Biochemical and Molecular Characterization of Pathogenic Vibrio Associated with White Feces Syndrome in Penaeus vannamei and their Antibiotic Susceptibility

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Abstract:
White gut disease (WGD) is an emerging threat to shrimp aquaculture, significantly impacting production and profitability. This study aimed to investigate the bacterial diversity associated with WGD in shrimp and antibiotic susceptibility. The total bacterial count (TBC) and total Vibrio count (TVC) were determined in digestive tracts and haemolymph of both Normal and WGD-affected shrimp. The TBC and TVC in the digestive tracts of WGD-affected shrimp were substantially higher (1.5×10^8 CFU/g and 1.41×10^7 CFU/g) compared to Normal shrimp (2×10^5 CFU/g and 0.43×10^4 CFU/g). Similarly, in haemolymph, TBC and TVC in WGD-affected shrimp (0.6×10^8 CFU/ml and 0.62×10^6 CFU/ml) were elevated relative to healthy shrimp (0.51×10^5 CFU/ml and 0.27×10^5 CFU/ml). A total of seven distinct bacterial isolates (V1-V7) were isolated from WGD-affected shrimp and characterized based on morphological, biochemical, and molecular analyses. Molecular characterization through 16S rRNA gene sequencing confirmed species as Providencia stuartii, Vibrio campbellii, Vibrio alginolyticus, Shewanella algae and Shewanella carassii. Shewanella carassii was identified as the same species but with different strains. Antibiotic susceptibility testing revealed isolates Vibrio campbellii, Vibrio alginolyticus, Shewanella carassii exhibited resistance to Penicillin-G, and Vibrio alginolyticus, Shewanella carassii resistance to Ampicillin whereas Providencia stuartii exhibited resistance to Tetracycline and Furazolidone. These findings highlight the pathogenic bacterial community associated with WGD and underscore the need for effective disease management strategies in shrimp aquaculture.

Key words: White gut disease (WGD), Shrimp, Aquaculture, Vibrio, Antibiotic,16S rRNA sequencing.

Introduction

In respoShrimp aquaculture is a vital global food sector driven by rising demand (FAO, 2020) and technological advancements (Boyd et al., 2018). India, a major producer, exported a record 17,81,602 MT of seafood in 2023-24, with vannamei shrimp production at 10,76,970 MT, 70% from Andhra Pradesh (MPEDA). Despite profitability, disease outbreaks, environmental issues, and market fluctuations pose major challenges (Lightner, 2011). Shrimp aquaculture faces major losses from bacterial and viral diseases, with white feces syndrome (WFS) causing up to a 60% production loss (Lightner, 1996; Tangprasittipap et al., 2013) and first detected in Thailand (2010–2011), leading to reduced feed intake and mortality (Chaweepack et al., 2015). characterized by floating white fecal matter, it resulting from hepatopancreatic microvilli sloughing into vermiform bodies, typically appearing 50–60 days post-stocking (Sriurairatana et al., 2014). It was initially linked to microsporidian infection, later studies did not support this hypothesis (Tang et al., 2016).

Vibriosis is a major threat to global shrimp aquaculture, affecting numerous penaeid species (Baticados et al., 1990). WFS is linked to V. anguillarum, V. alginolyticus, V. parahaemolyticus, V. harveyi, V. penaeicida, and V. campbellii causing severe mortality under stress (Lightner, 1988). WGD caused significant mortality, with outbreaks associated with V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, and V. splendidus (Janakiram et al., 2018). WFS-infected shrimp exhibited greater Vibrio diversity, with V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus, V. cholerae, and V. damsela found at twice the levels observed in healthy shrimp (Somboon et al., 2012). Identification of beneficial gut microflora in P. vannamei may aid disease prevention. Vibrio campbellii, a major shrimp pathogen, closely resembles V. harveyi but is genetically distinct (Austin & Zhang, 2006; Thompson et al., 2007). Gut microbiota play a key role in shrimp health, with Shewanella and Psychrobacter found in specific conditions (Ghonimy & Chang, 2024). WFS-affected shrimp show dominant Vibrio, Candidatus Bacilloplasma, and Acinetobacter, Shewanella was also dominant in WFS-affected shrimp, following Vibrio, Candidatus, Bacilloplasma, and Acinetobacter (Wang et al., 2024). Most research has focused on microbial communities in water and diet effects on intestinal microbiota (Zhang et al., 2014; Tang et al., 2014). Widespread antibiotic use in aquaculture has led to rising resistance, posing global concerns, especially with prophylactic use (Holmström et al., 2003; Chomwong et al., 2018). Vibrio harveyi's resistance challenges shrimp farms, while luminous vibriosis remains uncontrollable despite antibiotics (Jayasree et al., 2008, Shafiqur et al., 2009). Overuse of antibiotics fosters resistance and enables gene transfer between aquatic and human environments (Cabello, 2006). Proper culture management helps

prevent bacterial diseases (Flegel, 2012). Therefore, in the present paper efforts have been made to isolate and characterize the bacteria associated with white feces syndrome affected shrimp, P. vannamei.

Materials And Methods

Sample Collection

Normal and White Feces Syndrome(WFS) affected shrimps were collected from culture ponds near koppugundupalem, Rambilli, Visakhapatnam, Andhra Pradesh, India and brought to the laboratory under continuous oxygenated condition. Shrimp were anaesthetized on ice for 5-10 min, and aseptically dissected their digestive tracts (Hp & Gut). Pre-weighed digestive tracts (0.5g) were homogenized with 2% sterile NaCl solution.

Microbiological studies

Total bacterial count (TBC) and Total Vibrio count (TVC) (digestive tracts) & (Haemolymph)

Homogenized samples were serially diluted (10^{-1} to 10^{-6}) in sterile normal saline. One ml from 10^{-3} and 10^{-5} dilutions was added to Petri dishes, followed by lukewarm Zobell's Marine Agar (ZMA) for TPC (Fig.1) and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar for TVC (Fig.2). The plates were gently rotated in both clockwise directions, solidified, inverted, and incubated at 32±1°C for 24 hours. Colony counts were determined using a colony counter and expressed in CFU/g. Haemolymph (0.5 ml) was drawn from Normal and WFS-affected shrimp and enriched in 2 ml Nutrient Broth. Samples were then pour-plated on Zobell's Marine Agar (ZMA) for TPC and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar for TVC and incubated at 32°C for 18–24 hours. Colonies were counted (CFU/ml) and Total bacterial and Vibrio loads were estimated following standard methods (APHA 1992).

Predominant and morphologically different colonies were selected and purified on slants. The isolated bacteria were identified based on morphological, biochemical and molecular characteristics Bergey's Manual of Systematic Bacteriology up to species level (Kreig & Holt, 1984).

Molecular characterization of bacterial isolates through 16S rRNA

DNA Extraction and 16S rRNA PCR Amplification

Genomic DNA was extracted using the phenol-chloroform method (Sambrook et al., 1989). Bacterial cultures were grown in LB broth, lysed with lysozyme and Proteinase K, and DNA was purified. Extracted DNA was confirmed via 1% agarose gel electrophoresis (Fig.3). PCR amplification of 16S rRNA genes was performed using specific primers in a 40 µL reaction mix. The thermal cycling profile included 35 cycles: denaturation (95°C, 30s), annealing (53°C, 30s), extension (72°C, 1 min), and final extension (72°C, 10 min) (Roux, 1995). Amplified products were analysed on agarose gel under UV light.

PCR Purification, Sequencing, and Phylogenetic Analysis

PCR products were purified using the ExoSAP-IT® Kit and sequenced bidirectionally with the BigDye Terminator v3.1 Kit on an ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequences were identified using BLAST (NCBI), and the top ten matches were aligned with CLUSTAL W 1.6. A phylogenetic tree was constructed (Fig.5 to 11) using MEGA 7 (Kumar et al., 2015) with the Neighbor-Joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 replicates) ensured reliability (Felsenstein, 1985), and evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al., 2004).

Antibiotic sensitivity tests

The sensitivity of bacterial isolates to various antibiotics was tested following the method of (Bauer et al. 1996). Young cultures (18 hrs.) of the isolates were spread evenly over Mueller's Hinton Agar (MHA) dishes. Twelve commercially available Antibiotic discs (Himedia, Mumbai), Oxytetracycline(O), Norfloxacin(NX), Streptomycin(S), Ciprofloxacin(Cf), Gentamycin(Gen), Ampicillin (A), Furazolidone (Fr), Penicillin-G (P), Kanamycin(k), Chloramphenicol (c), Tetracycline (T) and Erythromycin (E) were placed over the spread culture carefully (6 discs per dish) and incubated overnight at 320C. Upon incubation, the inhibition zones obtained around the discs were measured by using Kirby Bauer scale(Fig.11).

Results And Discussion

Bacterial count

TBC and TVC in Normal shrimp digestive tracts were 2×10^5 CFU/g and 0.43×10^4 CFU/g, while in WGD-affected shrimp were 1.5×10^8 CFU/g and 1.41×10^7 CFU/g. In haemolymph, TBC and TVC in Normal shrimp were 0.51×10^5 CFU/ml and 0.27×10^5 CFU/ml, whereas WGD-affected shrimp TBC and TVC were 0.6×10^8 CFU/ml and 0.62×10^6 CFU/ml (Fig.3).

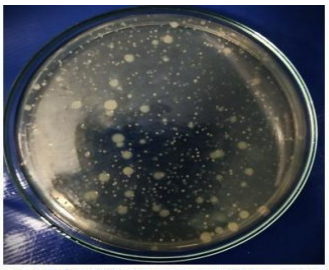


Fig.1 Bacterial colonies on ZMA Agar

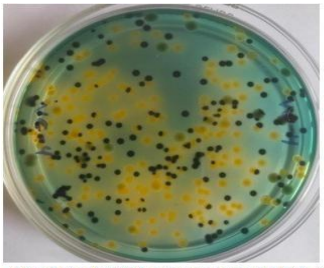


Fig.2 Bacterial colonies on TCBS Agar

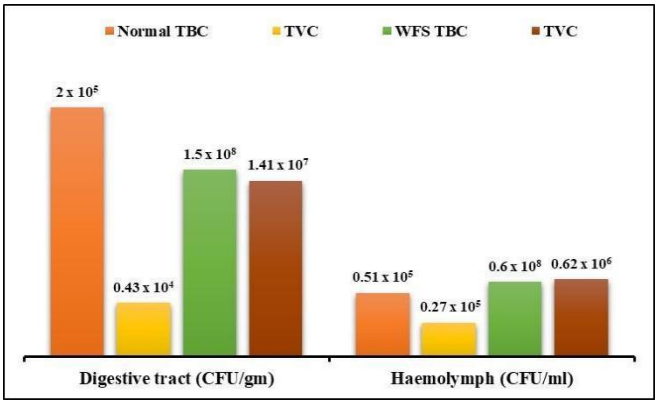


Fig .3 TBC and TVC of Normal and WFS shrimp

Morphological and biochemical characterization

Seven distinct bacterial isolates (V1 to V7) were obtained from the digestive tracts of White Gut-affected shrimp which exhibited green coloration, a shiny appearance, opaque optical properties, smooth texture, entire margins, and raised elevations. All seven isolates were examined under a microscope and subjected to the tests like Grams staining, Motility, Aerobic and Anerobic, Oxidative and Fermentative, Acid production from glucose, Oxidase, Catalase, Growth on TCBS, Nacl tolerance test, Temperature tolerance test, Voges Proskauer's test, Arginine, Salt tolerance 1%, ONPG, Citrate, Ornithine, Mannitol, Arabinose, Sucrose, Glucose, Salicin, Cellobiose following methods of Bergey's manual of Systemic Bacteriology (Kreig & Holt, 1984). Based on the above tests isolated bacteria were identified as V1-Providencia, V2-vibrio, V3-vibrio, V4-shewanella, V5-shewanella, V6-shewanella, V7- shewanella.

Molecular characterization

The seven isolates V1 to V7 were subjected to molecular characterization for confirmation of the species through 16s rRNA gene sequencing.

PCR amplification of 16s rRNA gene from isolates

The amplification of a fragment of the 16s rDNA gene was achieved using the 27F and 1492 primers. A single discrete PCR amplicon band measuring 1500 bp. (Fig.4)

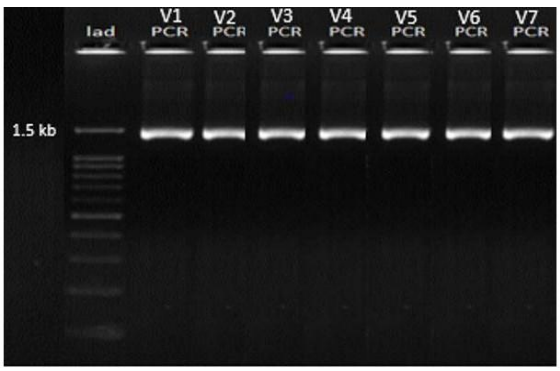
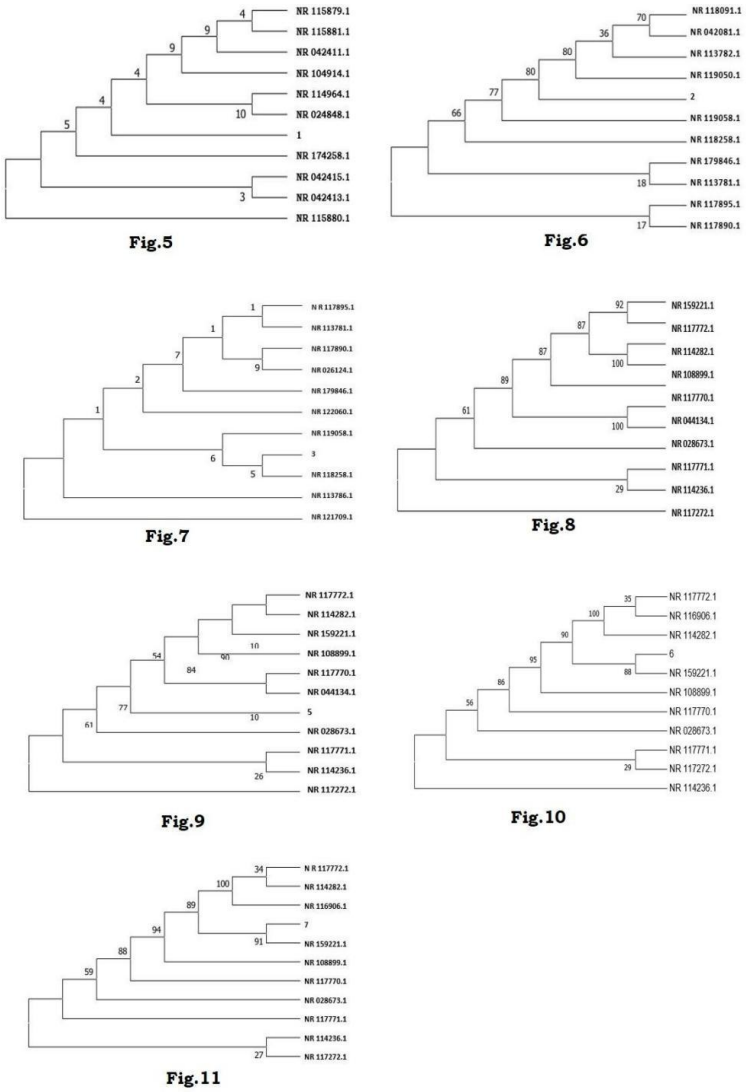


Fig.4 Photograph of gel showing the amplified DNA of 7 isolates

Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and

missing data were eliminated. There were a total of Minimum 1173 to Maximum 1521 positions in the final dataset of seven isolates. Phylogenetic Evolutionary tree was constructed using MEGA 7. BLAST analysis of the 16S rRNA gene sequences identified the bacterial isolates with high sequence identity: Providencia stuartii (96.03%), Vibrio campbellii (94.63%), Vibrio alginolyticus(96.89%), Shewanella carassii (99.43%, 99.08%, 99.86%), and Shewanella algae (96.96%).(Fig5-11)



Figs. 5-11. The phylograms of the Isolate V1, V2, V3, V4, V5, V6 and V7 and their similarity with Providencia stuartii, Vibrio campbellii, Vibrio alginolyticus, Shewanella carassii, Shewanella algae, Shewanella carassii, and Shewanella carassii.

Based on biochemical analysis, sequence homology, and phylogenetic studies, the bacterial isolates V1, V2, V3, V4, V5, V6, and V7 were confirmed as Providencia stuartii, Vibrio campbellii, Vibrio alginolyticus, Shewanella carassii, Shewanella algae, Shewanella carassii, and Shewanella carassii, respectively. The presence of Shewanella carassii in three isolates suggests the identification of different strains of the same species and submitted to NCBI gen bank and obtained accession numbers viz. PV174508, PV174516, PV174524, PV174536, PV174537, PV174538 and PV174540.

Antibiotic sensitivity

The inhibition zones observed in the sensitivity assay are illustrated were classified as highly sensitive (>20mm), moderately sensitive (15-20mm), sensitive (10-15mm), and R-resistant (0mm). (Figs.12-13).

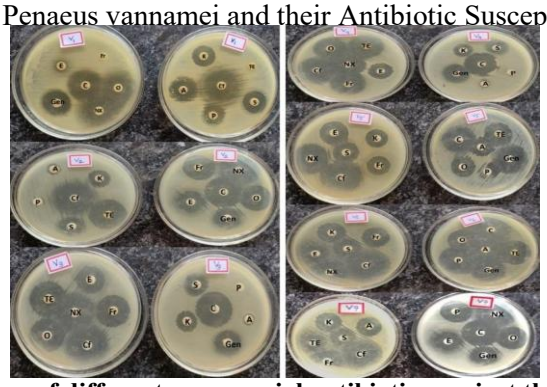


Fig.12 Inhibition zones of different commercial antibiotics against the microbial isolates V1-V7

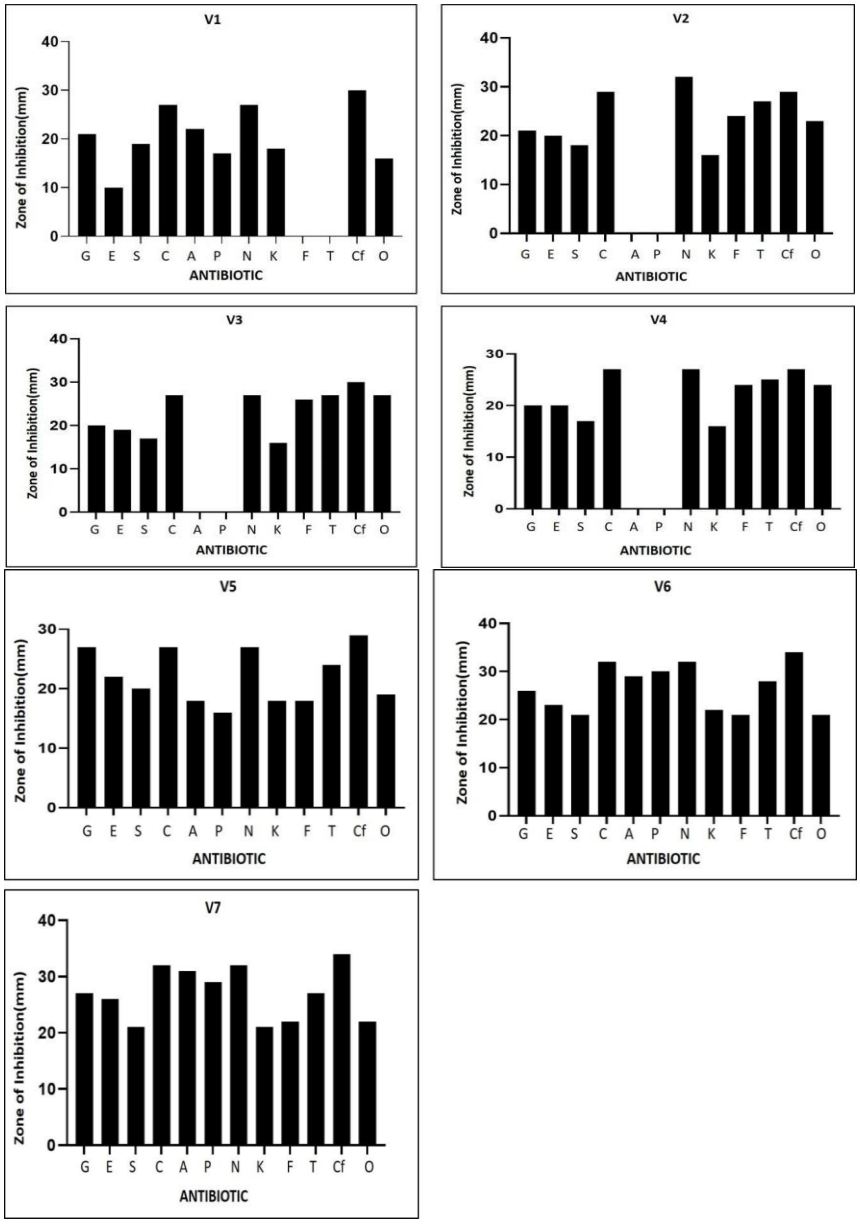


Fig.13. Antibiotic susceptibility test for bacterial isolates

***Gentamycin (G), Erythromycin (E), Streptomycin (S), Chloramphenicol (c), Ampicillin (A), Penicillin-G (P), Norfloxacin (N), Kanamycin (k), Furazolidone (F), Tetracycline (T), Ciprofloxacin (Cf), Oxytetracycline (O).**

Discussion

The shrimp industry faces significant challenges from disease outbreaks, particularly White Feces Syndrome (WFS), severely affects Penaeus vannamei, studies reported by Jayasree et al. (2006), has shown that Vibrio loads in shrimp tissues are significantly higher in diseased individuals. In this study, total bacterial counts (TBC) and total Vibrio counts (TVC) in the digestive tract of Normal shrimp were 2×10^5 and 0.43×10^4 CFU/g, respectively, while in White Gut-affected shrimp, they increased to 1.5×10^8 and 1.41×10^7 CFU/g. Similarly, in haemolymph, Normal shrimp showed TBC and TVC of 0.51×10^5 and 0.27×10^5 CFU/ml, whereas affected shrimp exhibited 0.6×10^8 and 0.62×10^6 CFU/ml, respectively. The widespread use of antibiotics in aquaculture has led to antibiotic-resistant bacteria, particularly Vibrio harveyi, complicating disease management (Jayasree et al.,2008); Karunasagar et al., 1994). Resistance of Vibrio species to Ampicillin and Penicillin-G is a growing concern Defoirdt et al., (2013). In this study, Vibrio campbellii, Vibrio alginolyticus, and Shewanella carassii showed resistance to Penicillin-G, while Vibrio alginolyticus and Shewanella carassii were also resistant to Ampicillin. Providencia stuartii exhibited resistance to Tetracycline and Furazolidone. However, Chloramphenicol was highly effective. whereas, Norfloxacin and Ciprofloxacin remained potent.

Cornejo-Granados et al. (2017) analyzed the microbiota of wild and farmed Pacific White shrimp using 16S rRNA sequencing, highlighting its role in characterizing microorganisms undetectable by traditional methods and the microorganisms that are inadequately distinguished by traditional techniques and offer enhanced characterisation require molecular analysis methods like 16S r-DNA sequencing (Drancourt et al., 2000). In this study, bacterial identification was performed using 16S rRNA sequencing, with phylogenetic analysis based on the Maximum Likelihood method (Kimura, 1980). BLAST analysis of 11 sequences (1173–1521 bp) showed 94.63%–99.86% identity with Providencia stuartii, Vibrio campbellii, Vibrio alginolyticus, Shewanella carassii, and Shewanella algae. Two strains of Shewanella carassii were identified.

Conclusion

These findings emphasize the importance of microbe's identification in shrimp providing a foundation for future research on pathogen management, disease control strategies and the pathogenesis of WFS, ultimately contributing to the development of more efficient and sustainable treatment approaches in shrimp farming.

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