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Strategy for DNA extraction and detection from insect pests in stored home grain samples

RESEARCH

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ABSTRACT

Stored grains are subjected to infestations with more than 60 species of insects, that responsible for millions of dollars loss and cause several health problems including allergies and gastrointestinal disorders. Traditional detection techniques are laborious, expensive and not sensitive to detect insect contamination at the egg and larvae stages. Therefore, alternative methods are needed for rapid and sensitive detection. One obvious approach is to develop a molecular approach utilizing genetic information of the potential insect species that infest grains for amplification of specific target gene fragment utilizing polymerase chain reaction [PCR]. In the present study, a number of known infested grain samples were used in standardizing a method to isolate larvae and adult insects that were based on centrifugation washing method and a filtration washing method. The isolated insects were subjected to DNA extraction and PCR amplification of defined regions of cytochrome oxidase I (COI) gene followed by sequencing to identify the different pest species. For PCR amplification new primers were designed and for this purpose the obtained COI sequences from different insects were aligned to design two sets of primers (named: COI-PCR4 and COI-PCR5) specific for the indicated insect mitochondrial COI gene. The designed primers were tested for their specificity and sensitivity. The suitability of PCR primers and DNA extraction methods were evaluated on eleven samples of commercial grains utilizing each primer set with the two extraction methods.

Keywords: Insect pests, grain, DNA extraction

Introduction

Grains are considered as the world's primary stable food and its seasonal harvesting obligates storage for different time periods either for short-term or long-term periods (Proctor, 1994; Rajashekar et al., 2010). During storage, grains are exposed to damage by microorganisms, mice and insect pests, which destroy about 10-20% of agricultural products annually (Dragisic Maksimovic et al., 2015; Rajashekar et al., 2010) (Holst et al., 2000). Improper maintenance of storage temperature and humidity leads to insect development, which then leads to biological and chemical damage (Chattha et al., 2015), For these reasons it is important to examine grains periodically for early detection of insects in order to minimize grain loss.

Over 60 species of insects can infest stored grains (Jian, 2019). Beetles (order *Coleoptera*), moths (order *Lepidoptera*) and mites (class *Arachnida*) are the most common species that live in human food grains (Aspaly et al., 2007) (Collins, 2012; Garcia-Cela et al., 2019; Waongo et al., 2019). The principle pests stages that cause

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damage are adult and larval stages of beetles and the larval stage of moths (Aspaly et al., 2007; Collins, 2012) (Abd El-Aziz, 2011; Hafiz, 1983). The presence of insect pests in grains causes quantitative loss due to direct feeding of insects which reduces grain weight, nutritional value and qualitative loss by contaminating the grains with insect excreta, pupal cocoons, dead bodies and odors (Collins, 2012). Many health problems are associated with insect infestations such as allergies, diarrhea and others (Arbogast et al., 2000; Athanassiou et al., 2017). Some of these pests act as vectors for aflatoxin producing fungi (Hell et al., 2000). Other insects have been involved in the transmission of pathogenic bacteria such as *salmonella* and *Enterococcus* spp. (Crumrine et al., 1971). Animals also may be affected if their feed were contaminated with insects and mites (Channaiah et al., 2010; Hell et al., 2000).

Visual inspection, sampling and sieving are widely used for insect detection in grains (Aspaly et al., 2007). Other methods were developed to detect hidden infestations including staining of kernels to detect eggs, density separation (Shi et al., 2016), x-ray micro-tomography (Toews et al., 2006), acoustical sensors technique (Mankin et al., 2010), near infrared spectroscopy (Perez-Mendoza et al., 2005), Enzyme-Linked Immuno-sorbent Assay (ELISA) (Dunn et al., 2008) and uric acid analysis (Wehling et al., 1984). The accuracy of these methods depends on insect species, their developmental stage and grain type (Abels and Ludescher, 2003; Dasmahapatra, 2010).

Molecular techniques were widely used to detect viruses, bacteria, fungi and insect pests with significant rapidity, reliability and allowed for large-scale analysis of multiple samples (Nowaczyk et al., 2009). This approach allows the detection of primary pests inside grain kernels after oviposition and during the early larval stages based on DNA barcoding using short DNA sequences from known region of the genome as a reference sequence for species identification (Abels and Ludescher, 2003; Dasmahapatra, 2010). DNA barcoding emerged as a rapid method for insect detection and identification by comparing unknown sequences against DNA barcodes for known species via distance-based tree construction or alignment searching (e.g., BLAST) (Min and Hickey, 2007; Virgilio et al., 2012). The standard sequence used was mitochondrial *cytochrome c* oxidase subunits *COI* and *COII*, *cytochrome b* or ribosomal DNA (Dasmahapatra, 2010; Virgilio et al., 2012).

The main focus of this study is to develop a reliable and specific molecular test for the detection of insect pests in home stored grains, standardization of suitable treatment for grains before DNA extraction and optimization of a convenient and efficient DNA extraction method suitable for insects found in plant seed.

Materials and Methods

Samples

A total of 11 grain samples including: corn, groat, lentils, rice, wheat, corn flakes, chickpeas, cumin, sesame, barely and animal feed were collected from local wholesale grocery stores. Samples were chosen randomly from sacks that comprised of 500 grams of large grains (<0.5cm) and 250 grams of fine grains (>0.5cm). Positive control samples were consist of rice, flour and barely samples that were heavily contaminated with fully developed larvae and adult insect pests. Insects' larvae and adult stages collected from infested flour, rice, and barely were isolated for DNA extraction and species identification.

Sample preparation for DNA extraction

All samples were processed using two procedures. 1-Centrifugation washing method: where 10 grams of grains were transferred to 50 ml sterile plastic tubes containing 20 ml distilled water followed by mixing for 2 minutes, then 10 ml of the turbid water were transferred into empty 50 ml plastic tube and centrifuged for 10 min at 4000 rpm. The supernatant was discarded and the pellet was collected for DNA extraction. 2-Filtration washing method: 50 grams of grains were transferred to sterile 100-200 ml glass beakers (according to grain size) containing 70 ml sterile distilled water, mixed for 2 min, then 40 ml of the turbid water were drawn and filtrated through 47 mm diameter and 8μm pore

size nitrocellulose membrane filters (Whatman Inc, Piscatway, NJ) using a vacuum filtration system. The membrane filters were left at room temperature to dry, punched and 4 small disks (0.5cm diameter) were taken for DNA extraction.

DNA extraction

The pellet or the filter discs were incubated in 1.5 ml tubes with 200 µl lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% triton hours. Equal volume of TE-saturated phenol (pH 8) was added to the aqueous solution, vortexed for few seconds and then centrifuged for 2 minutes at 14,000 rpm. The upper aqueous layer was transferred to new tube and DNA was precipitated by 0.2 M NaCl and 2.5 volumes of 100% cold ethanol. The mixture was incubated overnight at -20oC and centrifuged at 14,000 rpm for 10 minutes. The DNA pellet was left to dry then it was suspended in 100 µl of sterile double distilled water and stored at -20°C until further use.

Primers Design

At the beginning; three different PCR systems (COI-PCR1, COI-PCR2, and COI-PCR3 were designed that amplify *cytochrome oxidase I* (*COI*) gene of Diptera insects (Table 1), these PCR systems were used to amplify *COI* gene from extracted DNA of larvae and adult insects isolated from rice, flour and barely infested samples. The amplification products were subjected for DNA sequence analysis for pests species identification using BLAST generated comparison with their original *COI* gene DNA sequences. Based on new *COI* gene DNA sequence of the identified pests, new primers were designed from regions of highly conserved sequences of *COI* with the assumption to amplify *COI* gene from many other pest species (Table 1).

Polymerase Chain Reaction (PCR)

The amplification reaction was carried in 25 µl final volume using 2x concentrated green-*Taq* DNA polymerase (Thermo-Fisher, USA), 15 pmoles of each primer (reverse and direct), 2 µl of the original DNA extract or 5µl of the 1:10 diluted sample. The amplification protocol was run as follows: 5 min at 95 ^{o}C followed by 35 cycles of 30 seconds at 95 ^{o}C, 30 seconds at 50 ^{o}C, 1 min at 72 ^{o}C, and a final elongation step at 72 ^{o}C for 10 min. The amplified DNA fragments were resolved on 1.5% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA).

DNA Purification and DNA sequencing

PCR amplified DNA fragments were purified by PCR purification kit (Qiagene, Germany) according to the manufacturer instructions. The purified products were sequenced based on dye terminator method, using an automated DNA Sequencer machine (AB477).

Table1:DNA sequence information of the used primers

PCR system	Primer Direction	Primer sequence (5’-3’)	Amplicon size(bp)	Tm (c°)
COI-PCR1	Forward	TCATAAAGATATTGGAACTTTATAC	750	53.1
	Reverse	GATGTCCAAAAAATCAAAATAAAT		50.7
COI-PCR2	Forward	GGAACTGGGTGAACAGTTTATCCCCC	350	66.4
	Reverse	ATGTTGATAAAGAATAGGATCTCCTCC		60.4
COI-PCR3	Forward	AATAATATAAGATTTTGACTTCTTCC	350	52.8
	Reverse	TATAGTAATAGCTCCAGCTAAAACTGG		52.8
COI-PCR4	Forward	ATTGGAGGATTCGGAAATTGA	456	52.0
	Reverse	CCTCCTGCTGGATCAAAAAA		55.5

DNA Quantification

DNA positive controls were quantified using a *NanoDrop* instrument (Thermo Fisher scientific Inc, Waltham, Massachusetts, USA). Most of the positive control DNA samples were in the range between 50 to 300 ng/µl while the DNA extracted from grain samples ranged between 400 ng to 2 µg/µl.

Results

Preliminary screening of insect grain pests

DNA was extracted from isolated adult insects infesting barely and flour samples (Figure 1), followed by PCR amplification using the three *Diptera* based PCR systems (COI-PCR1, COI-PCR2, and COI-PCR3).

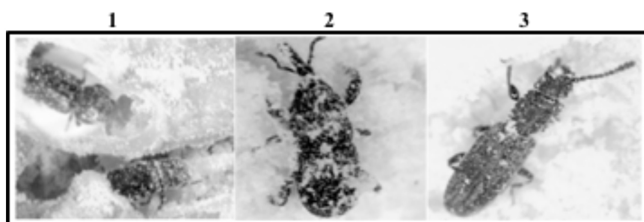

Figure 1: Different insect pest isolated from barely and flour infested samples which were identified according to their COI DNA sequence: (1) Barely: lesser grain borer (2) flour: granary weevil (3) flour saw-toothed grain beetle.

A successful PCR amplification was achieved by both COI-PCR1 and COI-PCR2 but not with the third PCR system (COI-PCR3) (Figure 2).

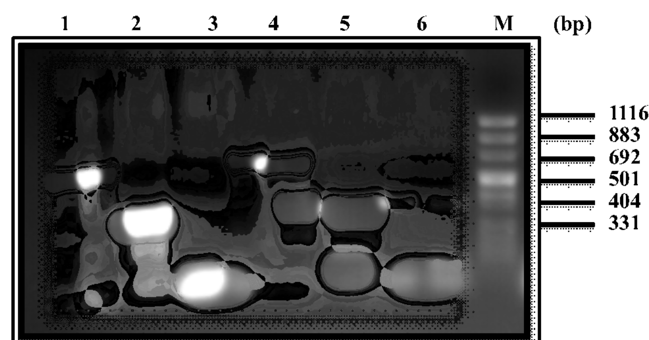

Figure 2: Agarose gel electrophoresis analysis of PCR amplified product of insect's DNA extracted from infested rice sample. 1 and 4: COI-PCR1, 2 and 5: COI-PCR2, 3 and 6: COI-PCR3. (M) Size marker.

DNA fragment about 700bp was amplified by COI-PCR1, and a fragment about 350bp was amplified by COI-PCR2. The amplified COI-PCR1 and COI-PCR2 DNA fragments from different unknown pests that were found in infested grains were sequenced. The sequence information was used to design more specific primers that are suitable to amplify *COI* gene from rice and grain pests (as described below).

Designing new specific primers

Based on the newly acquired *COI* DNA sequence information it was possible to identify pest type found in infested grain using BLAST sequence comparison. Table 2 shows a list of the major types of the identified pests and the similarity percentage to the obtained sequences. The DNA sequences of *COI* gene for the identified pests in table 2 were aligned using ClastalW2 software. The main purpose of this alignment is to identify a potential shared sequences for new primers that have the ability for a wider range of *COI* DNA gene amplification from many other pests species; and also a longer *COI* amplification that enables species identification after DNA sequence analysis. New direct and reverse primers that were used in PCR systems named: COI-PCR4 and COI-PCR5 were designed, these PCR systems amplify DNA segments of 456bp and 370bp respectively.

Table 2: Insect pest identified according to BLAST generated comparison

Pest scientific name	Pest common name	Accession number	Matching %
Plodia interpunctella	Indian meal moth	GU096541.1	99%
Oryzaephilus spp.	Grain beetle	KC407725.1	85%
Sitophilus spp.	Weevil	AY131101.1	76%
Rhyzopertha spp.	Grain beetle	KC407718.1	79%

Specificity analysis

The newly designed PCR systems (COI-PCR4 and COI-PCR5) were tested for their specificity. They did not amplify DNA extracted from insect free grains, plant leaves, and human DNA, even if 100 ng of DNA was used from each type of DNA (data not shown).

Sensitivity analysis

Serial dilutions ranged from 10ng to 1pg of DNA extracted from beetles were used to test the sensitivity of COI-PCR4 and COI-PCR5. It was clearly seen that COI-PCR4 is more sensitive than COI-PCR5; since it could amplify 1pg of the pests genomic DNA while the sensitivity of COI-PCR5 reached only to 10pg of DNA (Figure 3).

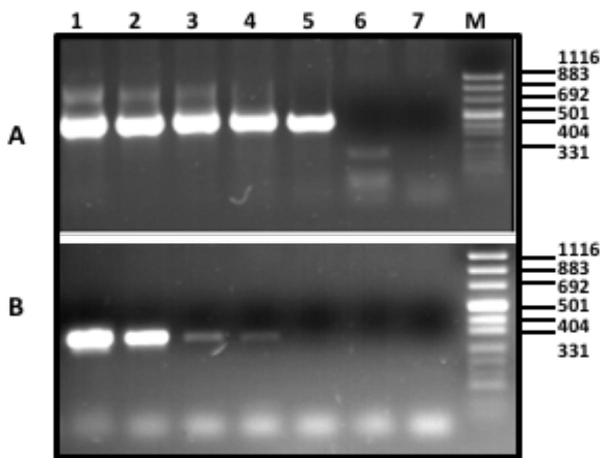

Figure 3: Sensitivity test of COI-PCR4 (A) and COI-PCR5 (B) targeting different concentration of beetle's pure genomic DNA isolated from barely. lane 1- 10ng, lane 2- 1ng, lane 3- 0.1ng, lane 4- 0.01ng, lane 5- 1pg, lane 6- 0.1pg, lane 7-Negative control, M: DNA size marker

Detection of pests in commercial collected grains

The two newly developed PCR systems (COI-PCR4 and COI-PCR5) were used to amplify pests' COI gene from 11 tested samples; after DNA extraction by filtration or centrifugation methods. PCR was performed with the extracted DNA and with DNA diluted 1:10. The results of the positive amplifications using both PCR systems with DNA extracted by the two indicated methods are summarized in table 3. COI-PCR4 system proved to be more efficient than COI-PCR5 in COI gene amplification from infected samples. Using this PCR system, it was possible to detect the presence of pests DNA in all 11 tested samples extracted by filtration washing method and after diluting 1:10. While using DNA extracted by the centrifugation method, it detects 10 out of 11 infected samples (Table 3, Figure 4).

The amplified COI fragment using COI-PCR4 system that target DNA samples from:

Table 3: Results of COI DNA amplification using COI-PCR4 and COI-PCR5 combined with centrifugation washing or filtration washing methods.

Extraction method	PCR system	Total positives original sample/ total	Total positives diluted (1:10)/ total
Centrifugation	COI-PCR4	6/11	10/11
	COI-PCR5	4/11	7/11
Filtration	COI-PCR4	9/11	11/11
	COI-PCR5	4/11	5/11

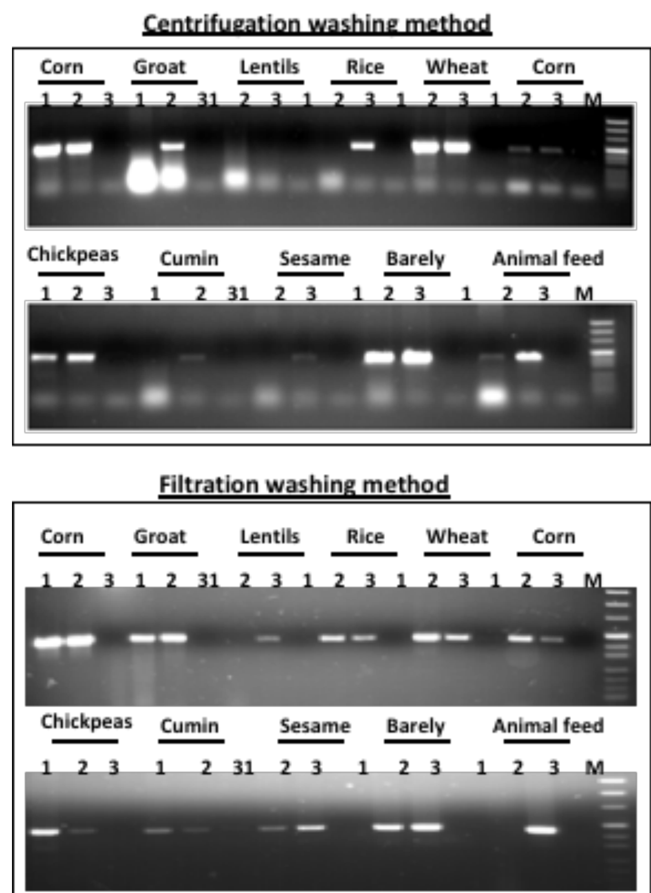

Figure 4: Agarose gel electrophoresis analysis of amplicons produced by COI-PCR4 primer system targeting DNA extracted from different types of grain samples by the centrifugation washing method (above) and filtration washing method (below). 11 samples were tested using three PCR reactions: 1- undiluted DNA, 2- diluted 1:10, 3- No DNA control. M: DNA size marker (bp).

corn, chickpeas, animal feed, rice and wheat extracted by centrifugation methodwere sequenced for species identification, the obtained

sequences were identified according to BLAST generated comparison as shown in table 4.

Table 4: insects identified according to BLAST generated comparison (amplification using COI-PCR4)

Pest scientific name	Source (sample)	Accession number	Matching %
Rhyzopertha spp.	Corn	KC407718.1	89%
Rhyzopertha spp.	Chickpeas	KC407718.1	74%
Samea spp. (*lepidoptera* spp.)	Animal feed	HM905018.1	84%
Rhyzopertha dominica	Rice	KC407718.1	99%
Rhyzopertha dominica	Wheat	KC407718.1	99%

Discussion

During shipping or storage periods grains are subjected to be infested by insect pests and mite, which cause economical losses by reducing grains quantity and quality. Hence, it is very important to adapt a sensitive method in order to detect insects at an early developmental stage or egg stage and implement control measures for the elimination of these insects and thereby reducing the loss of grains (Ngom et al., 2020). Insects can be found in many food components that if stored in cool dry conditions can slow further developmental stages that are hardly detected by the naked eye (Barrozo, 2019). In addition, many edible products are included in processed food as grind and milled materials infested with many types of insects that are hard to identify based on macro- or microscopic examinations (Abels and Ludescher, 2003; Hubert et al., 2018). Therefore, it is very important to develop a reliable sensitive diagnostic test that overcomes most classical insect detection methods even after processing. Consequently, detecting insects' genetic material provides a sensitive and specific examination tool for food and grain pests.

Previous studies using molecular approach dealt with one or two insect pests but lack the ability to detect several species of insects from different orders (Dasmahapatra, 2010). Our investigation focused on applying PCR amplification based on defined regions in the insects *cytochrome oxidase I* gene using general *COI* primers that are more specific for dipteral insects. Using this strategy, it was possible to detect and eventually identify different insects species in stored grains. DNA detection methods based on mitochondrial DNA is characterized by high sensitivity because of its many copies in the cell and approximately all mitochondrial genes sequences are known (Min and Hickey, 2007). The mitochondrial genes *COI* and *COII* subunits were used in standard molecular techniques to detect granary weevil in wheat flour (Ahrens et al., 2007). The DNA sequences of the amplified DNA fragments facilitated the design of more specific and sensitive amplification PCR systems for specific identification of insects in various grain samples.

Direct DNA extraction from grain samples by adding lyses buffer was not possible and several attempts were tried to overcome this problem, including the use of small sample size followed by short time incubation at high temperature for a quick extraction step. However, adapting this method for DNA extraction from plant or food materials for the purpose of insect examination was terminated since the small sample size can be misleading and many insect positive samples can be missed. These trials lead to the adaptation of two extraction procedures: 1- centrifugation washing and 2- filtration washing methods. Both protocols did not involve the addition of lyses buffer for long time in the presence of grain. This was achieved using a washing step with distilled water; and then collecting the wash water that contains particles; dust, eggs and fragments that were re-suspend in the lyses buffer. The filtration method relied on insect particles that will be returned on filter membrane with a pore size smaller than the smallest known pest egg (Wilson et al., 2003). Insect pest egg size ranges from 0.24-0.72 mm and the used membrane filter has a pore size of 8 μm. Another important complication was resolved after adopting these methods namely the dramatic reduction of plant DNA and proteins that may interfere in insects *COI* DNA amplification (Church et al., 2019).

All positive controls of insects DNA used in

our study were extracted from whole insects or their larvae that were directly removed from infested samples. Although it was possible to use previously identified *COI* DNA sequences for designing specific primers, we decided to have direct *COI* DNA sequences from our collected samples to identify specific types of insects isolated from local home or stores of grain samples (Min and Hickey, 2007). The developed COI-PCR4 and COI-PCR5 proved to be insect specific and did not amplify plant or human genomic DNA. These two systems also proved to be very sensitive, since it was possible to amplify 1pg of insect DNA template reflecting the sensitivity limits of these two systems. This sensitivity is equivalent to the detection of one egg found in 10 grams of grain sample (Ahrens et al., 2007).

Using classical PCR amplification, it is not possible to have quantitative results based on a single reaction. A quantitative procedure is needed to determine the insect accepted threshold level in grains according to the Federal Grain Inspection Service (FGIS) in the United States, which determines the number of insects allowed in grains (Fang et al., 2002). The combination of COI-PCR4 system and filtration system was sufficiently sensitive to detect even lower quantities than the allowed threshold level. Eventually, adapting these primers in a real-time quantitative PCR protocol would be even more sensitive for insect level detection.

The current developed molecular approach to detect insect pests in samples of stored grains were tested on eleven samples randomly collected from local stores. These samples were not purposely contaminated by insect pest for research purposes like previous studies (Hubert et al., 2018; Perez-Mendoza et al., 2005) but rather were processed as described using both centrifugation and filtration methods. The results based on using COI-PCR4 system in combination with centrifugation method shoed that ten of eleven collected grain samples were found to be positively infested. The results included positive amplification from corn, wheat, chickpeas, barely which gave very strong bands using the original and diluted samples, groat, rice and animal feed also gave strong bands for diluted samples only, lentils gave negative results whether using diluted or undiluted samples. The results indicated that using the filtration method for DNA extraction was more efficient and gave better results with the CO1-PCR4 system than the centrifugation method.

In conclusion, the data shown in this study represents a major step for the establishment of a rapid, sensitive and reliable molecular method for insect detection infested grains based on specific genetic marker information. Further work is needed to optimize the method for the specific identification of the various insects and develop a quantitative assay for the assessment the degree of contamination with the early developmental stages of insects for proper handling of contaminated grains. Furthermore, efforts will be directed to develop a multiplex test based on the present results for the detection of the vast majority of insects species known to infest grains. Eventually, this technique will hopefully open the way for the adoption of a national program to screen all imported and locally stored grains for periodic inspection to ensure the safety of grains for human consumption and prevents losses that can have a significant economical impact. Definitely, detection of insects contamination in grains at early stages will allow early interference to ensure the eradication of all contaminants utilizing effective and reliable methods that are used around the world for this purpose.

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