of antimicrobial agents. The mechanism of action of this antibiotic is to inhibit cell wall synthesis in various proliferating Gram-positive and distinct Gram-negative bacteria at an earlier stage (Popovic *et al.,* 2010). This is the first study to evaluate the efficacy of fosfomycin in controlling a serious bacterial infection in Nile tilapia such as streptococcosis.

Data generated within this study showed that the MIC assay values of fosfomycin against tested *S. iniae* were 12.5 and 25 ug/mL, with the former had a bactericidal effect for *S. iniae* B0035, JFM 0005, and NJB 02-011, while the latter for BNS 0014. These concentrations are comparable with those reported by Allerberger and Klare (1999) and Ribes *et al*. (2006), who found that the MIC of fosfomycin for *Streptococcus pneumoniae* and *Enterococcus* species was ≤ 64 ug/mL. The difference between the present results and those of Allerberger and Klare (1999) and Ribes *et al*. (2006) may be due to presence of fructose-6-phoshate in fosfomycin preparation (Adwifos®) that we used, which enhance the mode of action of fosfomycin (Michalopoulos *et al.,* 2011).

As a result within our study, oral administration of fosfomycin at a dose of 40 mg/kg body weight per day or more for 8 days was effective in increasing the survival of tilapia experimentally infected with *S. iniae* to 90 % or more, compared with 3.3 % for experimentally infected, non-medicated ones (Table 2). The significant reduction in mortality of the experimentally infected, medicated tilapia suggested that the serum fosfomycin concentration exceeded the MIC of the pathogen by which the margin necessary for an antibacterial to be effective in controlling a systemic infection (Gutierrez *et al.,* 2008).

Fosfomycin reduced mortality when it was administered as early as 24 hour after challenge. As a fact, the use of fosfomycim in veterinary is limited; therefore, there are currently no pharmacokinetic studies on fosfomycin in Nile tilapia. However, pervious studies with other animals (broilers: Aramayona *et al.,* 1997; Cattle: Sumano *et al.,* 2007; dogs: Gutierrez *et al.,* 2008) indicated that fosfomycin has good distribution into tissues achieving clinically relevant concentrations in serum, kidneys, inflamed tissues and cerebrospinal fluid and when meninges are inflamed it will cross the blood-brain barriers into cerebrospinal fluid in concentrations ranged from 10% to 60% of that found in the serum (Kuhnen *et al.,* 1987; Schintler *et al.,* 2009). Although the distribution of fosfomycin in tissues of Nile tilapia is unknown, if it is similar to that reported for other animals, that could explain the fosfomycin efficacy in streptococcal infection that known to produce mingoencephalitis (Eldar *et al.,* 1995; Shoemaker *et al.,* 2001). Moreover, fosfomycin, in addition to its antimicrobial activity, exerts immunomodulatory effects mainly on lymphocytes and neutrophil function. Pe´rez Ferna´ndez *et al.* (1995) found that fosfomycin enhances the phagocytic killing of invading *Staphylococcus aureus* by host cells.

Our investigation revealed the typical clinical signs attributed to streptococcosis same like that have been reported in the literatures (Eldar *et al.,* 1995; Evans *et al*., 2000; Agnew and Barnes, 2007; Bowker *et al*., 2010). In addition, the infection in this study was systematic as shown by positive isolation of *S. iniae* from different organs including, brain, kidney, liver and eye. On the other hand, the inability of fish to feed two days post infection emphasizes the critical important of monitoring and early intervention with the oral medicated feed. If inappetance begins, oral antibiotic therapy will be ineffective in reducing fish losses.

Considering its potential clinical efficacy, fosfomycin would appears as good option for controlling mortalities associated with streptococcosis infection in Nile tilapia, however, controlled field trials are recommended to establish its use in tilapia aquaculture.

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كفاءة الفوسفوميسين فى السيطرة على الإصابة بالمكورات السبحية فى أسماك البلطى النيلى

أجريت هذه الدراسة لمعرفة مدى فعالية (٢٥٪ فوسفوميسين) في السيطرة على عدوى المكورات السبحية أينيي في البلطي النيلي. وقد أظهرت تركيزات الحد الأدنى المثبطة من الفوسفوميسين ضد عدة معزولات من المكورات السبحية أينيي حساسية لـ ١٢,٥-٢٥ ميكروجرام / ملليلتر. كانت مستويات جرعة فوسفوميسين المختبرة ٤٠، ٦٠، و٨٠ ملجم من المادة الفعالة لكل كيلوجرام سمك في اليوم الواحد. بدأ تقديم الغذاء المحتوى على الفوسفوميسين بعد يوم واحد من إحداث العدوى عن طريق الغمر فى وجود المكورات السبحية أينيي واستمر استخدام الغذاء المعالج لمدة ثمانية أيام. كانت معدلات بقاء الأسماك على قيد الحياة من المجموعات المعالجة بالفوسفوميسين والمعرضة للإصابة هى ٩٠، ١٠٠ و ٩٦،٦٪ بمتوسط ٩٥,٥٣ ٪. لم تظهر جميع أفراد الأسماك المتبقية بعد التعرض للعدوى والعلاج ومجموعات المراقبة السلبية أي علامات سريرية، أو علامات مرضية كما أنه لم يتم عزل المكورات السبحية أينيي من أى منهما.