

Evaluation of potassium permanganate against an experimental subacute infection of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque)

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Abstract

An experiment was performed to evaluate the efficacy of potassium permanganate (KMnO_4) as a prophylactic and therapeutic treatment of an experimental subacute infection of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus*. Fish were cutaneously abraded and divided into five treatment groups: (i) challenged by waterborne exposure to *F. columnare* and not treated with KMnO_4 (positive control), (ii) challenged and simultaneously treated with KMnO_4 , (iii) challenged and treated with KMnO_4 at 1, 6 and 9 days post-challenge, (iv) not challenged and treated with KMnO_4 at 1, 6 and 9 days post-challenge (first negative control) and (v) not challenged and not treated (second negative control). The dosing of KMnO_4 was 2.0 mg L^{-1} above the potassium permanganate demand for 2 h duration. The survival of the group challenged and simultaneously treated with KMnO_4 (99%) was significantly higher than the positive control (78%) and was not significantly different from the negative control groups. The challenged fish treated with KMnO_4 post-challenge had 7% higher survival than the positive control (85% compared with 78%), but that difference was not statistically significant. The results demonstrate that KMnO_4 has a clear prophylactic value but

probably a marginal therapeutic value once the infection has established.

Keywords: channel catfish, experimental columnaris, *Flavobacterium columnare*, *Ictalurus punctatus*, potassium permanganate.

Introduction

Columnaris disease, caused by *Flavobacterium columnare*, exists worldwide and affects a wide variety of freshwater fish; no wild or cultured species, including ornamental fish, are known to be totally resistant to columnaris (Plumb 1999). It is one of the two most important diseases of channel catfish, *Ictalurus punctatus* (Rafinesque), the most extensively cultured fish in the United States, causing high mortality among infected fish (Wagner, Wise, Khoo & Terhune 2002).

Flavobacterium columnare causes a combination of external and systemic infections (Hawke & Thune 1992). The bacterium can be a primary pathogen but more commonly it is a secondary pathogen that affects hosts predisposed by stress or trauma. The environment and the condition of the fish are important in determining the rate and severity of infection. Channel catfish are susceptible to columnaris at temperatures from 15 to 30 °C and young fish are more severely affected than adults (Plumb 1999). Clinical signs of columnaris include frayed necrotic fins with greyish to white margins and depigmented and necrotic skin. *Flavobacterium columnare* attacks the fins, skin and gills of fish. The gill lesions have white to

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brown necrotic areas and viscera exhibit little or no pathology even when the infection is systemic (Plumb 1999).

In natural columnaris infections, individual fish in a population are likely to exhibit columnaris at different severity levels and pathogenic stages. The infections can be chronic with low severity and lingering mortality, acute with severe and rapid onset of mortality, or subacute with an infection severity and mortality between acute and chronic levels (Plumb 1999).

Potassium permanganate (KMnO_4) is an effective parasiticide and bactericide and kills skin and gill pathogens via its strong oxidizing properties (Noga 1996). The permanganate ion (MnO_4^-) is responsible for the strong oxidative power and imparts a light pink or purple colouration to water; MnO_4^- is reduced to MnO_2 as it oxidizes pathogens and causes their destruction. The reduced ion is relatively non-toxic, colourless, insoluble and biologically unavailable (Boyd 1979; Lasier, Winger & Bogenrieder 2000). As KMnO_4 reacts with organic matter, the amount needed for effective treatment is higher in organically rich water (Tucker & Boyd 1977).

The efficacy of KMnO_4 against acute columnaris has been examined in fathead minnows, *Pimephales promelas* (Rafinesque) (Jee & Plumb 1981), and channel catfish (Thomas-Jinu & Goodwin 2004; Darwish, Mitchell & Hobbs 2008). The studies of Jee & Plumb (1981) and Thomas-Jinu & Goodwin (2004) demonstrated a mortality reduction in columnaris-challenged fish treated with KMnO_4 in a static system, but it is unclear whether the efficacy is due to a reduction in the bacterial count in the water and on the host, thus preventing further infection, or due to a therapeutic effect of KMnO_4 on infected fish. The study of Darwish et al. (2008) showed that KMnO_4 was not effective against an experimental acute systemic infection in a flow-through system. No study has evaluated KMnO_4 efficacy against a subacute infection of columnaris in a flow-through system, or its efficacy as a prophylactic treatment.

The objectives of this study were to develop a subacute columnaris model exhibiting the clinical signs of the disease and to use the model to evaluate KMnO_4 efficacy *in vivo* against subacute infection and as a prophylactic treatment.

Materials and methods

Bacteria

Flavobacterium columnare (isolate LV359-01, donated by Andrew Goodwin, University of Arkansas at Pine Bluff, Arkansas) was cultured on Hsu-Shotts medium at 25 °C. The isolate was presumptively identified by the biochemical method of Griffin (1992) and definitively identified by the polymerase chain reaction (PCR) method of Darwish, Ismaiel, Newton & Tang (2004). The bacterial isolate was also genotyped into genotype II (Darwish & Ismaiel 2005).

Experimental design

Channel catfish fingerlings (mean weight 51.3 ± 2.7 g) were obtained from the Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, Arkansas. Using a randomization table generated by MINITAB program version 13 (MINITAB Inc.), groups of 10 fish each were randomly assigned to 25 continuously aerated flow-through tanks containing 60 L of well water; two groups per tank, i.e. 20 fish per tank. The flow rate was set at approximately 1 L min^{-1} and the temperature was maintained at 24.8 ± 0.25 °C. Fish were acclimatized to the experimental conditions for 7 days during which they were fed commercial channel catfish feed (Arkat Feed Co., Inc.). The fish were cutaneously abraded and the tanks were randomly assigned to one of five treatment groups (five tanks per treatment): (i) challenged by waterborne exposure to *F. columnare* and not treated with KMnO_4 (positive control), (ii) challenged and simultaneously treated with KMnO_4 , (iii) challenged and treated with KMnO_4 at 1, 6, and 9 days post-challenge, (iv) not challenged and treated with KMnO_4 at 1, 6 and 9 days post-challenge (first negative control) and (v) not challenged and not treated (second negative control).

Dissolved oxygen ($> 6 \text{ mg L}^{-1}$) and total ammonia nitrogen ($< 0.2 \text{ mg L}^{-1}$) were measured in five tanks daily (HACH DR/2010; HACH Chemical Co.). Each day, five different tanks were sampled so that in 5 days all 25 tanks were tested; this was repeated throughout the experiment.

Cutaneous abrasion of fish

At the end of the acclimatization period and prior to the challenge, the fish were cutaneously abraded. Fish of all treatment groups were scrubbed five times with dishwashing scrub pad to abrade the skin. The abraded area was the right lateral surface of the fish from the caudal end of the adipose fin to the base of the caudal fin.

Bacterial challenge protocol

Flavobacterium columnare (isolate LV359-01) was stored at -80°C in Hsu-Shotts broth (with 25% glycerin added) until needed. The bacterium was plated on Ordal's medium (Anacker & Ordal 1959). An isolated colony was used to inoculate a 5 mL start-up medium; *F. columnare* growth medium broth (FCGM; Farmer 2004) was inoculated and incubated for 24 h at 28°C . The start-up medium was used to inoculate 1 L of FCGM broth. The broth was incubated for 24 h at 28°C with orbital shaking of 200 revolutions per minute (New Brunswick Scientific). The purity of the culture was confirmed by streaking an Ordal's medium plate. The bacterial challenge was initiated 3 h after the cutaneous abrasion by placing the fish from each tank in a bucket containing 8 L of continuously aerated water and adding 87 mL of the bacterial broth with an optical density of 0.85 (measured at 550 nm wavelength). After 2 h of the bacterial challenge, the fish were removed from the challenge buckets and returned to their respective tanks. The non-challenged negative controls were similarly handled but not exposed to *F. columnare*.

Dead and moribund fish were necropsied and bacterial isolation was attempted from skin lesions and kidney on plates of selective Ordal's medium (Hawke & Thune 1992). At the conclusion of the experiment, 27 days post-challenge, four fish from each tank were subjected to necropsy and bacterial isolation from areas of abraded skin and from kidneys. The identity of the isolated bacteria was confirmed by PCR according to Darwish *et al.* (2004).

Potassium permanganate treatments

The potassium permanganate demand (PPD) of five random tanks was determined (Boyd 1979) and the average was calculated prior to the treatment. The KMnO_4 dose was calculated as the average PPD (0.5 mg L^{-1}) + 2 mg L^{-1} (Plumb 1999). Three of

the five treatment groups were treated with KMnO_4 as described previously. A stock solution of KMnO_4 was prepared by dissolving 1 g of KMnO_4 in 1 L of reagent grade water ($18.2\text{ M}\Omega\text{ cm}^{-1}$). For the treatment group that was challenged and simultaneously treated with KMnO_4 in the buckets, 20 mL of the stock solution was added to 8 L of water immediately after the bacteria were added; at the conclusion of the 2 h challenge, the fish were removed from the bucket and returned to their respective tank. For the treatment groups that were treated with KMnO_4 in the tanks, 150 mL of the stock solution was added to each treated tank after the water flow was turned off; at the conclusion of the 2 h dosing period, the water was flushed several times and normal water flow was restored.

The KMnO_4 concentration was determined immediately upon the addition of the stock solution and immediately prior to the conclusion of the 2 h exposure (Engstrom-Heg 1971). The Mn level in all the KMnO_4 -treated tanks was compared with the non-treated tanks to verify that flushing was effective and the exposure ceased. Water samples were collected from treated and non-treated tanks, preserved by adding HNO_3 to achieve 1% (v/v) and analysed for Mn with an inductively coupled optical emission spectrometer (Perkin-Elmer Optima 2000DV); the detection limit of the Mn method was 0.003 mg L^{-1} (APHA 1998).

Histology sampling and processing

To evaluate the scrubbing effect, the abraded area (skin and attached axial muscle) from three abraded and three non-abraded control fish (not included in the experiment) were processed for histology. Fish were sampled after being killed with an overdose of tricaine methanesulphonate (FINQUEL MS222; Argent Chemical Laboratories Inc.). The sampled tissues were immediately fixed in Bouin's solution for 24–48 h. Fixed tissues were rinsed with water and stored in 50% isopropanol until they were dehydrated in isopropanol, cleared in Hemo-De (Fisher Scientific) and embedded in Paraplast Plus (Oxford Labware). Tissues were sectioned (4–6 μm thick) and stained with haematoxylin and eosin (Luna 1968).

Statistical analysis

At the end of the experiment the survival percentages within tanks were arcsine-transformed.

Using the MINITAB program version 13 the data were subjected to one-way analysis of variance (Zar 1984; Sokal & Rohlf 1995) and differences among treatment means were determined according to Tukey (1953). Treatment effects were considered significant at $P \leq 0.05$.

The GRAPHPAD PRISM version 4 (GraphPad Software Inc.) was used to conduct logrank tests to compare the survival curves. The null hypothesis was that the treatments did not change the survival ($P \leq 0.05$).

Results

In vivo efficacy

Fish challenged and simultaneously treated with KMnO_4 had 99% survival which was significantly different from challenged fish not treated with KMnO_4 (78%). Challenged fish treated with KMnO_4 at 1, 6 and 9 days post-challenge had a higher survival rate (85%) than challenged non-treated fish (78%); however the difference was not significant (Table 1). According to the logrank comparisons the fish challenged and simultaneously treated with KMnO_4 had a survival curve similar to the non-challenged fish, but these curves were significantly different from challenged fish treated with KMnO_4 post-challenge or challenged fish not treated (Fig. 1). The survival curves of challenged fish treated with KMnO_4 post-challenge or challenged and not treated were not significantly different.

Potassium permanganate treatment

In the buckets, the measured KMnO_4 upon dosing was $2.08 \pm 0.08 \text{ mg L}^{-1}$ and immediately prior to the conclusion of the 2 h exposure was $0.48 \pm$

Table 1 Percent survival (mean \pm SE of the mean) of cutaneously abraded channel catfish (i) challenged by waterborne exposure to *Flavobacterium columnare* (PC), (ii) challenged and simultaneously treated with KMnO_4 (T 1), (iii) challenged and treated with KMnO_4 at 1, 6 and 9 days post-challenge (T 2), (iv) not challenged and treated with KMnO_4 at 1, 6 and 9 days (NC 1) and (v) not challenged and not treated with KMnO_4 (NC 2)

	Treatment group				
	PC	T 1	T 2	NC 1	NC 2
Survival % within tanks	78 ^B \pm 4	99 ^A \pm 1	85 ^B \pm 6	100 ^A \pm 0	99 ^A \pm 1

Mean values followed by different letters are significantly different ($P < 0.05$).

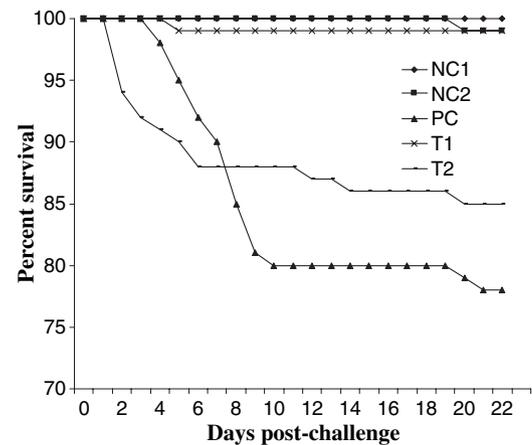


Figure 1 Survival curves of *Flavobacterium columnare*-challenged channel catfish showing the daily survival percentage. Fish were cutaneously abraded and divided into five treatment groups: (i) challenged by waterborne exposure to *F. columnare* and not treated with KMnO_4 (PC), (ii) challenged and simultaneously treated with KMnO_4 (T 1), (iii) challenged and treated with KMnO_4 at 1, 6 and 9 days post-challenge (T 2), (iv) fish not challenged and treated with KMnO_4 at 1, 6 and 9 days (NC 1) and (v) not challenged and not treated (NC 2). Each treatment group had 100 fish equally divided among five tanks.

0.01 mg L^{-1} . In the tanks, the measured KMnO_4 concentrations upon dosing at 1, 6 and 9 days post-challenge were 2.52 ± 0.06 , 2.44 ± 0.03 and $2.33 \pm 0.04 \text{ mg L}^{-1}$, respectively, and immediately prior to the conclusion of the 2 h exposure the concentrations were 2.24 ± 0.03 , 1.91 ± 0.04 and $2.07 \pm 0.03 \text{ mg L}^{-1}$, respectively. At the conclusion of the 2 h static exposure to KMnO_4 and after the tanks were flushed, the Mn level in the tanks was $0.036 \pm 0.004 \text{ mg L}^{-1}$; this represents 4.2% of the Mn applied in the KMnO_4 form.

Clinical signs and gross pathology

At 1 day post-challenge, challenged fish treated with KMnO_4 were more lethargic with rapid opercular movement than the positive control, i.e. challenged fish not treated. At 2–3 days post-challenge, the skin of the abraded area developed into focal ulcerative necrotizing dermatitis surrounded by depigmented skin, 0.2–2.2 cm wide. The dermis sloughed off revealing haemorrhagic and necrotic myositis. The fins were frayed and necrotic. As the infection progressed, the necrotizing myositis became more severe and discrete and diffuse multifocal depigmentation lesions of the skin often encompassed most of the body. Starting at 5–6 days post-challenge, in advanced cases of the

infection, the gills had focal or multifocal necrotizing branchitis with haemorrhages and yellowish mucoid material. At 8–10 days post-challenge the buccal mucosa was yellow-tinged. Moribund fish were lethargic with rapid opercular movements. No internal gross pathology was noted.

Bacterial isolation from fish

At 1 day post-challenge, there was one dead fish from the fish challenged and simultaneously treated with KMnO_4 ; attempts to isolate bacteria from this fish were negative. At 2 days post-challenge, there were six dead and moribund fish in the treatment group where fish were challenged and treated with KMnO_4 post-challenge. Of those six fish, bacterial isolation was positive both externally and internally in one fish and only externally in the other five. For 3–7 days post-challenge, the bacterium was isolated both externally and internally from all dead and moribund fish. For 8–21 days post-challenge, the bacterium from dead and moribund fish was isolated only externally from 47% and externally and internally from 53%. The identity of the *F. columnare* isolated was confirmed by PCR (Darwish *et al.* 2004). No *F. columnare* was isolated from killed fish at the conclusion of the experiment from the abraded area or the kidney.

Histopathology

The abraded area in the scrubbed fish had no epidermis and the superficial collagen fibres of the dermis were ruffled; the non-scrubbed fish appeared normal (Grizzle & Rogers 1976).

Discussion

In this study, fish challenged and simultaneously treated with KMnO_4 did not develop columnaris and KMnO_4 treatment at 1, 6 and 9 days post-challenge did not significantly increase the survival of the experimental subacutely infected fish. The results suggest that KMnO_4 would be a beneficial prophylactic treatment for columnaris in physically compromised or stressed fish, but at 1 day post-challenge the bacterial pathogenesis in challenged fish had advanced to the point that KMnO_4 therapeutic value appeared limited.

Potassium permanganate apparently reduced the number of bacteria in the water column and on the surface of the fish. Jee & Plumb

(1981) and Darwish *et al.* (2008) have demonstrated that KMnO_4 will reduce the number of bacteria *in vitro*; a reduction below an infective threshold will prevent the establishment of columnaris. Fish challenged and simultaneously treated with KMnO_4 exhibited no clinical signs of columnaris and attempts to isolate the bacteria were negative indicating no disease development.

The experimental infection in this study was subacute and mostly external in the early stages of the infection, unlike the acute infection produced by Darwish *et al.* (2008). The mortalities were lingering and low (22%) in the positive control fish. Treating with KMnO_4 at 1 day post-challenge did not significantly reduce the mortalities, and by 2 days post-challenge, the infection was external in all but one of the moribund and dead fish. The lack of significant efficacy could be partially explained by the limited accessibility of KMnO_4 to the infective bacteria. The histopathology of columnaris illustrated by Darwish *et al.* (2008) indicates that *F. columnare* infiltrates the dermis and lodges between the necrotic cells of the gills which makes the bacteria inaccessible to the oxidative power of KMnO_4 . The effectiveness of KMnO_4 application when columnaris is found systemically (6 and 9 days post-challenge) would certainly be limited (Noga 1996; Darwish *et al.* 2008).

Disease models are useful research tools that simulate a natural infection, but duplicating a natural infection will always remain a challenge because of the sheer complexity of the factors involved in the natural process. The mechanical abrasion probably enhanced the ability of *F. columnare* to attach and colonize at the abraded area (Bader, Nusbaum & Shoemaker 2003). An experimental infection was induced with lesions similar to those seen in natural infections (Plumb 1999), and the mortalities were not severe and did not start until 3 days post-challenge. Other columnaris disease models in the literature produced rapid and massive mortalities starting in 18 h (Thomas-Jinu & Goodwin 2004; Bader, Moore & Nusbaum 2006).

Potassium permanganate application could be beneficial in treating natural columnaris infections. In an infected population, individual fish are likely to exhibit columnaris in different stages of pathogenesis and some individuals may not be infected. Applying KMnO_4 will reduce the bacteria in the water column (Jee & Plumb 1981; Darwish *et al.* 2008) and the superficial bacteria on the host,

thus preventing the spread of the disease. In acutely infected fish, KMnO_4 accelerates mortality (Darwish *et al.* 2008) and the elimination of moribund fish will shorten the time *F. columnare* is shed in the water. As columnaris is typically a secondary infection occurring with other bacterial and parasitic pathogens (Plumb 1999), applying KMnO_4 in cases of mixed infections can have a beneficial effect. This is because the oxidative power of KMnO_4 is indiscriminate and can help eliminate or reduce other external pathogens contributing to the overall disease condition of the fish (Noga 1996).

The study of Jee & Plumb (1981) found that treating with KMnO_4 at 4 mg L^{-1} above the 15 min PPD reduced columnaris mortalities in fathead minnows, with 62–65% mortality in the treated fish compared with 86–100% mortality in untreated fish or those treated only with the PPD of the water. Similar results were also reported in channel catfish by Thomas-Jinu & Goodwin (2004) where the therapeutic dose (calculated according to Tucker 1989) reduced columnaris mortality from 100% to 69%. In both the studies of Jee & Plumb (1981) and Thomas-Jinu & Goodwin (2004) the fish were in static systems and there was no distinction between the prophylactic and the therapeutic effect of the KMnO_4 treatment. In this study, KMnO_4 was demonstrated to have a prophylactic effect (when challenged and simultaneously treated) but had limited therapeutic effect, with an insignificant reduction of mortality in the experimental subacute infection treated at 1, 6 and 9 days post-challenge. In static systems, fish are exposed to the bacteria for a longer period of time because the bacteria are not flushed out. Both the studies of Jee & Plumb (1981) and Thomas-Jinu & Goodwin (2004), together with this study, suggest that using KMnO_4 treatment in a columnaris-infected fish population will have an overall beneficial effect.

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