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Development of a polymerase chain reaction (PCR) assay targeted to the *dnaJ* gene of *Vibrio harveyi*, a bacterial pathogen in Asian seabass, *Lates calcarifer*

[1]Christopher M. A. Caipang, [2]Rolando V. Pakingking Jr.,
[3]Mary Jane S. Apines-Amar, [4]Fahrul Huyop, [2]Norwell B. Bautista

[1]Faculty of Biosciences and Aquaculture, University of Nordland, Bodø 8049, Norway; [2]Aquaculture Department, Southeast Asian Fisheries Development Center Tigbauan 5021, Iloilo, Philippines; [3]National Institute of Molecular Biology and Biotechnology, University of the Philippines Visayas, Miag-ao 5023, Iloilo, Philippines; [4]Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, University Technology Malaysia, 81310 Skudai, Johor, Malaysia.
Corresponding author: C. M. A. Caipang, cmacaipang@yahoo.com

Abstract. Partial sequence of the *dnaJ* gene of *Vibrio harveyi*, which was isolated from diseased juvenile Asian seabass, *Lates calcarifer* was identified. The partial sequence of *dnaJ* gene of *V. harveyi* was 447 bp and shared at least 77% identity at the nucleotide level with the *dnaJ* gene of other *Vibrios*. It was distinct from the *dnaJ* gene of other *Vibrios* but was closely related with the *dnaJ* gene of *V. rotiferianus* and *V. campbellii* having at least 90% nucleotide identity. PCR primers targeting this gene were designed to detect the pathogen in Asian seabass. The assay was specific to *V. harveyi* and the limit of detection was 100 pg of genomic DNA ml^{-1} or 100 fg of bacterial genomic DNA in a PCR reaction. This corresponded to a sensitivity of approximately 20 genome equivalents (GE) of *V. harveyi*. These results indicate that the *dnaJ* gene is a good candidate to develop primers for the PCR assay in detecting *V. harveyi* in fish.
Key Words: *Vibrio harveyi*, *dnaJ* gene, *Lates calcarifer*, PCR.

Introduction. The family Vibrionaceae consists of several species of bacteria that are pathogenic to fish, molluscs and crustaceans. *Vibrio harveyi* in particular, is a Gram-negative luminous bacterium that has caused severe mortalities in a wide number of species in aquaculture (Lightner 1993; Zhang & Austin 2000; Austin 2010; Cao et al 2010). In shrimps, this pathogen is implicated in the disease, luminous vibriosis, which is occurs mainly in all life stages of penaeid shrimp (Lavilla-Pitogo et al 1990; Liu et al 1996; Jayasree et al 2006). It has also resulted in mass mortalities in *Babylonia areolata*, one of the shellfish species cultured in China (Huang et al 2009; Cao et al 2010). In fish, this pathogen usually causes local hemorrhagic ulcers on the mouth or skin surface as well as necrotic lesions in the muscle and eye opacity (Muroga et al 1984; Leong 1992).

Among cultured marine fish species, *V. harveyi* has been isolated from diseased fish including Asian seabass, *Lates calcarifer* (Tendencia 2002), seahorse, *Hippocampus kuda* (Tendencia 2004), snapper, *Lutjanus guttatus* (Gomez-Gil et al 2006) and gilthead sea bream, *Sparus aurata* (Haldar et al 2010). The incidence rate of vibriosis in fish is higher in the summer when there is increased water temperature and abundance of organic load (Ortigosa et al 1989; Kin et al 1990; Carli et al 1993).

Due to the severity of the disease, early detection of the causative agent is necessary for the control of vibriosis. Several detection methods have been developed and the most popular technique is the use of molecular-based methods such as PCR. *V. harveyi* has several genes that are suitable candidates for its specific detection by designing PCR primers that allow amplification of the target gene sequence. Examples of

the genes that were used to identify *V. harveyi* from cultured organisms include, *toxR* (Conejero & Hedreyda 2003; Pang et al 2006), hemolysin or the *vhh* gene (Conejero & Hedreyda 2004; Parvathi et al 2009), *gyrB* (Thaithongnum et al 2006), an outer membrane protein, *vhhP2* (Sun et al 2009), some housekeeping genes such as *hsp60*, *gapA*, *recA*, *rpoA* and *pyrH* (Kwok et al 2002; Nishiguchi & Nair 2003; Thompson et al 2005) and the 16s rDNA (Oakey et al 2003; Fukui & Sawabe 2008). Despite of the availability of these gene markers for *V. harveyi*, accurate identification of this bacterial pathogen is sometimes imprecise and labour-intensive (Vandenberghe et al 2003). Recently, the *dnaJ* gene that encodes a heat shock protein 40 has been found to be suitable for the phylogenetic classification and identification of various *Vibrio* species (Nhung et al 2007).

The Asian seabass is a popular species for marine aquaculture particularly in net cages in most tropical countries (Tendencia 2002; Newton et al 2010; Ransangan & Manin 2010). Previously, a bacterial isolate was obtained from the diseased fish showing focal hemorrhages in the skin and exophthalmia and was later identified as *V. harveyi* by biochemical, phenotypic and physiological tests (Tendencia 2002). However, detection methods for this pathogen during the early stages of infection in the fish are lacking. Hence, this study identified a partial sequence of the *dnaJ* gene of *V. harveyi* and developed a PCR assay using that gene as a molecular marker for diagnosing vibriosis in Asian seabass.

Materials and Methods

Bacterial strains. Samples of *V. harveyi* grown on trypticase soy agar (TSA, Fluka, Germany) supplemented with 1.5% NaCl were kindly provided by the Fish Health Section of the Aquaculture Department, Southeast Asian Fisheries Development. The bacterium was isolated from diseased juveniles of Asian seabass that were cultured in marine cages in Central Philippines. Authenticity of the bacterium was verified following Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1984) and by sequencing of the 16s RNA. The other bacterial strains used in the study were purchased from various sources and routinely cultured on TSA.

Extraction of genomic DNA. Genomic DNA of the bacteria was extracted following the procedures described by Kulkarni et al (2010) with some modifications. Briefly, a loopful of the bacteria was scraped from the TSA plate and placed in a 1-ml microfuge tube containing DNA extraction buffer [10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea at pH 7.5 (Caipang et al 2004)]. The solution was added with 10 μl of Proteinase K (20 μg ml-1) and incubated for 1 hour at 37°C. Genomic DNA was extracted by phenol:chloroform:isoamyl (PCI) solution followed by precipitation with ethanol. The DNA pellet was resuspended in 100 µl of 1X TE buffer (pH 7.5) and stored at -20°C until use for PCR assays.

PCR amplification of *dnaJ* gene and sequence analyses. Partial fragment of the *dnaJ* gene of *V. harveyi* was amplified using degenerate primers designed by Nhung et al (2007). These primers resulted in the amplification of a PCR product with a size of approximately 558 bp. The PCR product from the amplification of the partial fragment of the dnaJ gene was treated with shrimp alkaline phosphatase (Takara, Japan) to remove traces of unbound phosphates during the PCR reaction. The treated PCR product was sequenced using Big Dye Terminator ver 3.1 following the procedures described by the manufacturer (Applied Biosystems, CA, USA) and comparative sequence analyses were performed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/). Multiple alignment of the partial sequences of the dnaJ gene of different was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Table 1 shows the *Vibrio* spp. and the accession numbers of their *dnaJ* gene that were used for the alignment. Phylogenetic tree was constructed using the Neighbor Joining Method with 1000 boostrap values of MEGA 4 (Tamura et al 2007).

Optimization of the PCR protocol. From the sequence of the fragment of the dnaJ gene of *V.harveyi*, two PCR primer sets were initially designed from the least conserved

regions of the gene to ensure high degree of specificity of detecting the target pathogen. The first set of primers (forward: 5'-TGATAATCTGCTGCGGATAAG-3'; reverse: 5'-GGCGCATTTGAACTTGACCG-3') amplified a PCR product having a size of 421-bp. The second set of primers consisted of the same forward primer and a reverse primer: 5'-ACAAGTCTCAGCTGAAGAAC-3', amplified a 384-bp PCR product.

The PCR reaction mixture consisted of 2 μl of each primer (5 pmol), 2 μl of 10x PCR buffer, 1.5 μl of 2 mM dNTP, 1 μl of 15 mM $MgCl_2$, 0.1 μl of *Taq* DNA polymerase (100 units) (Invitrogen, U.S.A.), 2 μl of the DNA template (1 μg ml^{-1}) and scaled up to 20 μl using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 sec and elongation at 72°C for 1 min; then a final elongation at 72°C for 5 min. PCR products (5 μl) were electrophoresed on a 0.8% agarose gel with 1% TBE electrophoresis buffer (pH 8.0) for 30 min, visualized using a gel documentation system (Biorad) and photographed.

The specificity of the PCR primers was evaluated using the optimized conditions as described earlier with other bacterial pathogens including *V. anguillarum*, *V. parahaemolyticus*, *Aeromonas salmonicida* and *Yersinia ruckeri*. To determine the sensitivity of the PCR assay, ten-fold serial dilutions of the bacterial genomic DNA were prepared at an initial concentration of 1 μg genomic DNA ml^{-1}. One microliter of the DNA template was used for the PCR following the optimized conditions.

Results and Discussion. A PCR product with a size of approximately 558-bp was amplified using degenerate primers for *dnaJ* gene of *Vibrio* spp. Direct sequencing of the PCR product showed that it had high homology with the dnaJ gene of Vibrio spp. and other closely related bacterial species. The partial sequence of the *dnaJ* gene of *V. harveyi* that was isolated from diseased Asian seabass consists of 447 bp (Fig. 1) and had an amino acid identity of at least 77% with the *dnaJ* genes of other *Vibrio* spp., and shared high nucleotide identity (> 90%) with *V. campbellii* and *V. rotiferianus* (Table 1). The nucleotide sequence of the dnaJ gene of *V. harveyi* that was isolated from Asian seabass is deposited in Genbank with an accession number of JF832351.

Phylogenetic analysis of the V. harveyi dnaJ gene showed that it is distinct from the *dnaJ* genes of other Vibrios (Fig. 2). It is closely related with the dnaJ genes of both *V. campbellii* and *V. rotiferianus*.

These findings are in agreement with a previous sudy done by Nhung et al (2007) where the authors demonstrated that the dnaJ gene of Vibrios is a good gene candidate for the molecular identification of different species of Vibrios. The dnaJ gene has a high degree of divergence compared with 16s rDNA thus making the former ideal in separating closely related Vibrios (Nhung et al 2007). For example, *V. mimicus* could not be differentiated from *V. cholerae* using the 16s rDNA (Chun et al 1999) as well as *V. scophthlami* from *V. ichthyoenteri* (Thompson et al 2002). In this study, the phylogenetic tree was constructed using the nucleotide sequences of dnaJ gene of the different Vibrios instead of using the amino acid sequences. Nhung et al (2007) contend that because dnaJ gene encodes a housekeeping protein, it consists of degenerative code that allows for silent mutations to take place. As a result, phylogenetic trees that are constructed based on amino acid sequences will have poor branching resolution compared to those constructed using the nucleotide sequences.

Using the partial sequence of the *dnaJ* gene of *V. harveyi*, we constructed two sets of PCR primers from the least conserved regions in order to detect this pathogen in Asian seabass. The primers that amplified a 421- bp PCR product were not specific (Fig. 3a). Aside from amplifying the *dnaJ* gene of *V. harveyi* it also resulted in the amplification of the *dnaJ* gene of *V. anguillarum* and *V. parahaemolyticus*. Hence, this primer set was not used in subsequent assays. On the other hand, the second primer set (forward: 5'-TGATAATCTGCTGCGGATAAG-3'; reverse: 5'-ACAAGTCTCAGCTGAAGAAC-3'), which amplified a 384-bp PCR product had a high degree of specificity. There was no amplification of the gene in the closely-related bacterial pathogens, *V. anguillarum* and *V. parahaemolyticus* as well as in other bacteria including *Aeromonas salmonicida* and *Yersinia ruckeri* (Fig. 3b). The second primer set was then used for the sensitivity assays.

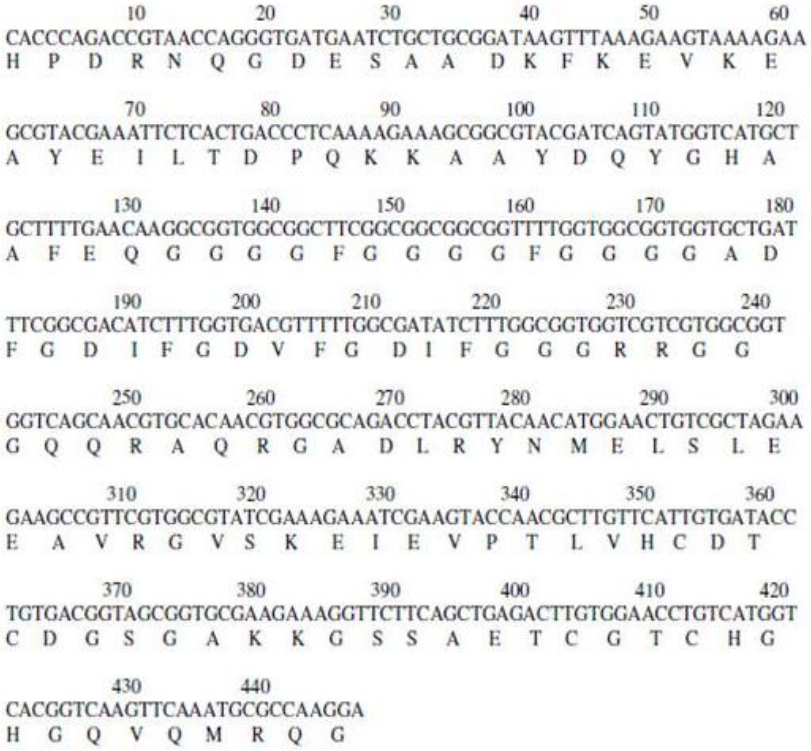

Figure 1. Nucleotide and amino acid sequence of the partial *dnaJ* gene of *V. harveyi* isolated from Asian seabass.

Table 1

Accession numbers of the different bacterial species used in the study and nucleotide identity of their *dnaJ* gene with the *V. harveyi* isolated from Asian seabass

Bacterial species	Genbank Accession Number	Nucleotide identity (%)
Vibrio harveyi (Vihar-1)	AB263039.1	99.0
V. rotiferianus (Viro)	AB263063.1	93.0
V. campbellii (Vica)	AB263024.1	90.0
V. natriegens (Vina)	AB263050.1	87.0
V. alginolyticus (Vial)	AB263020.1	87.0
V. scophthalmi (Viscop)	AB263066.1	85.0
V. vulnificus (Vivu-1)	BA000037.2	85.0
V. vulnificus (Vivu-2)	AE016795.3	85.0
V. parahaemolyticus (Vipa)	AB263057.1	84.0
V. nigripulchritudo (Vini)	AB263054.1	82.0
V. penaeicida (Vipe)	AB263059.1	82.0
V. coralliilyticus (Vico-1)	HM215579.1	82.0
V. coralliilyticus (Vico-2)	HM215575.1	82.0
Shewanella pealeana (Shpe)	CP000851.1	74.0
Salmonella enterica (Saen-1)	FN424405.1	75.0
S. enterica (Saen-2)	AP011957.1	75.0

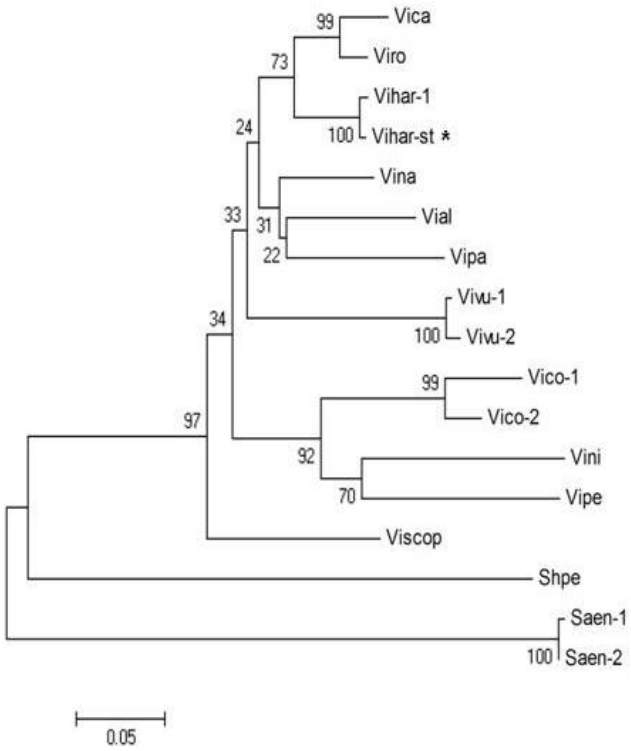

Figure 2. Phylogenetic tree of the *dnaJ* gene of the different *Vibrio* species and other bacteria. The Neighbor Joining method with 1000 bootstrap values was used for the analysis. The Genbank accession numbers and the bacterial species used for the analysis are indicated in Table 1. The *dnaJ* gene of the *V. harveyi* in the present study is marked with an asterisk.

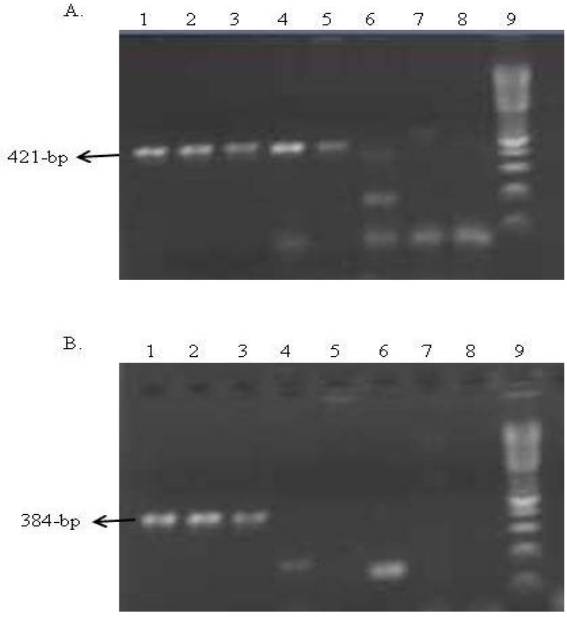

Figure 3. Specificity of the PCR assay using different primers targeting the *dnaJ* gene of *V. harveyi.* (A) primers for the amplification of a 421-bp PCR product, (B) primers for the amplification of a 384-bp PCR product. Lanes 1-3: *V. harveyi*, Lane 4: *V. anguillarum*, Lane 5: *V. parahaemolyticus*, Lane 6: *Aeromonas salmonicida*, Lane 7: *Yersinia ruckeri*, Lane 8: negative control (distilled water), Lane 9: 100-bp DNA marker. This is a representative of two independent PCR reactions.

In terms of the sensitivity of the PCR assay, ten-fold serial dilutions of the bacterial genomic DNA were prepared at a starting concentration of 1 μg ml^{-1}. The limit of detection of the PCR assay was 100 pg of genomic DNA ml-1 or 100 fg of genomic DNA

for every PCR reaction (Fig. 4). The genome size of *V. vulnificus* is approximately 5.3 Mbp (Chen et al 2003) and assuming that this genome size is used for the calculation of genome equivalents that can be detected by the PCR assay, then the sensitivity of this PCR assay for *V. harveyi* is approximately 18-20 genome equivalents (GE) per PCR reaction. For comparison, a PCR assay for the detection of *Francisella noatunensis* has a limit of detection of 50 GE per PCR reaction (Kulkarni et al 2011).

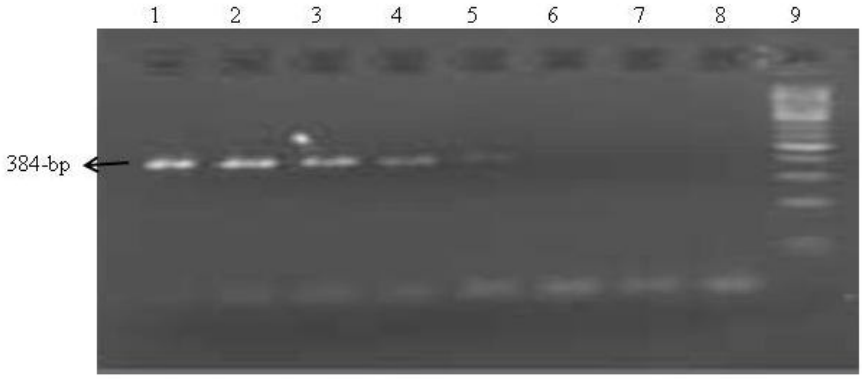

Figure 4. Sensitivity of the PCR primers (amplification of a 384-bp PCR product) to detect the *dnaJ* gene of *V. harveyi* isolated from Asian seabass. Amplification was done at 40 cycles at an annealing temperature of 55°C in a 20-μl PCR reaction. Lane 1: 1 μg DNA ml^{-1}, Lane 2: 0.1 μg DNA ml^{-1}, Lane 3: 10 ng DNA ml^{-1}, Lane 4: 1 ng DNA ml^{-1}, Lane 5: 100 pg ml^{-1}, Lane 6: 10 pg DNA ml^{-1}, Lane 7: 1 pg DNA ml^{-1}, Lane 8: negative control (distilled water), Lane 9: 100-bp DNA marker. This is a representative of four independent PCR reactions.

The results of our PCR assay for the detection of *V. harveyi* in Asian seabass reinforces the notion that this assay provides a specific and highly sensitive method for diagnosing the disease during the early stages. In the case of *V. harveyi*, several PCR assays targeting various genes of this pathogen have been developed. Examples of these *V. harveyi*-specific genes or sequences were the *toxR* (Conejero & Hedreyda 2003; Pang et al 2006), hemolysin or the *vhh* gene (Parvathi et al 2009), *vhhP2* (Sun et al 2009) and the 16s rDNA (Oakey et al 2003). In our study, we have successfully identified a partial sequence of the *dnaJ* gene of *V. harveyi* and used this gene as a molecular marker to develop primers for the detection of the pathogen.

It is well-known that there is a high degree of variation within *V. harveyi* isolates, and proof of this is the wide varaibility of the vhh gene in different isolates of this bacterial species (Parvathi et al 2009). Hence, the PCR assay should be targeted to a specific isolate in order to have an accurate detection method. In the Philippines, *V. harveyi* is known to infect shrimps (de la Peña et al 2005) as well as it has been isolated from seahorse (Tendencia et al 2004) and seabass (Tendencia 2002). Except for the development of different PCR assays to detect *Vibrio harveyi* in shrimps (Caipang & Aguana 2011), there is no PCR assay that has been developed and standardized to detect *V. harveyi* that was isolated from fish. As such, this PCR assay is the first to detect *V. harveyi* in aquacultured fish in the Philippines.

Conclusions. We have sequenced a fragment of the *dnaJ* gene of *V. harveyi* and designed specific primers targeting this gene for the detection of the bacterial pathogen in Asian seabass. The results also showed that *dnaJ* gene is a good candidate for distinguishing *V. harveyi* from other bacteria and can be potentially used as a marker to differentiate the isolates within *V. harveyi* populations.

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Authors:
Christopher Marlowe A. Caipang, Faculty of Biosciences and Aquaculture, University of Nordland, Bodø 8049, Norway, cmacaipang@yahoo.com.
Rolando V. Pakingking Jr., Aquaculture Department, Southeast Asian Fisheries Development Center Tigbauan 5021, Iloilo, Philippines, rpakingking@seafdec.org.ph
Mary Jane S. Apines-Amar, National Institute of Molecular Biology and Biotechnology, University of the Philippines Visayas, Miag-ao 5023, Iloilo, Philippines, mary_jane.amar@up.edu.ph.
Fahrul Huyop, Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, University Technology Malaysia, 81310 Skudai, Johor, Malaysia, fzhutm@gmail.com
Norwell Brian Bautista, Aquaculture Department, Southeast Asian Fisheries Development Center Tigbauan 5021, Iloilo, Philippines, nbbautista@seafdec.org.ph.
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