Asian Fisheries Science **22**(2009):1045-1057
ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2009.22.3.014

Asian Fisheries Society, Selangor, Malaysia

Turmeric (*Curcuma longa*) Treatment for Vibriosis in Indian Major Carp *Labeo rohita*

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Abstract

Diseased *Labeo rohita* (26 ± 0.4 cm) were treated with turmeric (*Curcuma longa*), which can cure Vibriosis using the dipping method. *Labeo rohita* were collected from the wild (Tamirabarani River, Tamil Nadu). The enumeration of bacteria isolated from diseased fish ranged from $2.3 \pm 0.8 \times 10^5$ to $6.5 \pm 0.5 \times 10^7$ cfu•g^{-1}. The various bacteria isolated were *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas* sp. and *Vibrio anguillarium.* The antimicrobial activity of medicinal herbal extracts of turmeric (*C. longa*), neem (*Azadirachta indica*) and *Aloe vera* (both ethanol and distilled water extracts) was noticed against the isolated bacteria. The maximum zone of inhibition of the antimicrobial activity was noticed in turmeric extracts against all the bacteria. Healthy *L. rohita* (18 ± 0.45 cm) were injected with Gram-negative bacterium *V. anguillarum* (10^6 cfu•ml^{-1}) to observe the signs of disease. The signs were noticed externally on the 5th day after injection. Hemorrhagic spots appeared at the site of injection and the lesions progressed subsequently. The infected *L. rohita* individuals were treated with the application

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of turmeric powder suspension (2g•L^{-1} water) using the dipping method. The infected individuals were allowed to swim in the turmeric water for 10 min daily until the lesions healed completely (21 days), which was considered as the treated group. Another set of injected fish that were not given the dip treatment was classified as the untreated group. The control groups were injected with physiological saline. The hematological parameters of control, treated and untreated group were monitored on days 0, 7, 14 and 21 which showed significant differences ($P<0.05$). The total leucocyte count (WBC: 10^4 mm^{-3}) significantly ($P<0.05$) increased from initial (3.25 ± 0.43) to final (3.31 ± 0.47) in treated *L. rohita*. The total erythrocyte count (RBC: $10^6 mm^{-3}$) decreased in the untreated group from day 7 (2.58 ± 0.13) to day 14 (2.06 ± 0.03) and decreased thereafter up to day 21 (1.93 ± 0.09). There was no increment in RBC count until day 21, except for minor fluctuation observed between the initial (day 0: 2.58 ± 0.13) and the final (day 21: 2.74 ± 0.12). The hemoglobin (Hb: g•dl^{-1}) and packed cell volume (PCV: %) counts significantly ($P<0.01$) decreased on day 14, but increased on day 21 in the treated group. When compared with the control and untreated groups, the total protein level significantly ($P< 0.05$) increased on day 21 (5.21 ± 1.25) in treated fish. The antibody agglutination titre value was higher in the untreated group than in the treated and control groups. In the treated group, the titre value increased from day 1 (72 ± 2.7) to day 14 (160 ± 1.52) and decreased later till day 21 (144 ± 3.5). The application of medicinal herb turmeric through dip treatment elicited wound healing and subsequent control of Vibriosis in *L. rohita.*

Introduction

Aquaculture production becomes more intensive. The incidence of diseases including various infectious diseases has increased and led to significant economic losses. Disease in fish is one of the crucial factors that inhibit the expansion of aquaculture. Various chemotherapeutants have been used for the treatment or prevention of diseases. However, the use of antimicrobial agents in aquaculture has resulted in more resistant bacterial strains. These resistant bacterial strains could have a negative impact on the therapy of fish diseases or human diseases and the environment of the fish farms (Smith et al. 1994). An alternative method of protection is the use of medicinal herbs that can inhibit colonization and exert inhibitory effects against undesired microorganisms. Nowadays herbs or herbal products play a significant role in aquaculture. Many kinds of herbal medicines have been used in China to control fish diseases and have produced satisfactory results (Rajendran 1990). About 10 herbs are most commonly used in China to treat diseases like enteritis, gill rot, white head and white mouth diseases (Rath 2000). Fish are in constant interaction with bacteria present in the water around them and also in their food. In fish farms, rearing conditions may enhance the proliferation of opportunistic bacteria which may cause diseases. Handling, crowding, lack of hygiene and feed-

ing with nutritionally imbalanced diets and other forms of stress are some of the main factors that influence the occurrence of diseases. A major common disease in cultured marine fish is Vibriosis, caused by *Vibrio anguillarum*, and Pastecuellosis caused by *Photobacterium damselae* (Pederson et al. 1997).

Vibrio infections usually occur in fish from various water bodies like marine, estuarine and freshwater. This fact has been reported throughout the world. Significant mortality (≥ 50) was observed in fish culture facilities under progress due to disease outbreak. Common names of Vibrio infections in fish are likewise called "red pest", "salt-water furunculosis", "red boil" and "pike pest" (Reed and Francis-Lloyd, 2002). Turmeric is a well known indigenous herbal medicine having many significant biological activities. It is an antiviral, antibacterial, antifungal and an excellent anti-inflammatory herbal product (Baum and Ng 2004; Gul et al. 2004). *C. longa* exhibits anti-tumor activities (Ruby et al. 1995) and prevents cancer (Somasundaram et al. 2002).

The present paper describes the isolation and identification of the bacteria isolated from diseased *L. rohita* and the antimicrobial activity of the herbal extract against the isolates. It also describes the potential recovery of *V. anguillarium*-infected *L. rohita* by herbal treatment and associated hematological changes.

Materials and Methods

Isolation and identification of fish pathogen

A total of 123 infected *L. rohita* with symptoms of wound, septicemia and reddish scratches on the body were collected from the Tamiraparani river-fed systems, Tamil Nadu. One gram of muscle tissue from infected individuals were taken and homogenized with sterile water. The homogenates were centrifuged at 1000 rpm for 10 min. The supernatants were subjected to serial dilution and inoculated in tryptone soya agar (Himedia). The inoculated plates were incubated at 28°C for 24-48 h to count the total heterotrophic bacteria. The colony forming units (CFU) were determined under Quebec darkfield colony counter. After quantitative analysis, isolated bacteria were identified as to genus or species by various morphological and biochemical tests using the criteria provided in Bergey's Manual of Systematic Bacteriology (Holt 1986).

Extract preparation of medicinal herbs

The rhizomes of *C. longa* were boiled and dried under a shade at room temperature and was ground to fine powder. Four grams of the powder was dissolved in 5 ml of sterile water and ethanol separately. They were centrifuged at 2000–5000 rpm for 15 min and the supernatant was collected in sterile eppendorf tubes to study the antimicrobial activity.

Five grams of fresh neem (*Azadirachta indica*) leaves were collected and washed several times in water. Then they were immersed in 0.1% (w/v) mercuric chloride solution for 3 min for surface sterilization and washed in sterile water. The washed leaves were ground in a mortar and pestle with 10% (v/v) ethyl alcohol and filtered in a double layered cheese cloth. The extract was collected in sterile eppendorf tubes. This is called "ethyl alcohol neem leaves extract". A similar preparation of this extract using sterile water is called "distilled water neem leaves extract".

Aloe vera extracts were prepared using the gel of *A. vera.* The extract was collected in sterile tubes and 5 ml of ethyl alcohol and sterile water were added separately. Their uniform suspension was centrifuged at 1500 – 2000 rpm for 15 min.

Antimicrobial activity of medicinal herbs against fish pathogens

Antibacterial activities of different fractions of turmeric were detected by agar well diffusion method, agar disc diffusion method and agar dilution method (Cheeshbrough 2000). Sterilized Muller Hinton agar (Himedia) plates were prepared. The bacterial isolates were swabbed in each plate and marked, respectively. Three wells were made in each plate using a sterile gel cutter. Among the three wells, one was kept as control which contained sterile water. The second and third wells were added with 100 µL of ethanol and distilled water extract of turmeric, respectively. A similar procedure was adapted for the remaining two extracts of neem and *A. vera*. After an incubation period of 24-48 h at 37°C all the plates were observed for zone formation, followed by identification. The minimum inhibitory concentrations (MIC) of selected antimicrobial agents were determined as described by Schmidt et al. (2000).

Experimental fish

L. rohita (18 ± 0.45 g) were collected from the Tamirabarani river fed systems, Tamil Nadu. They were transported to the Microbiology Laboratory of K.R. College, Tamil Nadu in oxygenated polythene bags (capacity: 15 L) allowed in cement tanks (3x1x1 m) and acclimatized for 3 weeks under laboratory condition. During the acclimatization period, the

individuals were fed with commercial pellet feed (CP Aquafeed, Chennai) and the water was changed once in two days. Water temperature, dissolved oxygen and pH were maintained throughout the experimental period.

Growth of Vibrio anguillarum

V. anguillarum was cultured in tryptone soya agar and harvested in tryptone soya broth. The broth was incubated overnight in a shaker for 12 h at 22°C and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was discarded and the bacterial pellet was washed three times with saline (pH 7.2). The bacteria were prepared to 10^6 cfu•ml^{-1} and determined using Neubauer hemocytometer slide (Yadav et al. 1992).

Infectivity and turmeric dip treatment

After acclimatization 20 fish were randomly selected and distributed into 50 L plastic trough. Three duplicates were maintained for each treatment. Two sets of fish were intramuscularly injected with 100 µL of *V. anguillarium* at the concentration of 10^6 cfu•ml^{-1} by using a 1 ml tuberculin syringe to induce ulcer (Brenden and Huizinga 1986). One set of injected individuals was dipped in turmeric solution (2 g of turmeric powder dissolved in 1 L distilled water) twice a day for 10 min. It was continued until the wound healed. It was classified as a treated group. Another set of injected fish was not exposed to dip treatment. It was classified as untreated group. The control group was injected with physiological saline. Blood was collected from the treated, untreated and control fish once a week (on days 7, 14 and 21) to study the hematological parameters *viz*., total leukocyte count, total erythrocyte count, hemoglobin content and antibody agglutination titre value. The mortality was noted and recorded every day before and after injection.

Blood collection

Blood was collected serially using 1 ml tuberculin syringe with 24 gauge needle by puncturing the caudal vein at regular time intervals after immunization. Blood was collected in heparinized small serological tubes.

Hematological analysis

Total erythrocyte count (RBC: x 10^6 mm^{-3}) was determined in 1:20 ratio dilution of the blood sample in Hayem's fluid and total leucocyte count (WBC: x 10^4 mm^{-3}) at 1:200 ratio dilution of the blood sample in Turke's solution using neubauer hemocytometer. Hemoglobin (Hb: g•dl^{-1}) was determined following the Cyanhaemoglobin Method. Twenty micro-

liter blood was drawn from a heparinized capillary tube, mixed in 5 ml of cyanhaemoglobin reagent (Hycel) and the mixture was measured at 540 nm under a spectrophotometer (Hesser 1960; Yokoyama 1960; Larsen and Snieszko 1961; Larsen 1964; Houston 1990). The packed cell volume level (PCV: %) was read after centrifugation for 10 min. After reading the PCV, the erythrocytes were discarded and the total plasma protein (TP: $g \cdot dl^{-1}$) was determined. The rest of the blood sample was allowed to clot and sera samples were collected in order to study the bacterial agglutination titre value following the method of Thompson et al. (1993) and Roberson (1990).

Statistical analysis

Data were presented as mean ± standard deviation of the number of fish per group. Hematological parameters were analyzed using student *t*-test to compare the difference in values among control, treated and un-treated fishes.

Results

The isolated fish pathogens were identified as *V. anguillarum, E. coli, A. hydrophila* and *Pseudomonas* sp. based on their biochemical reactions. The results of microbial identification test are given in table 1. The enumeration of microbes from diseased *L. rohita* ranged from $2.32 \pm 0.8 \times 10^5$ to $6.5 \pm 0.5 \times 10^7$ $cfu \cdot g^{-1}$. *V. anguillarum* was available in higher (47%) concentration than the other isolated bacteria. The zones of antimicrobial activity of medicinal herbs of turmeric, neem and *A. vera* against *V. anguillarium, Pseudomonas* sp., *E. coli and A. hydrophila* by distilled water and ethanol extracts are given in tables 2 and 3. The ethanol extract of *C. longa* showed better zone formation against all isolated bacteria than the distilled water extract. Neem extract showed higher zone formation against *A. hydrophilla*. The minimal inhibitory concentration of distilled water extract and ethanol extract were 9 ± 2 and 11 ± 1, respectively. No zone formation was observed in the control group. All the three medicinal herbs showed less antimicrobial activity against *E. coli.*

Table 1. Isolation and identification of 1) *Aeromonas hydrophilla*, 2) *Pseudomonas* sp., 3) *Vibrio anguillarium*, 4) *Escherichia coli*, from infected *L rohita*

Test	1 (26.8%)*	2 (13.77%) *	3 (47%) *	4 (12.50%) *
Gram staining	-	-	-	-
Motility	+	+	+	+
Pigment	-	-	NP	-
Oxidase	+	+	+	-
Catalase	NP	+	NP	+
H_2S	+	NP	NP	-
Nitarate reduction	+	+	+	+
Gelatin liquefaction	-	-	+	-
Simmon citrate	+	-	+	+
Urease	-	-	-	+
Indole	+	-	NP	-
Voges-Proskauer	+	NP	+	+
Decarboxylase				
Ornithine	NP	NP	-	NP
Lysine	NP	NP	-	NP
Arginine	+	-	+	NP
TSI				
Slant/butt	K/A	K/K	-	A/A
Gas	-	-	+	+
H_2S	-	-	+	-
Growth at:				
37^0C	+	+	+	+
0% Nacl	+	+	+	+
2% Nacl	+	NP	+	NP
4% Nacl	NP	NP	+	NP
8% Nacl	NP	NP	NP	NP
Growth in McConkey agar	+	+	+	+
Acid from:				
Arabinose	+	-	+	+
Glucose	+	-	NP	+
Glycerol	+	NP	NP	+
Inositol	-	NP	V	NP
Lactose	+	NP	NP	+
Maltose	+	+	+	+
Mannose	+	-	+	+
Mannitol	+	-	+	+
Saccharose	NP	NP	+	+
Sorbitol	-	NP	+	+
Xylose	NP	NP	-	+

*: Percentage indicates number of isolates.

Table 2. Antimicrobial activities of the distilled water extracts of *C. longa*, *A. indica* and *A. vera* against fish pathogens

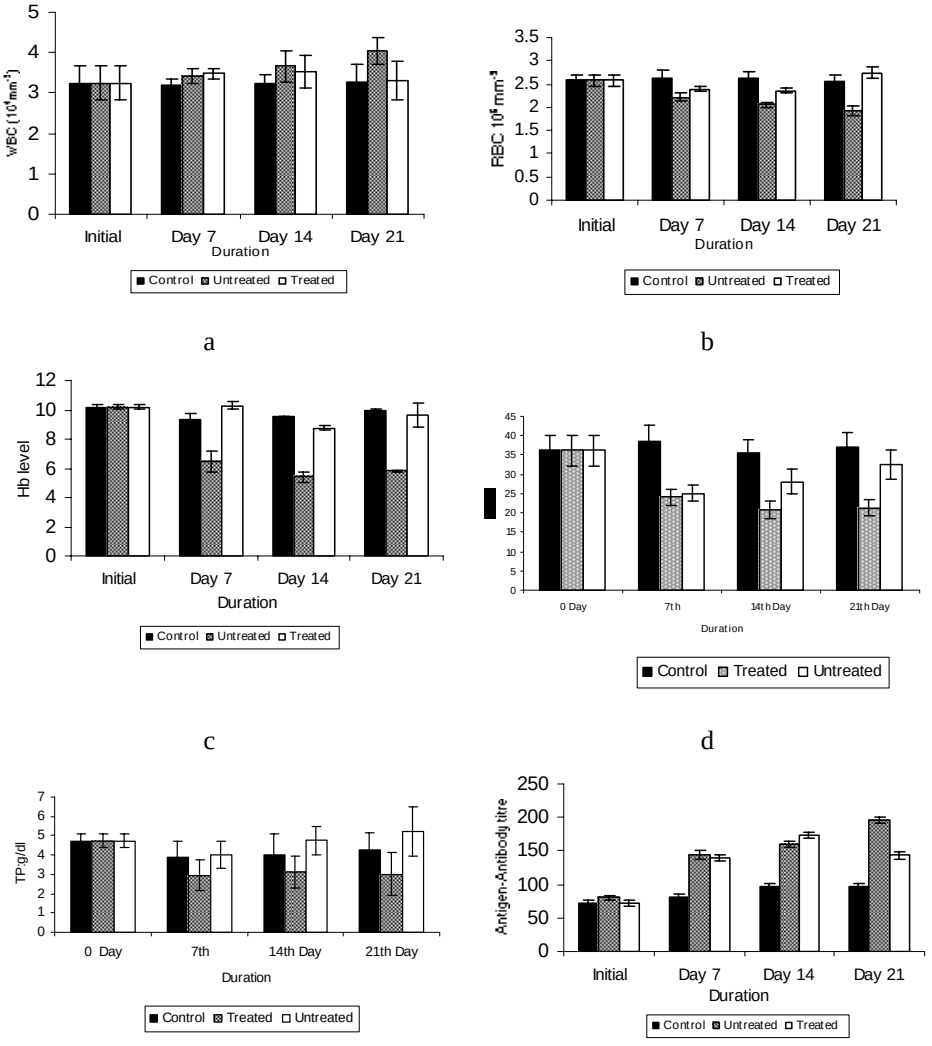
Name of fish pathogens	Zone of inhibition (mm)			
	Control (Distilled water)	Turmeric (*C. longa*)	Neem (*A. indica*)	*A. vera*
V. anguillarium	-	10 ± 2	7 ± 1	3 ± 2
A. hydrophila	-	8 ± 0	9 ± 2	4 ± 1
Pseudomonas sp.	-	6 ± 1	3 ± 1	-
E. coli	-	2 ± 1	-	3 ± 3

Table 3. Antimicrobial activities of the ethanol extracts of *C. longa*, *A. indica* and *A. vera* against fish pathogens

Name of fish pathogens	Zone of inhibition (mm)			
	Control (Distilled water)	Turmeric (*C. longa*)	Neem (*A. indica*)	*A. vera*
V. anguillarium	-	12 ± 2	8 ± 3	7 ± 1
A. hydrophila	-	8 ± 2	11 ± 1	5 ± 3
Pseudomonas sp.	-	6 ± 1	4 ± 2	-
E. coli	-	4 ± 2	3 ± 1	2 ± 0

The values of various indices of the *V. anguilarum* against *C. longa* treated, untreated and control fish are indicated in figures 1a to f. The TLC of treated fish initially increased on day 7 (3.48 ± 0.81) when compared with untreated and control fish. On days 14 and 21 the WBC level significantly ($P<0.05$) increased in treated fish compared with the untreated and control fish. At the same time the WBC level of the treated fish was found equally similar with that of the control fish during the initial period (day 0: 3.25 ± 0.45). On the other hand, the TEC in untreated fish decreased (2.20 ± 0.11), when compared with treated groups (2.39 ± 0.05) and control fish (2.63 ± 0.18) and from day 7 onwards it decreased gradually. In *C. longa* treated fish, the RBC level increased to a maximum level of 2.74 ± 0.12 (day 21). The hemoglobin content of infected untreated fish was observed from day 7 to day 21 and it ranged between 10.2 ± 0.19 and 5.83 ± 0.07, when compared with control (9.3 ± 0.45 to 10 ± 0.03) and treated fish (10.3 ± 0.23 to 9.68 ± 0.81). The PCV level in treated fish increased from day 7 to day 21 (25.12 ± 2.00 to 36.63 ± 3.75), when compared to untreated fish. The PCV level in infected untreated fish significantly decreased ($P< 0.01$) to a minimum of 21.25 ± 2.10 on day 21 (Fig. 1d). The total protein level in infected fish decreased initially in the treated fish (Fig. 1e) and further significantly ($P<0.01$) decreased to a minimum of 3.10 ± 0.83 and 3.00 ± 1.25 on days 14 and 21, respectively. In the case of treated

fish the maximum protein level was obtained on day 21 (5.21 ± 1.25). It was significant (P<0.05) when compared with control and untreated fish. Antibody agglutinating titre was found in the untreated group up to day 21, as the dip treated fish showed a meagre level, which was nearly too its initial level (Fig. 1f).



Figures 1a to f. Hematological parameters of peripheral blood in *Labeo rohita*, white blood cell (WBC: $x10^4$ mm^{-3}), red blood cell (RBC: $x10^6$ mm^{-3}), hemoglobin (Hb: $g \cdot dl^{-1}$), packed cell volume (PCV: %), total protein (TP: $g \cdot dl^{-1}$) and antigen-antibody agglutination titre (Log^{-10}) obtained on days 0, 7, 14 and 21

Discussion

In our study, *A. hydrophila, E. coli, Pseudomonas* sp. and *V. anguillarium* were isolated from the infected fish. De Figueiredo and Plumb (1977) isolated four different bacteria from infected snakehead (*Channa striatus*) in Thailand namely *A. hydrophila, Aquaspirillum* spp., *Pseudomonas* spp. and *Streptococcus* spp. Further they reported that *A. hydrophila* strains isolated from the infected fish were highly virulent, showing severe dermomuscular necrotic lesions in catfish and snakehead (Lio Po et al. 1998). In the present study *V. anguillarium* affected *L. rohita* severely. Further *L. rohita* showed the difference in susceptibility to the four bacterial isolates (Table 1). In the present study the bacterial load of rohu was $2.3 \pm 0.8 \times 10^5$ to $6.5 \pm 0.5 \times 10^7$ cfu$\cdot g^{-1}$, but it was slightly lower than the earlier report of Manohar (2007) in tilapia (5.5×10^6 to 9.8×10^9 cfu$\cdot g^{-1}$).

Many herbs can be used as therapeutics because they have the medicinal properties to prevent disease and possess antimicrobial properties (Harikrishnan et al. 2003). The antibacterial activity exhibited by *C. longa* and *Tridax procumbens* against *E. coli, Proteus vulgaris, Pseudomonas aeruginosa* and S*taphylococcus aureus,* vary from one another. This is due to the resistant nature of the pathogens. Both alcoholic extract and distilled water extract showed better results against *P. vulgaris* and *P. aeruginosa* (Lai and Roy 2004; Arun Singh 2005). In the present study the ethanolic extract of turmeric showed more antimicrobial activity than the distilled water extract of turmeric against *V. anguillarium*. Neem extract was shown to be the most effective against *A. hydrophila* than with the other species. *A. vera* was less effective against all pathogens. Turmeric (ethanol extract) showed better results against *V. anguillarium* than the other fish pathogens. *V. anguilarum* used in the present study was moderately virulent (10^6 cfu$\cdot$fish^{-1}) in experimentally infected rohu. The isolate was highly virulent in carp held in aquaria with LD_{50} of $10^{4.6}$ cfu$\cdot$fish^{-1} in rohu and catla (Azad 1998). This could be attributed to the fact that the pathogen is basically ubiquitous in fresh and brackish water (salinity of 15-20 ppt) environment (Hazen et al. 1978). In the present study the WBC level in treated fish initially increased from the control level on day 14 up to a maximum, whereas it decreased thereafter. Erythrocytic necrosis virus (ENV) infected fish have also shown abnormal, dense, and compact WBC that reached the highest level after 72 h (Haney et al. 1992). In almost all infected fish, the homeostatic processes are extended beyond the normal limits due to stress (Pickering 1981). In *C. longa* treated fish, the TEC

count increased from day 7 to day 21. The hemoglobin and PCV level of infected fish went down to the control value from day 7 to day 21, but the Hb level in the treated fish increased slightly on day 21. The decreased hemoglobin content may be due to the result of swelling of RBC as well as poor mobilization of hemoglobin from the spleen and other haemopoeitic organs in *Ictalurus punctatus* (Scott and Rogers 1981). These facts support the present finding that significant decrease in erythrocyte and hemoglobin content is possibly due to hypochromatic anaemia caused by the bacteria. The total protein level significantly increased in turmeric treated fish. Similarly Harikrishnan et al. (2003) found that when compared to control, the protein level significantly ($P<0.05$) increased in neem extract treated *Cyprinus carpio* infected with *A. hydrophila*.

Conclusion

Herbal medicines employed to treat Indian major carp against vibriosis contain soluble and particulate components both of which may generate protective immune responses. After dip treatment (with *C. longa* aqueous powder), fish exhibited a significant increase in WBC, RBC and hemoglobin.

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Date Received: 25 March 2007; Date Accepted: 30 May 2008 (MS 07/36)