Digital PCR assay for the detection and quantification of shrimp adulteration levels

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Introduction

Food fraud damages local economies and deceives consumers. For instance, Atlantic white shrimp (*Litopenaeus setiferus*) is one of the most valuable domestic shrimp species in the United States (U.S.) with a distinct taste and naturally abundant nutrients. Hence, it is widely preferred amongst consumers in the U.S. Due to low supply, it commands a higher price point. On the other hand, Pacific white shrimp (*Litopenaeus vannamei*) is predominantly produced by aquaculture and is extensively farmed outside. Due to cheaper labor costs and fuel subsidies, the Pacific white shrimp has a lower price point. This significant price gap is the driving factor for the substitution, where domestic Atlantic white shrimp is replaced with imported Pacific white shrimp. Commonly, species are entirely substituted. However, recent market sampling data have shown that the shrimp is being sold as a mixture of domestic Atlantic white shrimp and Pacific white shrimp.

As with many forms of food fraud, morphological methods to identify Atlantic white with imported Pacific white shrimp are difficult, especially as shrimp is most commonly traded in processed forms that remove morphological identifiers, such as peeled, deveined, battered and cooked. Thus, DNA-based methods are widely used for identifying shrimp species. Methods developed in our lab and published by others include DNA barcoding, qPCR and PCR-lateral flow-based detection. However, these methods are only suited for detection. Often, Atlantic white and Pacific white shrimp are mixed, and current methods have limited capability of accurately quantifying the substitution levels.

Nanoplate-based digital PCR (dPCR) is an advanced technique for the accurate quantification of nucleic acids. Unlike traditional methods, dPCR provides an absolute measurement of DNA content without relying on standard curves. This enhances the precision and sensitivity.

In recent years, dPCR has been effectively utilized for the quantitative detection of various animal-derived components – including beef, chicken and salmon in both processed and mixed food products. To date, no study has been done to quantify Atlantic white or Pacific white shrimp by dPCR. Quantification is needed as there have been reports of adulteration to fraudulently boost economic gains. Therefore, we designed this method to identify and quantify Atlantic white and Pacific white shrimp and estimate weight percentages using dPCR.

Materials and methods

2.1 Sample preparation

Raw, peeled and deveined Atlantic white shrimp and Pacific white shrimp were obtained from a local supplier in Houston, TX, USA. Samples were stored at −20°C until further use. Frozen samples were defrosted in the refrigerator. Different mixtures of known percentages of Atlantic and Pacific white shrimp, i.e., 5%, 20%, 40%, 60%, 80%, and 100% were prepared in filter stomacher bags to a total mass of 50 g. The samples were mixed and hand-crushed for 1 minute, followed by a dilution with 200 mL of peptone buffer saline solution. The PBS shrimp mixture was homogenized using the Stomacher homogenizer at 230 rpm for 30 s. A 10 mL liquid from the homogenized samples was collected and stored at −20°C.

2.2 DNA extraction

Genomic DNA was extracted from pellets obtained from 2 mL of each homogenized sample by centrifuging at 20,000×g for 2 minutes. DNA was extracted using the DNeasy® Blood & Tissue Kit (QIAGEN, MD, USA), following the manufacturer's instructions. Concentration and purity of the extracted DNA were measured using a Nanodrop® 2000 spectrophotometer (Thermo Fisher, MA, USA). Samples were diluted to a 10 ng/µL working stock for PCR application.

2.3 Determination of primer annealing temperatures

A gradient qPCR was used to standardize the annealing temperature of Atlantic and Pacific white shrimp primers. The gradient PCR was performed on a LightCycler® 96 instrument (Roche Diagnostics Corp., Indianapolis, IN, USA), using a SYBR® Green master mix and 0.40 µL of forward and reverse PCR primers, 20 ng/µL of extracted DNA and 7.6 µL of water. The qPCR amplification profile consisted of an initial denaturation step at 94° C for 30 s, followed by 35 cycles of denaturation at 95° C for 30 s, annealing between 55-63°C for 30 s and extension at 72°C for 20 s. The melt curve step after amplification consisted of 95°C for 10 s, 65°C for 60 s and 97°C for 1 s. The annealing temperatures showing reproducible results and specific melting peaks were used for further assay development. These annealing temperatures were also used for dPCR.

2.4 dPCR procedure

The dPCR assay was performed on an 8-well 26k Nanoplate (QIAGEN, Hilden, Germany). A 50 µL dPCR reaction mixture, which consisted of 5 µL of DNA, 4 µL of *L. setiferus* and *L. vannamei* forward and reverse primers, 12.4 µL QIAcuity® EG PCR Kit and 28.6 µL of nuclease-free water, was prepared. Out of the 50 µL reaction mixture, 45 µL was loaded into each nanowell. The singleplex amplification process was carried out with an initial denaturation of 95°C for 2 minutes, followed by 35 cycles of amplification at 94°C for 30 seconds, annealing at 57°C (*L. setiferus*) and 62°C (*L. vannamei*) for 30 seconds and extension at 72°C for 20 seconds. The imaging profile consisted of the green channel (EvaGreen) with an exposure duration of 250 ms and a gain of 6. Finally, the data obtained was analyzed by QIAcuity Software Suite (version 2.5.0.1). Digital PCR data was analyzed by setting a common threshold for each dPCR run. The threshold bar was manually positioned slightly above the negative control and non-template control groups.

2.5. Inclusivity and Exclusivity Testing

To determine the cross reactivity of the assay with commonly traded shrimp species on the US market, DNA from 30 samples spread across 6 species were randomly selected from FSU seafood specimen library and tested using the dPCR assay according to the procedure in detailed section 2.4 using a 24-well 26k nanoplate (QIAGEN, Hilden, Germany). These samples included Argentine red shrimp (Pleoticus muelleri) (n = 4), Atlantic white shrimp (*L. setiferus*) (n = 7), pink shrimp (*Penaeus duorarum*) (n = 4), black tiger shrimp (*Penaeus monodon*) (n = 4), Pacific white shrimp (*Litopenaeus vannamei*) (raw and cooked) (n = 7), and Royal red shrimp (*Pleoticus robustus*) (n = 4). dPCR sample concentration values below 0.7 cp/µL were considered negative.

2.6 Assay Validation with Market Samples

Twenty-five shrimp samples were purchased from restaurants located in Louisiana (USA). One sample was raw and the remaining were cooked, fried or grilled. DNA was extracted from the raw blended sample following the procedure in 2.2 and used for the dPCR in 2.4. Crude DNA was extracted from the remaining 24 samples using our lab DNA extraction protocol, diluted 1/10 and used in the dPCR reaction. These market samples were simultaneously tested with our published PCR-lateral-flow assay for *L. setiferus* and *L. vannamei*, and the results were compared.

Results and discussion

DNA yield and purity ranged from 8.7–49.6 ng/µL and an A260/280 of 1.5–2.11, respectively, indicating good quality for PCR reactions. From the gradient qPCR reaction, annealing temperatures of 57°C (*L. setiferus*) and 62°C (*L. vannamei*) were found to be best suited. From dPCR, at a threshold of about 60 RFU, both samples had no fluorescence for non-template and negative controls, i.e., 100% *L. setiferus* or 100% *L. vannamei* lane (Figure 1A

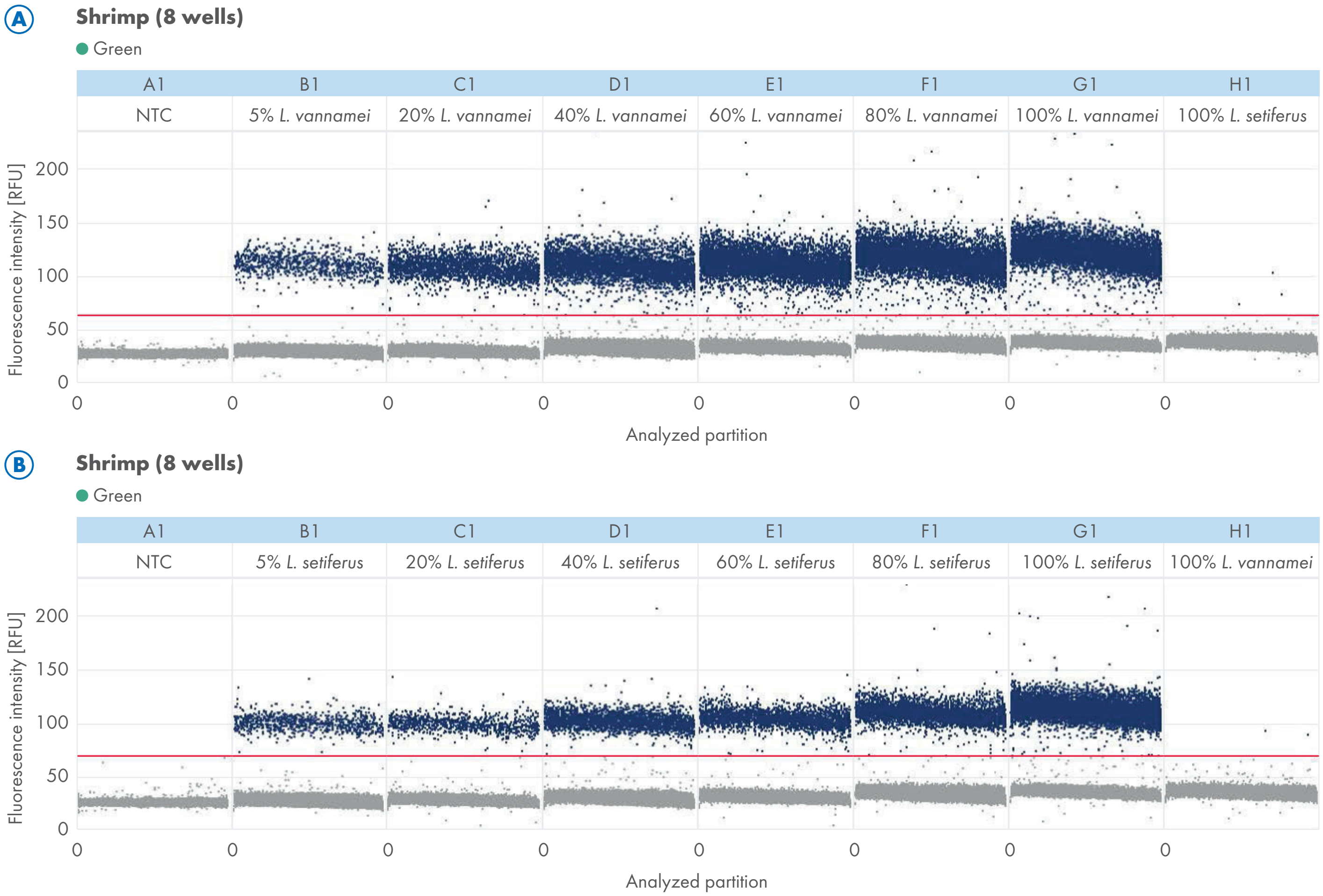

Figure 1. dPCR detection for *L. vannamei* assay (Pacific white shrimp) (**A**) and *L. setiferus* assay (Atlantic white shrimp) (**B**) showing fluorescence values against the number of analyzed partitions for various weight proportions of NTC, 5, 20, 40, 60, 80, 100%, respectively.

and B). There was a good separation between samples of the selected weight percentages, with a significant increase in the fluorescence signals as weight percentages increased (Figure 1).

The concentration (cp/µL) of dPCR amplicons showed an increasing trend as the weight percentages of *L. vannamei* and *L. setiferus* increased from 5% to 100%.

For inclusivity and exclusivity testing, a few of the 30 non-target and NTC negative control samples generated very few positive partitions (<0.64 cp/µL) and remained below the predetermined negative cut-off (0.70 cp/µL). In total, DNA barcoding validated both dPCR assays standardized in this study, showing 100% inclusivity and exclusivity.

The dPCR assay did not show any inhibition due to the presence of inhibitors in crude DNA extracts from cooked shrimp samples. For the assays validation with market samples, the dPCR results for cooked shrimp samples purchased at market matched with PCR lateral flow test results. The suspected raw blended samples showed a trace level of *L. vannamei*, which may have been caused by using the same scoop for two different shrimp kept next to each other at the market.

Conclusion

In this study, we standardized a dPCR-based method for the identification and quantification of Atlantic white shrimp and Pacific white shrimp. The dPCR signal increased as the amount of target shrimp was increased. Pacific white shrimp is exported in large quantities to many western countries, where it is used to undercut the domestic Atlantic white shrimp species, making it a global problem. The dPCR assay developed in this study can be used by regulatory agencies to secure the food chain from fraud for the detection and quantification of Pacific white shrimp substitutions in retail and wholesale shrimp samples. Thus, it will have a significant impact on domestic shrimpers.