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Original Article

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Abstract

Florfenicol (FF) and Chloramphenicol (CAP) were tested for their *in vitro* antimicrobial activity against 102 Vibrio isolates from clinical cases. The Minimum Inhibitory Concentration (MIC) for both antimicrobials was ≤ 8 μg/ml suggesting that all of the tested isolates were susceptible to both antimicrobials. The observed MIC range of FF (0.5-4.0 μg/ml) was more potent than that of CAP (0.5-8.0 μg/ml). The activity of both antimicrobials was not substantially influenced by the addition of sea water (5 ppt salinity) to the test system. When considering the best type of antimicrobial for food producing animals and *in vitro* antivibrio activity, FF is a good prospect for the treatment of shrimp Vibrio pathogens. Accordingly an *in vivo* study was conducted on 3-month old black tiger shrimps, *Penaeus monodon* (14.5 ± 1.0 g) which were fed for 5 days with FF medicated feed at 0.8 g of FF per kilogram of feed, and 2.5% of the shrimps body weight. Florfenicol-amine (FFA), the marker residue of FF, was measured in the hepatopancreas and muscle using High Performance Liquid Chromatography (HPLC) and UV detection. The FFA concentration in samples from control shrimp was zero using these methods. The maximum concentration of FFA (C_{max}) detected in the hepatopancreas after 1 hour (T_{max}) was 0.7 μg/g tissue, and C_{max} in the muscle was 0.05 μg/g tissue 4 hours (T_{max}) after the initial medication. The average concentrations of FFA analyzed every 24 hours after medication were 0.54±0.04 μg/g in the hepatopancreas and 0.15±0.02 μg/g in the muscle. By the seventh day, following the cessation of feeding the medicated feed, the drug residue in the shrimp hepatopancreas and muscle, was lower than any detectable limits for the methods used (0.01 μg/g).

Keywords : Black tiger shrimp, minimum inhibitory concentration, florfenicol, residues.

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บทคัดย่อ

การให้ยาฟลอเฟนิคอลผสมอาหารแก่กุ้งกุลาดำ

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ศึกษาประสิทธิภาพของ florfenicol (FF) เปรียบเทียบกับ chloramphenicol (CAP) ในการยับยั้งเชื้อ Vibrios ก่อโรคในกุ้งกุลาดำ (*Penaeus monodon*) จำนวน 102 isolates พบว่าความเข้มข้นต่ำสุดของ FF และ CAP ที่สามารถยับยั้งการเจริญของเชื้อ Vibrio ที่ใช้ทดสอบ (Minimum Inhibitory Concentration; MIC) คือ 0.5-4 μg/ml และ 0.5-8 μg/ml ตามลำดับ และ MIC ของยาต้านจุลชีพทั้งสองชนิดไม่เปลี่ยนแปลง เมื่อทดสอบประสิทธิภาพของยาในอาหารเลี้ยงเชื้อที่ผสมน้ำทะเลความเค็ม 5 ส่วนในพันส่วน (part per thousand, ppt) เนื่องจาก FF เป็นยาต้านจุลชีพที่อนุญาตให้ใช้ในสัตว์เลี้ยงเพื่อการบริโภค จึงทดสอบการให้ยา FF ในกุ้งกุลาดำอายุ 3 เดือน (น้ำหนักตัวเฉลี่ย 14-15.5 g) โดยการให้ยาผสมอาหารในขนาด 0.8 mg FF ต่อกิโลกรัมอาหาร ติดต่อกัน 5 วัน ให้อาหารวันละประมาณ 2.5% ของน้ำหนักตัวกุ้ง วิเคราะห์ปริมาณ florfenicol-amine (FFA) ตกค้างในเนื้อเยื่อของกุ้งกุลาดำระหว่างการให้ยาและภายหลังการหยุดยาโดยวิธี High Performance Liquid Chromatography (HPLC) ใช้ UV detector ไม่พบ FFA ในเนื้อเยื่อกุ้งกุลาดำกลุ่มควบคุม ส่วนกุ้งที่ได้รับยาตรวจพบความเข้มข้นเฉลี่ยสูงที่สุดของ FFA (C_{max}) ใน hepatopancreas เท่ากับ 0.7 μg ต่อกรัมเนื้อเยื่อ ที่เวลา 1 ชั่วโมง (t_{max}) และในกล้ามเนื้อเท่ากับ 0.05 μg ต่อกรัมเนื้อเยื่อ (C_{max}) ที่เวลา 4 ชั่วโมง (t_{max})หลังจากได้รับยาครั้งแรก ความเข้มข้นเฉลี่ยของ FFA ตลอด 24 ชั่วโมงของการให้ยามีค่า 0.54±0.04 μg ต่อกรัมใน hepatopancreas และมีค่า 0.15±0.02 μg ต่อกรัมในกล้ามเนื้อ และความเข้มข้นของ FFA จะค่อยๆ ลดลงหลังหยุดการให้ยาจนไม่สามารถตรวจพบได้ในวันที่ 7 หลังการหยุดยา

คำสำคัญ : กุ้งกุลาดำ ความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญของเชื้อที่ใช้ทดสอบ ฟลอเฟนิคอล ยาตกค้าง

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Introduction

The use of antimicrobials as a prophylactic medication against shrimp Vibriosis is a common practice in Thailand. The current practice, however, may have limited usefulness because of the lack of antimicrobial susceptibility data and the possible antagonistic effects of components in sea water to antimicrobial activity (Park et al., 1994; Torkildsen et al., 2000). In addition, an increase in acquired antibacterial resistance by some Vibrio pathogens has ensured the need for antibacterials that will combat these pathogens. Florfenicol (FF) is a broad-spectrum, primarily bacteriostatic, antibacterial with a range of activity similar to that of chloramphenicol (CAP). Florfenicol is chemically different from CAP and lacks the functional group associated with the induction of human aplastic anemia (Adams, 1995;

Plumb, 2002). The compound has been specifically developed for veterinary use and human aplastic anemia that is associated with CAP has not been clinically reported. Maximum Residue Limits (MRLs) of FF have been established for all food-producing species, including bovine, ovine, caprine, porcine, poultry and fin fish (EMEA, 2002). In aquaculture, FF demonstrates potent activity against a wide range of fish pathogens *in vitro* and *in vivo* (Samuelsen et al., 2003). The drug has been authorized in many countries for use in aquaculture, including Canada, Japan, Norway and the United Kingdom. However, medication with FF to treat Vibriosis in black tiger shrimps has not been reported. To establish the correct dosage regimes and the prudent use of a drug, data on the pathogen's susceptibility to the drug in use is regarded as fundamental knowledge. This study was therefore initiated to examine the Minimum Inhibitory Concentrations (MICs) of FF for Vibrio strains isolated from diseased black tiger shrimps in Thai culture facilities. The possible antagonizing effect of sea water on the compound was also evaluated to assess it's therapeutic use in a marine environment. Besides the study on FF, the *in vitro* efficacy of CAP was evaluated and compared. Although CAP is a potent antimicrobial against Vibriosis in humans and animals, the drug is prohibited in food-producing animals due to its potential toxicity to humans (Prescott and Baggot, 1994). A replacement for this drug is needed for food–producing animals. As FF is a synthetically produced antibacterial that has been specifically developed for veterinary use, the present study was designed to examine it's use as a replacement for CAP in shrimp culture.

Materials and Methods

Antivibrio activity-Minimum Inhibitory Concentration (MIC)

The procedures described here are in accordance with the international recommendations provided by the National Committee of Clinical Laboratory Standards (NCCLS). Mueller Hinton Agar (MHA) containing 1% NaCl plates, with serial two dilutions with the two antimicrobial agents, were inoculated with a standardized inoculum of the test strains (10^7 Colony Forming Unit, CFU/ml). Using a standard multipoint inoculator (NCCLS), bacteria from mature cell cultures were allocated at approximately 10^4 CFU/spot on the surface of the MHA. After 18-20 hr incubation, the MIC was recorded as the lowest concentration of antimicrobial with no visible growth of bacteria. Quality control and purity control of the methods were regularly performed for each test. Additional tests on Meuller Hinton Agar, dissolved in sea water (5 ppt salinity), were performed to evaluate any possible effects of components in the sea water that might affect the antivibrio activity of FF.

Bacterial strains

Test strains: Vibrio isolates derived from clinical cases of diseased shrimps were obtained from the culture collection of the Department of Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The collection has culture specimens from disease cases occurring between 2001 and 2004, mainly from the western and southern parts of Thailand. Previously, the identification of the isolates was performed by using conventional biochemical methods described in either the FDA Bacteriological Manual (Elliot et al. 1992) or the API system (BioMerieux, France). One hundred and two Vibrio strains isolated from the hepatopancreas of the diseased shrimps were 7 *V. alginolyticus*, 14 *V. cholerae*, 14 *V. damsela*, 27 *V. fluvialis*, 30 *V. parahaemolyticus* and 10 *V. vulnificus* strains that were used. All bacteria strains were stored in maintenance broth containing 40% glycerol, at -70°C. Before each experiment for the

MIC was carried out, the stored bacterial strains were transferred to Tryptic Soy Agar (TSA, Difco Laboratories, USA) supplemented with 1% NaCl. After incubation at 22°C for 18 hr, the inocula were transferred to Tryptic Soy Broth supplemented with 1% NaCl and the cell density was adjusted to McFarland standard 0.5 or approximately 10^8 CFU/ml. The inocula were then diluted ten-fold in sterile normal saline, giving a final cell density of approximately 10^7 CFU/ml.

Quality control strains: Additional bacterial organisms were obtained from the American Type Culture Collection for use as quality controls: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Vibrio parahemolyticus* ATCC 17802

Antimicrobial agents

The antimicrobials that were tested were Florfenicol (FF) (Takeda Chemical Ind., Ltd., Japan) and Chloramphenicol (CAP) (Sigma Chemical Co., USA.). A serial two-fold dilution of antimicrobials dissolved in absolute methanol was processed with distilled water as suggested by NCCLS, giving a series of tested concentrations from 32 to 0.5 µg/ml.

Quantitative analysis of Florfenicol-amine (FFA)

Shrimp experiment

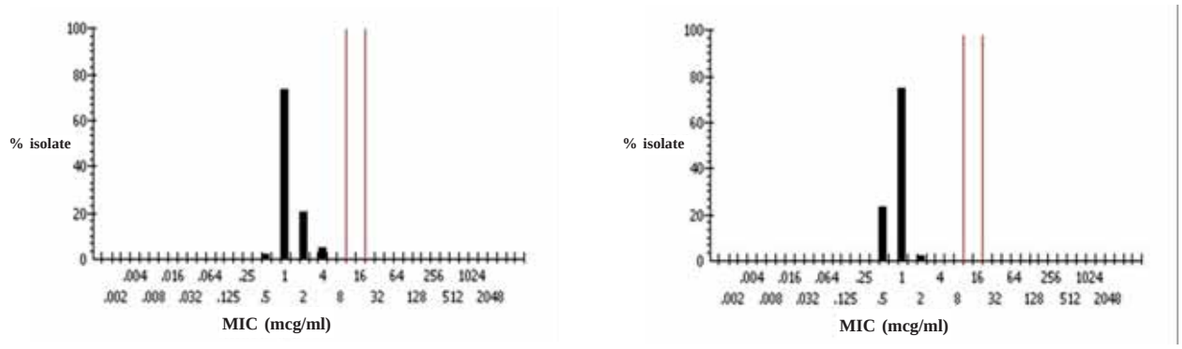
Black tiger shrimps *Penaeus monodon* (14.5±1.0 g) were acclimatired in different aquaria (n=18/ 90 L aquarium) for 14 d. Water quality parameters were maintained at a total ammonia nitrogen concentration of < 1.0 mg/L; nitrite nitrogen < 0.1 mg/L; temperature 27-28°C; pH 7.5-8.5; 30 ppt salinity and dissolved oxygen 7-8 µl/L. Shrimps were fed 3 times daily (5 am, 1 pm and 9 pm) with food amamting yo 2.5% of their body weight. The Florfenicol-medicated pellets were prepared by manually mixing of 0.8 g FF per kilogram of feed. The pellets were coated with fish oil and allowed to dry before use. The hepatopancreas and the muscle (middle segment) of the shrimp were sampled at 0.5, 1, 2, 4, 8.5, 12, 16.5 and 24 hours and every 24 hours thereafter, following the initial medicated feed, and 1, 3, 5, 7, 9 days after the 5-day treatment. Five shrimps were randomly pooled for each sample and 2 samples (2x5 shrimps) were analyzed to obtain the mean concentrations. Tissue samples were stored at -70°C until analysis.

Analysis of shrimp tissues

The concentration of Florfenicol-amine (FFA) in the shrimp tissue was analyzed by High-Performance Liquid Chromatography (HPLC) as previously reported by Wrzesinski et al. (2003). The chromatographic system was Shimadzu HPLC LC10 with an ultraviolet-visible detector SPD-10A (λ 220 nm). The chromatographic column was a 250 mm x 4.6 mm column packed with Zorbax (C8, 5 µm particles) and 12.5 mm x 4.6 mm guard columns. The mobile phase consisted of 10 mM potassium phosphate (pH 4.0) and 100% acetonitrile, which was pumped at a flow rate of 1.0 µl/min. The analyses were performed at 25°C.

Results

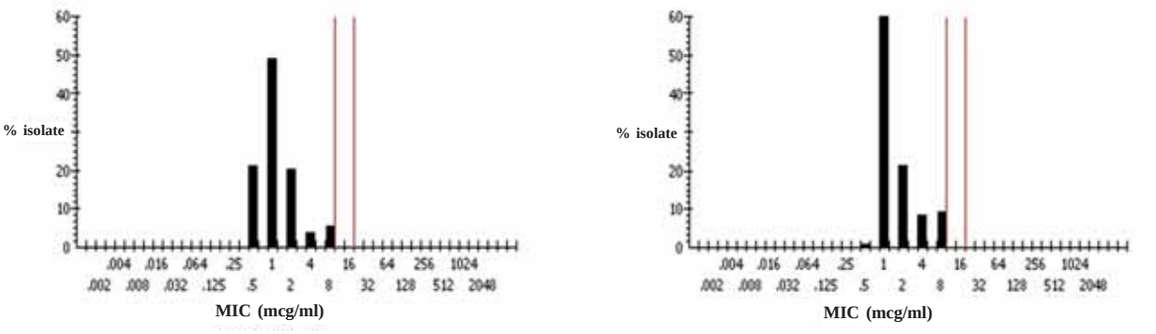
The values of the MICs of FF and CAP against 102 Vibrio isolates associated with the diseased black tiger shrimp are presented in Table 1. The MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the isolates tested. The data showed that both FF and CAP were effective against the Vibrio isolates being tested (MIC_{50}, 1 µg/ml). However, FF had a better overall MIC range than did CAP and moderate differences in MIC values were noticed between strains tested on MHA dissolved in distilled water, with 1% NaCl added and MHA dissolved in sea



Florfenicol with 1% NaCl added

Florfenicol in sea water (5 ppt salinity)

Florfenicol : Distribution of isolates to the different MIC values tested on MHA dissolved in distilled water with added 1% NaCl (left) and MHA dissolved in sea water (5 ppt salinity) (right)



Chloramphenicol with 1% NaCl added

Chloramphenicol in sea water (5 ppt salinity)

Chloramphenicol : Distribution of isolates to the different MIC values tested on MHA dissolved in distilled water with added 1% NaCl (left) and MHA dissolved in sea water (5 ppt salinity) (right)

Figure 1 Frequencies of Minimum Inhibitory Concentrations (MICs) observed for FF and CAP.

water. The test on MHA dissolved in distilled water, with added 1% NaCl, showed a MIC range of 0.5-2 µg/ml for FF while the CAP MIC range was 0.5-8 µg/ml. The MIC range observed with a test on MHA dissolved in sea water for FF was 0.5-4 µg/ml, a more narrow MICs than CAP (0.5-8 µg /ml). The frequencies of MICs observed for both compounds indicated that the distribution of isolates with different MIC values was towards the right (i.e. higher value) due to the addition of sea water to the test medium (Fig. 1). The MIC distribution figures also correlated with the estimated values of MIC 50% and MIC 90% with both compounds showing a similar value at MIC 50%, but an increasing value at MIC 90% in the sea water test.

Table 1 Minimum Inhibitory Concentrations (MICs, µg/ml) of antimicrobials against 102 Vibrio isolates associated with shrimp disease[a].

Antimicrobials	MIC_{50}		MIC_{90}		MIC range	
	DW [b]	SW [c]	DW [b]	SW [c]	DW [b]	SW [c]
Florfenicol	1	1	1	2	0.5-2	0.5-4
Chloramphenicol	1	1	2	4	0.5-8	0.5-8

[a] Data was analysed by using WHONET 5 Laboratory Database Software. Geneva.

[b] Tests were established on Mueller Hinton Agar dissolved in distilled water with added 1% NaCl.

[c] Tests were established on Mueller Hinton Agar dissolved in sea water (5 ppt salinity).

Concentrations of Florfenicol-amine (FFA) in the hepatopancreas and muscle tissue of shrimps given 0.8 g FF/kg feed daily, and feeding at 2.5% of body weight daily for 5 days, are shown in Fig. 2. Within 24 hour of medicated feeding, the mean peak concentration of FFA showed a maximum concentration (C_{max}) in the hepatopancreas of 0.7 µg/g tissue, and was observed at 1 hour later (T_{max}); The C_{max} in muscle was 0.05 µg/g tissue, and was found 4 hours after the initial medication. Mean peak concentrations every 24 hours during the 5 day treatment were 0.54±0.04 µg/g in the hepatopancreas and 0.15±0.02 µg/g in the muscle, suggesting that the dosage sustained relatively consistent FF concentrations in both tissues. However, a mean concentration comparable to an *in vitro* MIC_{50} (1 µg/ml) was not achieved in either tissue. FFA was found in both tissues on the fifth day following the cessation of feeding medication (0.23 µg/g in the hepatopancreas and 0.04 µg/g in the muscle), the residue had less than detectable limits (0.01 µg/g) by the seventh day.

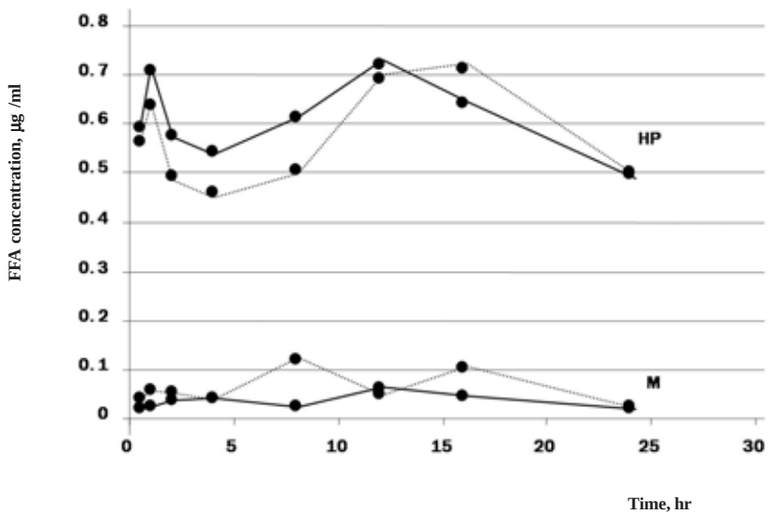


Figure 2 Florfenicol-amine (FFA) concentration analyzed in the hepatopancreas (HP) and the muscle (M) of shrimps after feeding the medication (0.8g FF/kg feed). The daily feeding regimen was 2.5% of the body weight daily subdivided 3 times, every 8 hours. (——) The first day of administration; (·····) the second day of administration.

Discussion

The results of FF MIC testing from this investigation are within the range reported from a screening of MIC on 9 Vibrio isolates from diseased shrimps studied by Mohney et al. (1992), who obtained values of 0.5-4 µg/ml. However, the MIC values of FF against the pathogenic fish vibrio *V. anguillarum* presented a narrow range of 0.4-0.8 µg/ml (Fukui et al., 1987) and 0.2-0.8 µg/ml (Zhao et al., 1992). When a medium based on sea water was used in this study, the MIC range of FF, for the same strains, extended from0.5-2 µg/ml to 0.5-4 µg/ml, indicating moderately reduced antibacterial activity when FF was dissolved in sea water. No effect of sea water was observed on the MIC range of CAP in the same study. The changes in MIC values due to the addition of sea water to the medium have been reported for several antimicrobial agents, resulting in implications for MIC testing (Lunestad and Samuelsen, 2001). Our observation that CAP and FF did not show a significant increase in MIC values when tested on sea water based medium, compared to 1% NaCl supplemented medium, is similar to the finding of Torkildsen et al. (2000). The minimal influence of sea water components on antimicrobial activity is necessary for optimal therapeutic procedures, particularly when the compound is used in a marine environment.

The oral dosage used in the study was based on the MIC testing results. The oral administration of a diet containing 0.8 g FF/kg feed, and 2.5% body weight daily intake, would allow an approximate intake of 20 µg FF/g body weight, corresponding to 10 times the MIC_{90} of 2 µg/ml. Shojaee AliAbadi and Lees (2000) suggested that the optimal dosage regimen for a bacteriostatic drug, should maintain concentrations at the site of infection, in excess of MIC_{90}, for the entire medication period. We quantified the concentration of FF in shrimp tissues using the method described by Wrzesinski et al. (2003). The quantifying procedure included acid hydrolysis to convert FF and its known metabolites to FFA. Pharmacokinetic studies of FF have been reported in many fish species, and generally plasma concentrations have been used to describe drug bioavailability in fish (Samuelsen et al., 2003; Yanong and Curtis, 2005). The concentration of FF in shrimp haemolymph was not determined in this study, because shrimp haemolymph clots effectively and the application of anticoagulant has been shown to interfere with the analytical results (unpublished data). The hepatopancreas is a major metabolic organ in shrimps, located in the cephalothorax region and exposed to haemolymph in the body cavity. Because of its physiological character and as it is the target site for shrimp Vibriosis, the total amount of FF, expressed as FFA, was quantified in the hepatopancreas rather than the haemolymph.

In the present study it appeared that FF was rapidly absorbed and that FFA in the hepatopancreas was found within an hour of the initial medication and sustained at 0.5-0.6 µg/g throughout the medication period. Despite rather stable concentrations in the hepatopancreas, concentrations were lower than the MIC_{90} value of 2.0 µg/ml. The lower bioavailability observed in this study may be attributable to loss of the drug when in solution, after feed is placed in the aquarium. Analysis of dissolution studies on feed samples *in vitro*, showed that about 30% of FF sprayed onto feed was lost in the water 15 min after immersion at 30°C (data not shown). Losses of compounds through leaching have been acknowledged in many studies, particularly when the compound was sprayed on to feed or top-dressed (Yanong and Curtis, 2005). Medicated feeds with the medication mixed in during feed preparation, are recommended for greater stability, than top-dressed feeds. The use of drugs possessing high bioavailability should be considered to minimize leaching and any environment impact.

In conclusion, the MIC data obtained in this study suggests that black tiger shrimp vibirio pathogens are susceptible to FF and CAP. Due to the haemotoxic effect of CAP in humans, the use of CAP in veterinary medicine is restricted to non-food use. Despite the potentially serious effects that improper use could have on human health, the use of CAP continues to be reported as a treatment drug of choice within the international shrimp culture community. The present study suggests a modified analogue of CAP, FF, is an alternative antimicrobial for penaeid shrimp culture. The absorption of FF following administration of FF top-dressed pellets was fairly rapid. The concentration in the hepatopancreas, 0.7 μg/g (C_{max}), occurred 1 hour (T_{max}) after administration. However inadequate concentrations of FFA in the hepatopancreas, associated with losses of the compound through leaching, necessitates further studies to ascertain the best concentration level. The dosing regimens with FF properly incorporated into feed should be confirmed before any recommendation can be made for the prophylactic or therapeutic treatment of shrimp Vibriosis with this compound.

Acknowledgements

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