Mitigation of Enterocytozoon hepatopenaei (EHP) in shrimp Aquaculture through the application of neem leaf extract

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Abstract

The effects of *Enterocytozoon hepatopenaei* (EHP) infection in shrimp farming were investigated, and a sustainable intervention approach involving Neem aqueous extract (NAE) was proposed. Extended exposure to NAE demonstrated enhanced antioxidant defenses and immune responses, effectively mitigating the oxidative stress associated with EHP infection. Moreover, the application of NAE showed promise in reducing the proliferation of EHP spores within shrimp hepatopancreas, indicating its potential for combatting EHP infection in shrimp aquaculture.

Introduction

India is a prominent player in global aquaculture, particularly in shrimp production, ranking second worldwide and serving as the leading exporter of black tiger shrimp (*Penaeus monodon*) and whiteleg shrimp (*Litopenaeus vannamei*). Despite these achievements, the emergence of EHP, a microsporidian parasite, poses a substantial threat to shrimp farming in India and beyond. EHP affects shrimp growth, causing development retardation among individuals and hindering overall productivity. Clinical symptoms include growth retardation, lethargy, decreased feed intake, and soft shells. EHP does not generally lead to mass mortalities but increases vulnerability to other diseases and negatively impacts shrimp quality. EHP has been documented in numerous global shrimp farming regions, including India, where prevalence rates reach 50% in certain provinces like Shandong and Jiangsu, China. The disease has also been confirmed in *L. vannamei* populations in India.

Challenges in Controlling EHP

Detecting EHP infection poses a challenge due to its latent nature, lacking immediate mortality or discernible external manifestations. This delay in identification hinders timely intervention, enabling the parasite's unchecked dissemination. Currently, viable commercial treatments for EHP are unavailable, and potential interventions, like prebiotics and probiotics, necessitate further refinement and testing. EHP spores exhibit remarkable resistance to environmental variables, such as salinity fluctuations, disinfectants, and temperature changes, contributing to their prolonged persistence and elevating the risk of subsequent reinfection in aquaculture cycles. Shrimp that recover from EHP infection may harbor spores asymptomatically, serving as inconspicuous carriers and potential infection sources for healthy shrimp. The intricate life cycle of EHP, encompassing both intracellular and extracellular stages, complicates effectively targeting all phases through control measures.

Current EHP Controlling strategies

EHP-infected shrimp exhibit no visible symptoms, complicating timely treatment and facilitating the spread of the disease. Effective treatment methods for EHP are currently unavailable. To manage EHP, it is crucial to ensure that broodstock maturation and hatchery facilities are thoroughly cleaned and disinfected using sodium hydroxide solution (25 gms /L) followed by acidified chlorine (0.2 gms /L). Screening broodstock for EHP before admission to facilities is crucial. While PCR testing of post larvae (PL) is recommended to identify and prevent EHP transmission, this technique may not be accessible to all farmers, requiring expertise for its implementation. Disinfecting ponds with quicklime (CaO) and maintaining appropriate pH levels can help eliminate EHP spores between cultivation cycles. The thick-walled spores of EHP are challenging to inactivate, emphasizing the importance of stringent biosecurity measures and proper pond preparation to prevent EHP contamination in aquaculture ecosystems. Ongoing research on EHP strives to unravel its complete life cycle and host-pathogen interactions and formulate efficient control strategies. The existing knowledge gap delays the development of suitable and sustainable control methods against EHP infection. Additionally, researchers explore alternative avenues, such as leveraging natural compounds from plants to enhance shrimp resistance against EHP infection autonomously.

Neem as a Potential Treatment against EHP

Neem (*Azadirachta indica*) has been studied extensively for its potential bioactive properties due to limonoids, terpenoids, flavonoids, and saponins. These compounds have shown antifungal, antibacterial, anti-inflammatory, and antiviral activities. Investigation into using NAE against EHP spores demonstrates promise in boosting shrimp immunity and potentially mitigating EHP infections. NAE was prepared following Agbenin and Marley's procedure. Fresh neem leaves were sterilized, rinsed, dried, ground into a fine powder, soaked in distilled water, and filtered before storage at -4°C. The extract was concentrated and tested for toxicity. NAE toxicity was assessed using zebrafish larvae and shrimp post larvae (PL 10), with 5-25 mg/L concentrations for zebrafish and 20-100 mg/L for shrimp larvae. Toxicity analysis in adult shrimp included 30-55 mg/L concentrations. Adult shrimps were exposed to NAE at determined concentration of 40 mg/L for 15 days for therapeutic efficacy evaluation against EHP infection.

Scientific Approaches to Study EHP and Neem

To investigate the effectiveness of NAE against EHP, researchers utilize diagnostic tools such as enzymatic quantification from post-exposure of hepatopancreas samples (superoxide dismutase, catalase, prophenol oxidase, and nitric oxide levels) and staining techniques like Calcofluor-white (CFW) and Hematoxylin and Eosin (H&E) were used for spore detection and histological examination. PCR sensitivity tests were performed for EHP gene detection.

Results

NAE underwent preliminary screening in zebrafish larvae to assess its impact at varying concentrations. Zebrafish larvae exhibited high mortality at concentrations excluding five mg/L, with complete mortality observed within 24 hours. Notably, larvae tolerated the 5 mg/L dosage for 24 hours. Shrimp larvae exposed to higher concentrations (60-100 mg/L) also experienced significant mortality after 24 hours, with 20 and 40 mg/L groups demonstrating similar survival rates to the control after 36 hours. Adult shrimp were employed to confirm effects observed in larvae, and toxicity testing revealed that concentrations of 30-40 mg/L led to 100% survival, while higher concentrations (45-55 mg/L) resulted in mortality within 24 hours. The optimum NAE dosage for subsequent exposure studies was 40 mg/L based on concentration versus percentage lethality analysis.

Continuous exposure of shrimp to 40 mg/L NAE for 15 days, with water replacement every three days, revealed improved antioxidant activity. Analysis of immunological parameters in post-treatment shrimp demonstrated that NAE exposure stimulated antioxidant defense mechanisms, influencing non-enzymatic and enzymatic antioxidants. Superoxide dismutase activity significantly increased in NAE-treated shrimp infected with EHP, indicating a reduction in reactive oxygen species levels compared to EHP-infected shrimp. Catalase activity also increased in NAE-treated shrimp, reaching levels comparable to the control group. Nitric oxide activity, essential for immune responses, increased significantly in NAE-treated, EHP-infected shrimp, approaching control group levels. Prophenol oxidase activity, a vital component of the phenol oxidation pathway, increased dramatically after NAE administration, surpassing both control and EHP-infected groups.

Staining techniques, including Calcofluor-white (CFW), revealed fewer EHP spores in NAE-exposed shrimp compared to the model group. Histopathological examination of hepatopancreas sections exhibited fewer dark blue-dyed spores in NAE-exposed shrimp than EHP-infected shrimp, while no spores were observed in the control group. Polymerase chain reaction (PCR) sensitivity analysis confirmed the specificity of the EHP PCR method, with the targeted 358 bp band exclusively observed in DNA samples from EHP-infected shrimp. NAE exposure appeared to result in a lower level of EHP infection or a reduced quantity of EHP DNA in the analyzed samples. These findings suggest the potential efficacy of NAE as a treatment option for controlling EHP infection in shrimp.

Continuous exposure of shrimp to 40 mg/L NAE for 15 days, with water replacement every three days, revealed improved antioxidant defense via increased superoxide dismutase (SOD) and catalase (CAT) activity. NAE aided in neutralizing superoxide radicals, protecting against oxidative damage induced by EHP infection. Additionally, NAE normalized nitric oxide (NO) activity, potentially enhancing immune responses. Bioactive components in neem, such as triterpenoids and flavonoids, contribute to these effects. Histological examination of shrimp hepatopancreas indicated fewer EHP spores in NAE-exposed shrimp, supporting its role in EHP eradication. Calcofluor-white staining revealed reduced spores in NAE-exposed shrimp feces. Polymerase chain reaction (PCR) sensitivity testing suggested NAE may influence EHP infection or DNA quantity, suppressing EHP replication and proliferation. Identified compounds in Neem methanol extract (NME) possess antimicrobial, antioxidant, and anti-inflammatory attributes, potentially inhibiting EHP proliferation and mitigating associated inflammatory responses (Fig. 1). Further research is needed to validate the effectiveness of these compounds in the context of EHP infection in shrimp. Overall, NAE shows promise in enhancing antioxidant defense immune response and combating EHP in shrimp aquaculture.

Conclusion

NAE shows promise in enhancing antioxidant defense and immune response while combating EHP infection in shrimp aquaculture. Through various scientific approaches, NAE demonstrated significant potential in boosting shrimp immunity, reducing EHP spore development, and improving antioxidant activity.

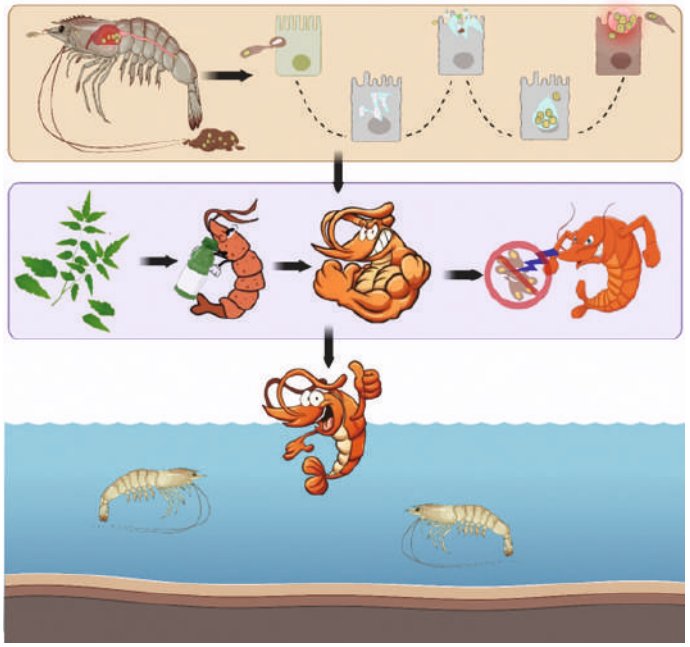

Fig. 1 Mechanism of EHP infection and NAE treatment therapy in the EHP infected shrimp.