DEVELOPMENT OF A PCR BASED PROTOCOL FOR WSSV SCREENING FOR MAJOR CRUSTACEANS INHABITING IN CULTURED SHRIMP FARM

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ABSTRACT

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The array of prevalence of white spot syndrome virus (WSSV) in cultured tiger shrimp, Bagda (*Penaeus monodon*) and non cultured *Metapenaeus monoceros* & crab, *Scylla serrata* from the coastal region of Bangladesh was determined by a two-step polymerase chain reactions (PCR)-based screening protocol using two set of oligonucleotide primers. The product of the first-step PCR of total genomic DNA isolated by a single-step DNA preparation without involving hazardous organic solvents from the muscle and hepatopancreas of sample from apparently diseased free pond and diseased pond served as the templates for the second-step PCR. The ability of identify a single piece of target DNA with high specificity, easiness, and cost-effectiveness of this method do not depend on using a patented WSSV detection kits as in elsewhere in the country. Up to date this is the first scientific report on applying PCR-based WSSV screening protocol in a diversity of species in Bangladesh. The ground-breaking works in Bangladesh and the results suggest that a PCR screening of WSSV infection for shrimp brood and larval stocks and other potential virus carriers could be an effective tool in battle against the fatal virus that has almost devastated the shrimp commerce of the country.

Key words: White spot, virus, DNA, primer

INTRODUCTION

Outstanding knowledge on the wide spreading of WSSV (white spot syndrome virus) over a farm, broadly over an area are the initiative against this destructive virus that could restrict a huge shrimp production loss (Hossain, 2001) finally could retain the overall economic sustainability of a farm. A recent major outbreak of WSSV infection in China, Japan, Taiwan, Bangladesh, Thailand, India (Otta *et al.,* 2003; Cen, 1998; Zhan and Wang, 1998; Pongmaneerat *et al.,* 2001; ASCC, 1996), resulted in a high reduction in shrimp production, has raised major concerns in aquaculture around the world. WSSV is a member of a new virus family (Van *et al.*, 2001) with high virulence (Yoganandhan *et al.*, 2003) first discovered in Taiwan in 1992 (Chen 1995), its vituperative nature contains a wide spreading host range as crayfish , *Penaeus monodon, Macrobrachium rosenbergii, Metapenaeus monoceros*, crabs (eg., *Scylla serrata)*, copepods, lobsters etc (Lo *et al.*, 1996a; Supamattaya *et al.*,1998; Cai *et al.*, 1995; Takahashi *et al.*, 1994; Peng *et al.*, 1998; Wongteerasupaya *et al.*, 1995); multi transportation mode as both vertical (Lo *et al.* 1999) and horizontal (ASCC, 1996) transportation since it can transmit through brood to offspring, plankton to shrimp, food prepared from infected animals to cultured shrimp.

Due to the lack of methods to cure its infection; no alternative(s) if infect once, emphasize should be intense on its prevention by lay outing its presence and the level of infection (Hossain, 2001). WSSV takes some time to express itself but once after its expression, infected animal die within three to eight days resulting high mortality (Corbell *et al.*, 2001). Expression of WSSV infection furnished with some characters as white to reddish to discoloration(Corbell *et al.*, 2001; Chou *et al.*, 1995) over the head and carapace, low appetite, gather near the embankment etc.; those are not distinct from some other viral infection as the infection of bacterial white spot syndrome (Wang *et al.*, 2002). WSSV is a completely known virus with regard to its proteomic analysis (Canhua *et al.*, 2002), genomic studies (Yang *et al.*, 2001) the morphological characteristics (Chou *et al.*, 1995), the general biological properties (Wongteerasupaya *et al.*, 1995; Lo *et al.*, 1996b) and developed diagnosis method. Though it is a recognized virus in sense of its known host range, molecular knowledge and diagnosis method, its virulent nature and unexpected effect leads to early dead overshadow the mentioned success. Hence require a rapid and sensitive diagnosis protocol that will be able to screen the virus in its various hosts at every life stage. There are various diagnosis developed by Durand *et al.*,1996; Takahashi *et al.*, 1994 ; Kim *et al.,* 1998; *et al.*, 1997; Corbell *et al.*, 2001; Tapay *et al.*, 1999 among them PCR is the most rapid, sensitive (Lightner and Redman, 1998; Lo *et al.,* 1996c), specific (Tan *et al.*, 2001) and reliable method (Tang and Lightner, 2000).

The present experiment was conducted using PCR protocol accompanished with a simple procedure consists of isolation of DNA, PCR preparation then subjected to polymerase chain reaction in a thermal cycler followed by 35 cycles. The competitor and the target DNA in a polymerase chain reaction are similar in size and share the same primer recognition sites, thus ensuring similar amplification efficiency for both (Gilliland *et al.*, 1990). Thus PCR procedure can quantify very low amounts of nucleic acid, more accurately it can identify a single piece of target DNA if present in the sample.

The present study was therefore carried out to optimise the screening protocol in a wide host species spectrum (*P. monodon, Metapenaeus monoceros* and *Scylla serrata)* in Bangladesh context with regard to on-farm preparation of their tissues for rapid extraction of DNA, optimal thermal cycling condition and ingredient compositions for PCR. The simplicity and cost-effectiveness of this protocol rely on using a single-step DNA preparation without involving hazardous organic solvents as well as two pairs of oligonucleotide primers highly specific for WSSV gene. PCR protocol used in this experiment shows an admirable result as virus was detected both in nested and non-nested reaction and in all sample in a short period of time illustrating efficiency to investigate into a wide host range that could be pioneer for the farmer.

MATERIALS AND METHODS

Sample

Adult tiger shrimp, bagda (*Penaeus monodon), Metapenaeus monoceros* and *Scylla serrata* from diseased (animals containing external disease symptoms and being death for some reasons) pond and apparently disease free pond (having no external disease symptoms) were collected from Shamnagor under Shatkhira district, a remote area of coastal region of Bangladesh devoted to viral diseases endlessly. Moreover, animals of diseased pond investigated as infected and uninfected sample. About 100 mg each of muscle and hepatopancreas of selected animals was dissected out by sterilized, separate surgical blades to avoid cross contamination. The 18 dissected tissue samples were kept in 18 correspondingly labelled 1.5-ml tubes containing 500 µl of DNAzol, a guanidinium isothiocyanate (Cox, 1968) based buffer (Invitrogen, Life Technologies, USA) that enable a single step DNA extraction and transported in an ice bag to the laboratory for the extraction of total genomic DNA.

Extraction of DNA

Collected tissue samples flooded in DNAzol were homogenised by separate sterilised tissue grinder and centrifuged at 13,000 rpm for 15 min with a table top centrifuge machine (Eppendorf; model: 5415D). Four hundred micro litres of supernatant was transferred to fresh and new 1.5-ml tubes containing equal volumes of cold 100% ethanol. The two solutions were mixed by inverting the capped tubes for a few times and kept for 10 min to allow the precipitation of DNA. The mixture was then subjected to centrifugation at 13,000 rpm for 30 min. The precipitated pellet of DNA was obtained by removing supernatant alcohol. The DNA pellet thus obtained was washed with 500µl of 70% ethanol, kept for five minutes and centrifuged at 13000 rpm for another five minutes. Finally, the pellets of DNA were obtained at the bottom of tube by removing supernatant alcohol and were allowed to evaporate any traces of alcohol at room temperature followed by dissolving in 100µl sterile distilled water.

Quantification and qualification of extracted DNA

Absorbance at 260 nm using a spectrophotometer against distilled water blank was applied to determine the concentrations of DNA samples while their integrity was studied by agarose gel electrophoresis at 50 v for 40 min with a 0.5% agarose gel incorporating ethidium bromide at a concentration of 0.5 µg/ml in TAE buffer (40 mM Tris- Acetate & 1mM EDTA, pH 8.0) as described by Sambrook *et al.* (1989). DNA was visualized under UV illumination and the resulting banding pattern was captured by a digital camera. DNA size was determined by comparison with a DNA size marker (λ / *Hind* III • *Eco*R I double digest; Nippon Gene, Japan).

Design of primers

Two pairs of oligonucleotide primers of 22 bp and 21 bp named as LoF1, LoR1 and LoF2, LoR2 (PROLIGO Primers & Probes, Australia) specific for WSSV DNA sequences were used. These primers correspond to a cloned, 1461-bp, *Sal*I-digested WSSV genome fragment as described by Lo *et al.* (1996b). First pairs of primers, LoF1 and LoR1 designed to serve as outer primers targeted to amplify WSSV genomic DNA while the remaining primers serve as inner, nested primers that act on amplified DNA resulting from the first pair of primers. The nucleotide sequences of these primers are shown in Figure 1 with their estimated location and orientation with regard to a hypothetical WWSV genome.

PCR conditions

***Control reactions*:** In both PCR run, DNA extracted from *Penaeus monodon* reported to be WSSV positive by a commercial testing agent in Madras in India (personal comm.) used as positive control, whereas from a hypogenetically distant species walking catfish, *Clarias batrachus* were used as negative control template. Instead of any template DNA, reaction mixture with distil water were used as reagent control.

***First step PCR*:** 20-µl reaction mixture, a combination of appropriately diluted approximately 40 ng sample tissue DNA as the templates with 20 pmol of LoF1 and LoR1 primers, 20 nmol of dNTP mixture, 2 µl of 10× AmpliTaq™ buffer and 0.5 unit of AmpliTaq™ DNA polymerase (Roche Molecular System, USA) in 0.2 ml thin-walled PCR tubes were applied for each sample for PCR assessment using an automated thermal cycler (Mastercycler Personal, Eppendorf, Hamburg, Germany). PCR reaction times were 3 min at 94^0 C for the first round followed by 35 cycles of denaturation for 30 sec at 94°C, 10 sec of annealing at 58°C, and 1 min of extension at 72°C. The last extension step at 72°C was extended for 5 min. Following completion of the thermal

cycling, a sample of 10 µl from each amplified PCR products was analysed electrophoretically by running on a 1% agarose gel while preparing for the second round of amplification (nested PCR).

***Second step PCR*:** In this case, instead of tissue DNA an aliquot of 2 µl from the first PCR product was used as the DNA template together with the nested primer pair, LoF2 and LoR2. The rest of the PCR condition was the same as described above.

PCR product analysis: Aliquots of the PCR products were analyzed by 1% agarose gel electrophoresis visualised under UV transillumination and the amplified product size was determined by comparison with a 1 kb plus DNA ladder (Invitrogen, Life Technologies, USA).

RESULTS AND DISCUSSION

PCR based screening for the existence of WSSV in different crustaceans of shrimp farm starts with a vast spectrum intended to simple DNA extraction to DNA materials amplification. Total genomic DNA extraction accompanied with a single step DNA extraction reagent, DNA_{Zol} which talent to solubilize all cellular components yet allows selective precipitation of DNA in the presence of alcohol (Cox, 1968) also improves the release of DNA into solution. Resulted electrophoretic patterns of the extracted genomic DNA samples are shown in Figure 2 (A and B). As shown in the figure a DNA band at about 20 kbp position were produced by *Penaeus monodon*, *Metapenaeus monoceros* and *Scylla serrata* tissue samples with varying degree of intensity and smearing pattern. The smearing indicate the presence of numerous smaller size DNAs resulting from the mechanical shearing of the centrifugation process and pipetting involved. The smearing might contain cellular RNAs as well which, however, should not interfere with amplification of a target DNA virus.

The final yield of purified DNA ranged between 0.88 to 1.35 mg with the highest yield being from hepatopancreas of *Penaeus monodon* and the lowest from muscle tissues of *Scylla serrata*. The average $A_{260/280}$ ratio of the extracted DNA materials was 1.68 ±0.22, which was quite enough for semi-preparative diagnostic PCR (Sambrook *et al.,* 1989) because the PCR is so sensitive and requires little initial DNA templates thus very small–scale DNA isolation are suitable. Various protocols developed to extract DNA for the PCR detection of WSSV in animal sample by different workers (Lo *et al.*1996b; Nonaka *et al.,* 1998) include use of proteinase K and organic solvents such as phenol, chloroform and iso-amyl alcohol to digests cellular protein require 1 to 8 hrs. (Hoelzel, 1998). The present method of extraction using DNA_{ZOL} provides an advanced isolation method as because it solubilizes all cellular components yet allows selective precipitation of DNA in the presence of alcohol as well as the main ingredient of DNA_{Zol}, guanidinium isothiocyanate is a strong denaturant of proteins capable of dissolving cytoplasmic and nuclear membranes. Thus combines both reliability and efficiency with simplicity of the isolation protocol as well as it is a fast method and permits isolation of genomic DNA from a large number of samples in less than an hour following tissue grinding. Although there are an option to remove the interfering RNA molecules by an additional step of RNAse treatment followed by washing with ethanol. DNA isolated by this method can thus be used for applications such as Southern analysis, dot blot hybridization and molecular cloning.

In two pairs of used oligonucleotide primers, the outer primer pair (LoF1 and LoR1) amplifies a 1,447 bp fragment from the WSSV genome of 3, 05,107 bp while the second pair of primers (LoF2 and LoR2) amplifies a 941 bp internal sequence to the first set of primers. The electrophoretic patterns of PCR products using the first primer set are shown in Figure 3 (A and B). A 1,447-bp DNA band indicative of the presence of WSSV was observed only in the diseased pond sample and positive control sample (Figure 3, lane 1 through 9 of 'A' and lane 10 through 12 of 'B') suggesting moderate to high WSSV infection while rest of the sample tested were either free from the virus or the infection level was very low. The look of band in the positive control sample consequent to 1,447-bp did not result from PCR artifacts as no such band could also be obtained from the sample containing unrelated, vertebrate DNA as the template or from the sample lacking the template DNA. Subsequent amplification (nested PCR) of the first round PCR product as template with the nested primer pair, LoF2 and LoR2 followed by agarose gel electrophoresis demonstrated the presence of WSSV in all the tested samples including the positive control one, as evidenced by an expected DNA fragment of 941-bp with varying degree of intensity (Figure 4). Visualization of a specific DNA fragment corresponding to the expected 941bp in samples including the positive control but not in the negative control and reagent control (no template DNA) confirmed the existence of WSSV in the samples tested.

In this experiment, specificity, ability to amplify a conserved sequence and high sensitivity of PCR protocol was exhibited by the following way: 1) since no product was generated when the same primer set was used to amplify negative control sample in both non-nested and nested PCR; 2) Amplification of disease free pond samples (Figure 4, lane 13, 14, 15, 16, 17, 18 and 19 of 'B') WSSV DNA was only observed in the nested PCR reaction step; 3) no amplified product in the control reaction containing water instead of any template DNA. Tan *et al.* (2001) describe that only a single step PCR is sufficient to amplify WSSV specific DNA from moribund shrimp or shrimp having clinical signs of disease. In the present experiment, contamination of disease

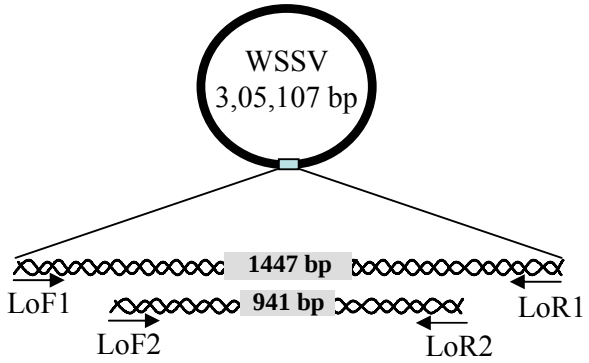
free pond samples with WSSV was only confirmed by a two-step (nested PCR) reaction as because the WSSV infection in this stage falls into light or carrier stage characterized by a low viral load of a few hundred viral copies, detectable only by sensitive methods such as nested PCR (Tan *et al.,* 2001). However, for healthy shrimp with no clinical syndrome a second round of PCR (nested PCR) is required to confirm the presence of virus as nested PCR has been observed to increase the sensitivity of detection of WSSV by 103 to 104 fold (Lo *et al.,* 1996a).

An investigation only into the dead/ dying or healthy shrimp with obvious sign of infection is of no significance as because the viral infection in this stage can not be quarantine. In viewing this fact the present experiment was aimed to establish the protocol that could be routinely applied to detect virus in various crustaceans of shrimp farm in all life stage as brood to offspring. Hence we purposively preferred apparently healthy shrimp (*Penaeus monodon*), *Metapenaeus monoceros* and *Scylla serrata* with moribund shrimp (*Penaeus monodon*), *Metapenaeus monoceros* and *Scylla serrata.*

The shrimp production region of Bangladesh has been believed to be a harbor of virus that causes crop devastation in a consistent and sporadic manner. Sampling in the present experiment covered a range as *Penaeus monodon*, *Metapenaeus monoceros* and *Scylla serrate* with healthy and declining conditions suggesting a complexity of infection in the sampling site. The complexity of infection suggesting that every investigated animal's type is highly vulnerable to WSSV which can be extended to other uninvestigated animals. The infection pattern as some in early or latent stage while rests are in highly infection stage, emphasis that the contamination time for two different ponds is not same and the route of contamination may be the brooders, larvae they used, food, water and plankton that served in a linked system.

To this date, the outbreaks nature and mortality pattern due to WSSV infection are of mysterious knowledge. Thus the health management attempt has become a chance and suggesting to invention of a revolutionary prevention and control methods to control the spread of the WSSV disease. Knowledge on the presence of virus in every possible invention route as post larvae, food, brood, supplied water, plankton as well as other inhabitant of shrimp farm will greatly help the farmer to understand the risk associated with stocking infected larvae and appropriate management measures for their culture. The PCR protocols described in this present communication for scrutinized judgment of viral presence should not dependent on the mortality rate and clinical signs but can be applied at any time will aid farm managers in decision making in culture management, perhaps allowing positive steps to be taken to reduce losses when WSSV strikes.

In Bangladesh, this PCR based WSSV screening protocol committed to be applied in a wide scale and the results suggest that a PCR screening of WSSV infection for shrimp brood and larval stocks and other potential WSSV carriers could be an effective tool in fight against the deadly virus that has almost devastated the shrimp commerce of the country.

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LoF1: 5´-ACTACTAACTTCAGCCTATCTA-3´
LoR1: 5´-TAATGCGGGTGTAATGTTCTTA-3´
LoF2: 5´-GTAACTGCCCCTTCCATCTCC-3´
LoR2: 5´-TACGGCAGCTGCTGCACCTTG-3´
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Figure 1. The nucleotide sequences of two primer sets with their estimated location and orientation with regard to a hypothetical WSSV genome.

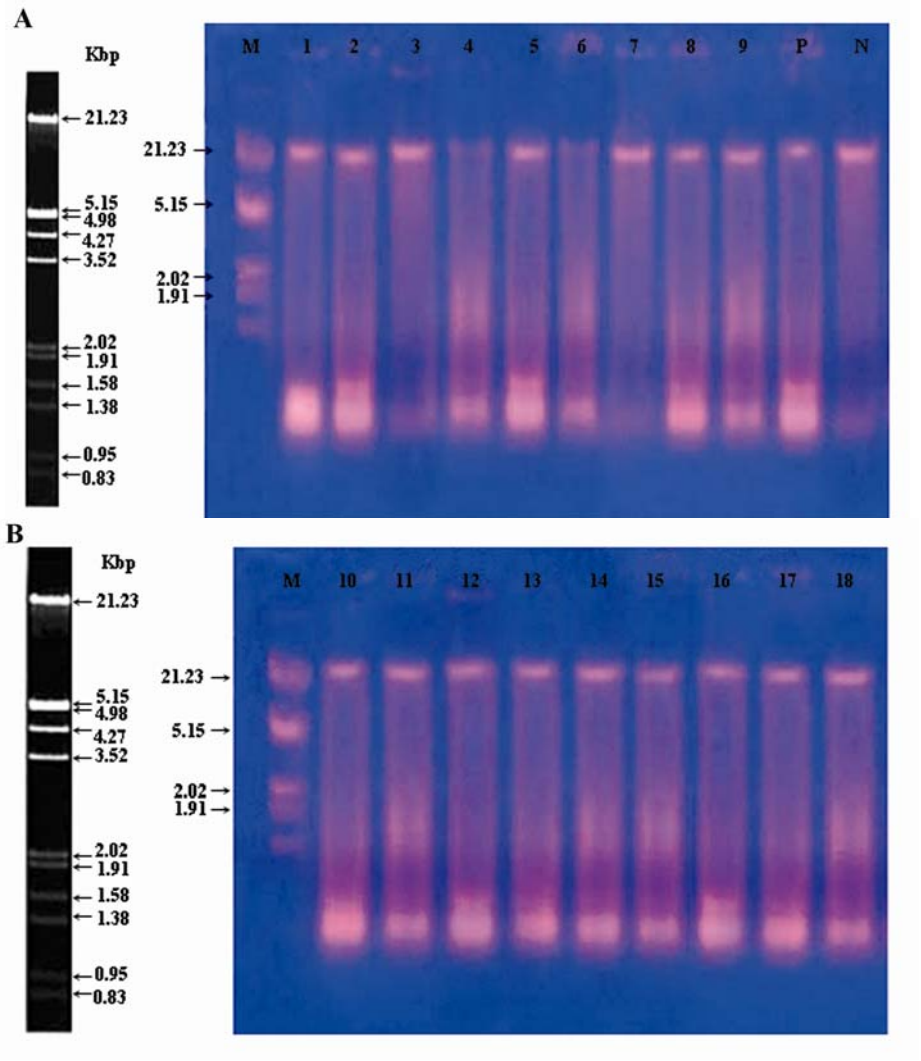

Figure 2. UV visualization of extracted total genomic DNA following electrophoresis in a 0.5% agarose gel. Lanes 1, 2, 3, 4, 5, & 6 of 'A' and lanes 7, 8 & 9 of 'A' and 10,11 & 12 of 'B' corresponded with the diseased pond infected and uninfected animals, respectively, therefore, represent the total DNA isolated from muscle and hepatopancreas of *Peneaus monodon*, *Metapenaeus monoceros* and *Scylla serrata* individuals, respectively. Lanes13, 14, 15, 16, 17 and 18 of 'B' analogous with the diseased free pond representing the total DNA isolated from muscle and hepatopancreas of *Peneaus monodon*, *Metapenaeus monoceros* and *Scylla serrata* individuals, respectively. Lane 'P' corresponds to control DNA isolated from shrimp diagnosed to be WSSV infected by a commercial testing agent in Madras, India while lane 'N' corresponds to that isolated from a phylogenetically distant species, *Clarias batrachus*. Lane M, *Hind* III • *Eco*R I double digest of λ DNA as a size marker.

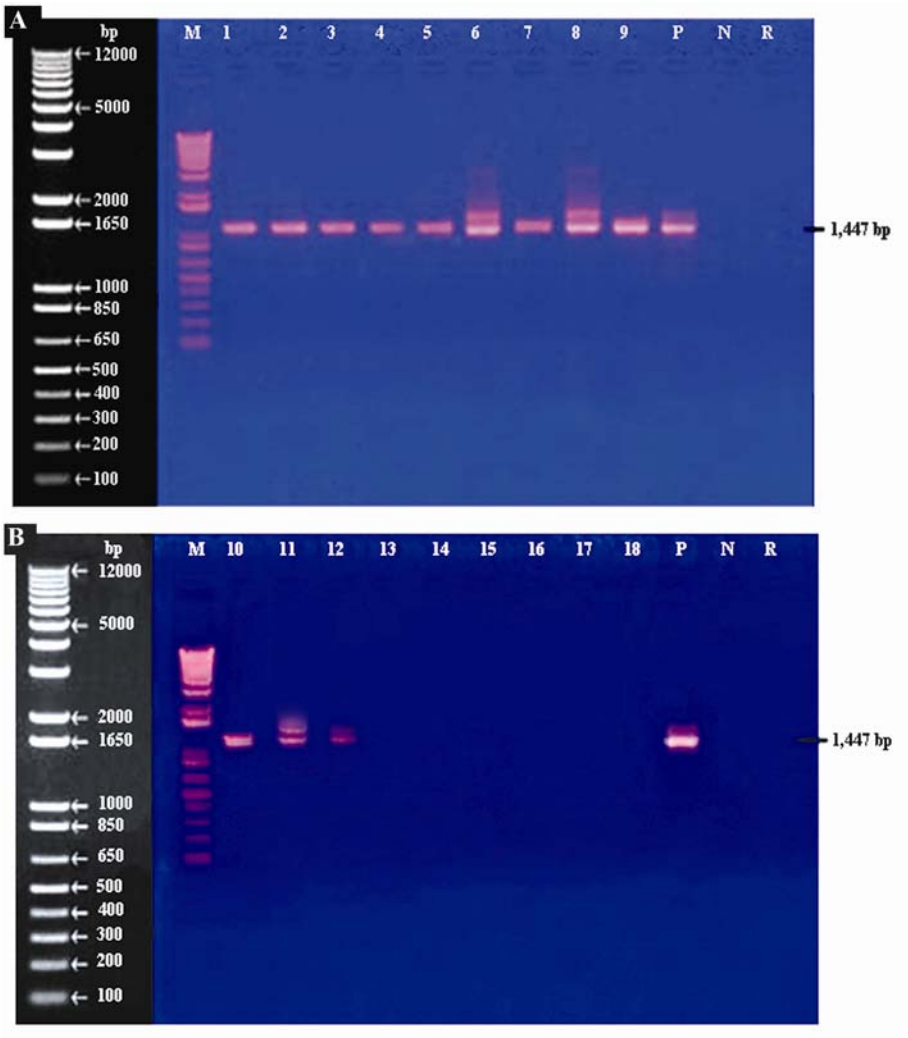

Figure 3. UV visualization of the first step PCR product following electrophoresis in a 1% agarose gel. Lanes 1 through N (A through B) represent samples as cited in Figure 2 while lane 'R' correspond to PCR product containing H_2O instead of template DNA. Lane M, DNA size marker

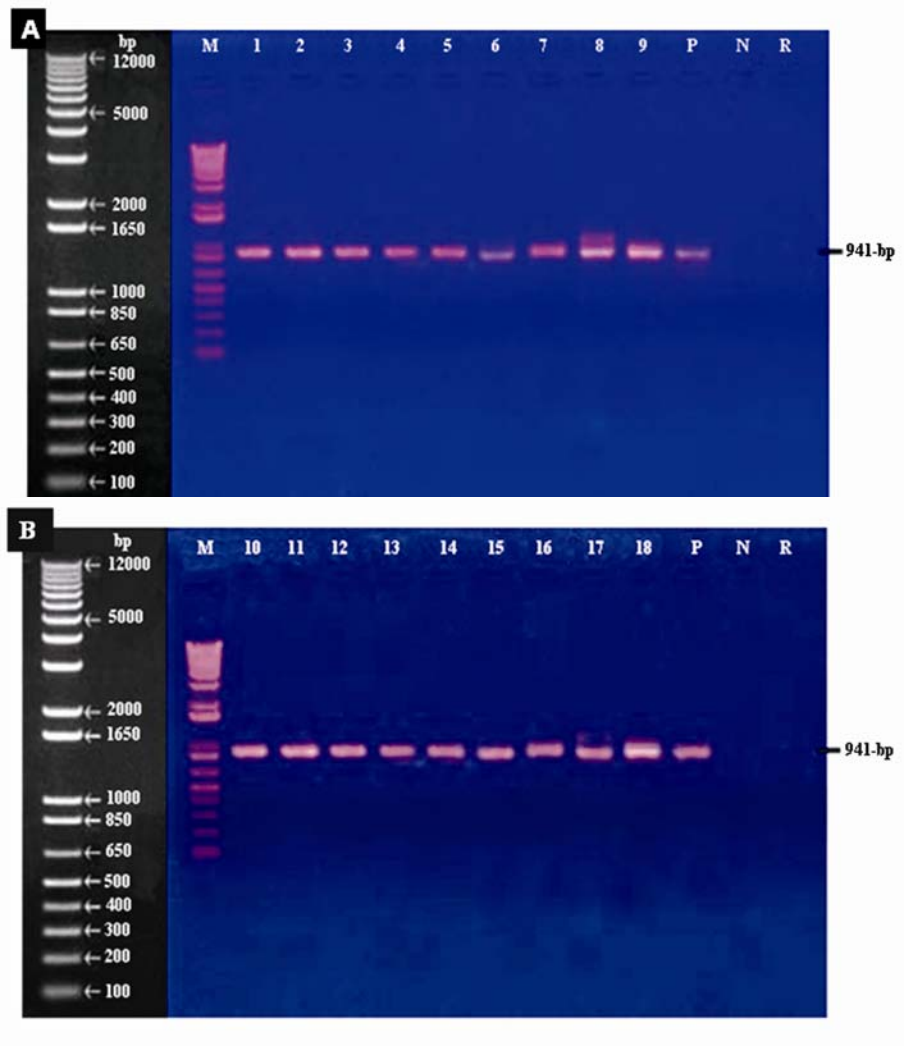

Figure 4. UV visualization of the second step PCR product (nested PCR) following electrophoresis in a 1% agarose gel. Refer to Figure 3 for description of the samples in each lane.

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