PAPER

The effect of gold nanoparticles on the diagnostic polymerase chain reaction technique for equine herpes virus 1 (EHV-1)

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Nano-biotechnology has been a noticeable research area because of its successful applications in molecular diagnostics and therapy of various genetic and microbial diseases. Although the polymerase chain reaction (PCR) technique is one of the most highlighted and promising applications in the molecular diagnosis field, it suffers from some drawbacks that affect its efficiency. For instance, as a diagnostic technique for equine herpes virus-1 (EHV-1), conventional PCR could lead to false negative results due to the low viral titer in some samples, which leads to the necessity to improve its sensitivity. In this study, we carried out experiments to determine the effects of 15 nm unmodified citrate-coated gold nanoparticles (GNPs) on the key PCR reactants in order to see if these would enhance the overall outcomes of the reaction. Our results showed that, after optimization of the GNPs, oligonucleotide primers and Taq polymerase concentrations, a specific high yield amplification with a detection limit of 10^2 DNA copies could be reached compared to the 10^5 to 10^4 detection limit of conventional PCR. Thus, the developed and optimized GNPs-assisted PCR technique could be used for a more efficient, highly sensitive molecular detection of EHV-1.

1. Introduction

The polymerase chain reaction (PCR) is a breakthrough technique that was first explored and improved by Kary Mullis in 1983.[1,2] It employs an *in vitro* DNA amplification system that mimics the *in vivo* DNA replication by the simultaneous primer extension of the complementary strands of DNA based on a set of repeated temperature cycles.[3] Nowadays, it has become one of the most important and reliable techniques in molecular diagnosis,[4] biotechnology, cloning,[5] fingerprinting,[6] microarray[7] and genetic analysis[8] fields. Although it is a very sensitive technique, it suffers from some specificity and efficiency drawbacks[9,10] as it constitutes an error-prone *in vitro* reaction that lacks an *in vivo* DNA replication control mechanisms, such as the single stranded DNA binding protein (SSBP), which results in a highly specific satisfactory replication.[11] To overcome these problems, some additives are added to the reaction mixture, such as dimethylsulfoxide[12] (DMSO), amidoamines,[13] bovine serum albumin[14] (BSA), dithiotheritol[14] (DTT) and glycerol.[14] Furthermore, novel modifications in PCR techniques have been developed to accomplish higher reaction efficiency, *e.g.* hot start PCR[15] and touchdown PCR.[16]

The evolution of the nanotechnology field since 1980 and interdisciplinary applied research have led to great applications in molecular biology and biotechnology. Recently, the addition of different types of nanoparticles to PCR has attracted significant attention due to their desirable chemical and physical properties.[17,18] Nanoparticle-assisted PCR, or the nano-PCR technique, has been investigated for enhancing the efficiency of the PCR technique.

Gold nanoparticles (GNPs) were first introduced as a new additive in PCR to avoid non-specificity even at lowered annealing temperatures[19] and to enhance the overall reaction. There are two hypotheses that exist that might explain how the enhancement occurs in PCR when GNPs are added: the first suggests that the enhancement occurs due to the surface interactions of the GNPs with the PCR reactants, while the other suggests that the enhancement occurs due to the heat transfer by the high conductivity and high heat ability of GNPs.[19,20] Until now the actual mechanism of the enhancement still a mystery under investigation. However, in related studies, it was reported that GNPs-assisted PCR could be used for the more sensitive diagnosis of various disease causative agents, such as some bacteria[21] and viruses.[22–24]

Equine herpes virus 1 (EHV-1) is a DNA virus that infects the equine species, with a worldwide distribution and where the infection can be subclinical or can cause serious economic loss in the equine industry as can cause sporadic abortion, neonatal death, equine herpes myeloencephalopathy (EHM) and upper respiratory diseases.[25]

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In the present study, we highlight the effects of 15 nm unmodified citrate-coated GNPs on the key components of the diagnostic PCR technique for EHV-1. The sensitivity of the developed GNPs-assisted PCR was determined in comparison to the conventional PCR technique in order to achieve a more efficient diagnostic tool for EHV-1.

2. Experimental section

2.1 Reagents

Hydrogen tetrachloroaurate(III)trihydrate and trisodium citrate dihydrate were purchased from Alfa Aesar (UK). EHV-1 vaccine was supplied from Duvaxyn® (Australia). EHV-1 oligonucleotide primers were purchased from Metabion (Germany). GoTaq® G2 Flexi DNA polymerase was purchased from Promega (USA). Agarose, Tris base, boric acid and EDTA were purchased from Sigma Aldrich (USA). GeneJET Genomic DNA Purification Kit was purchased from Thermo Scientific™ (Germany). Ethidium bromide was supplied from Serva Electrophoresis (USA) and DNA marker from GeneDirex® (USA).

2.2 GNPs synthesis

Gold nanoparticles were synthesized using the improved Turkevich–Frens synthesis method.[26] Briefly, after boiling an aqueous solution of 0.02% $HAuCl_4$ (50 mL) with rapid stirring, 5% sodium citrate (1.176 mL) was added under lower stirring velocity. The colour of the aqueous solution changes from pale yellow to grey, black, deep violet and finally to ruby red. After reaching the ruby red colour, the solution was boiled for an additional 2 minutes to ensure that there is no further colour change, then it was allowed to cool down to room temperature. The synthesized GNPs were characterized by a JEOL-JEM-2100 Transmission Electron Microscope (TEM) and BMG SPEC-TROstar Nano UV-visible spectrometer.

2.3 Preparation of DNA template

EHV-1 vaccine DNA was used as a template for the PCR techniques. The extraction of viral DNA was done using the GeneJET Genomic DNA Purification Kit according to the supplier manual. The DNA was eluted with 50 μL of elution buffer and its concentration and purity were measured with a BMG SPEC-TROstar Nano UV-visible spectrometer; then it was stored at −20 °C for further use.

2.4 Establishment of conventional PCR for EHV-1 diagnosis

To amplify the 188 pb species-specific fragment for EHV-1, a pair of oligonucleotide primers specific for glycoprotein B gene (Table 1) was used. PCR was carried out in a 15 μL reaction mixture composed of 5× Taq DNA polymerase buffer (3.3 μL), 10 mmol dNTPs (0.6 μL), Taq DNA polymerase (1.25 units), 10 pmol primers mix (1 μL) and DNA template (1 μL), then the reaction was completed to 15 μL with PCR grade water. The PCR program was run as follows: initial denaturation step at 94 °C for 5 min, then 35 cycles of: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; then, a final extension at 72 °C for 5 min. 6 μL of PCR product was loaded and migrated onto 1.5% agarose gel containing 0.5 pg of ethidium bromide dye. The gel electrophoresis was run under constant voltage (85 V) for 45 min. After electrophoresis, the gel was visualized, photographed and analysed by Bio-Rad Gel Doc XR documentation system. The size of the resulted bands was evaluated compared to 50 bp DNA markers. For the optimization of this conventional PCR, different annealing temperatures ranging from 55 to 60 °C and different primer concentrations ranging from 0.33 to 1.67 μM were used.

2.5 GNPs-assisted PCR optimization

2.5.1 GNPs concentration optimization. Different concentrations of GNPs (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2 nM) were introduced to the original PCR reaction under the same conditions and PCR program to highlight the general effect of GNPs on PCR yield and sensitivity. The PCR products were analyzed and photographed as previously described.

2.5.2 Primer, Taq polymerase and BSA concentrations optimization. Different primers, Taq polymerase and BSA concentrations varied from 0.167–1.67 μM, 1.25–7 units, 0.2–1 μg μL^{-1}, respectively, were added to the reaction after the optimization of the GNPs concentrations. The reaction was carried out using the same PCR program to estimate the effect of GNPs on each reactant. The PCR products were analyzed and photographed as previously described.

2.5.3 Sensitivity. To compare the sensitivity of GNPs-assisted PCR and conventional PCR, 10-fold serial dilutions of the template EHV-1 DNA were used.

2.6 PCR product quantification

Bio-Rad Gel Doc XR documentation system software was used to quantify each band intensity resulting from the migration of the amplified PCR product on 1.5% agarose gel. The measured band intensities were expressed in adjusted volumes (Int.).

3. Results and discussion

3.1 Characterization of the synthesized gold nanoparticles

GNPs sized 15 ± 3 nm were produced by the improved Turkevich–Frens method. The synthesized GNPs were indicated by the appearance of a ruby red colour. The TEM image showed that the synthesized GNPs had a relatively regular size with a spherical morphology (Fig. 1A). The UV-vis spectroscopy showed a λ_{max} specific peak at a wavelength of 521 nm (Fig. 1B).

3.2 Optimization of the conventional PCR technique

For better PCR outcome, the PCR conditions were optimized relative to the annealing temperature and primer concentration.
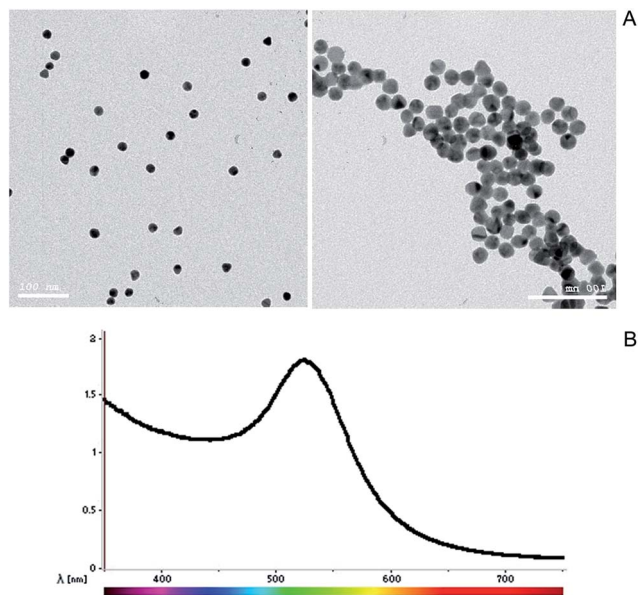
Table 1 Oligonucleotide primers used for EHV-1 specific fragment amplification by PCR

Name	Sequence (5′-3′)	Location	Product size	Ref.
FC3	ATACGATCACATCCAATCCC	gB 2699–2718	188 pb	27
R1	GCGTTATAGCTATCACGTCC	gB 2886–2867		

Fig. 1 (A) TEM image of the synthesized GNPs with a relatively regular size and spherical morphology. (B) UV-visible spectroscopy specific peak at a wavelength of 521 nm.

The effect of these factors was determined by the band intensity of the migrated PCR product on 1.5% agarose gel. The highest PCR yield was detected at an annealing temperature of 60 °C (Fig. 2A) and a primer concentration of 0.67 μM (Fig. 2B).

3.3 Effect of different GNPs concentrations on conventional PCR

To determine the best GNPs concentration that give the highest PCR yield, PCR was carried out with different GNPs
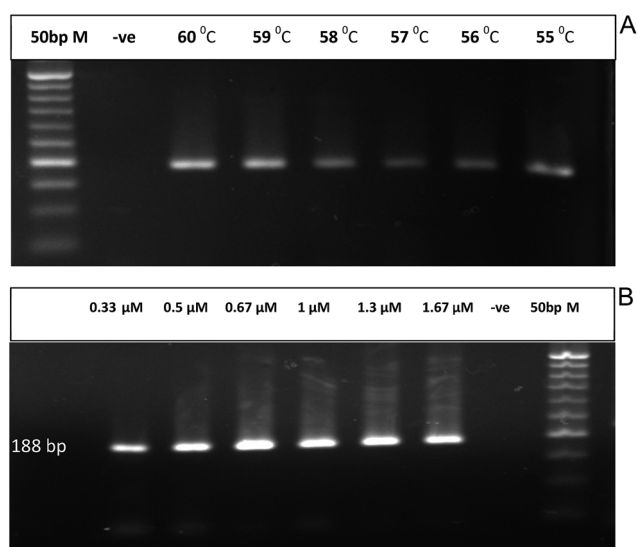

Fig. 2 (A) Conventional PCR amplification of 188 bp specific fragment for EHV-1 at different annealing temperatures ranging from 55 to 60 °C and compared to a negative control (−ve) and 50 bp marker. (B) Conventional PCR amplification of 188 bp specific fragment for EHV-1 at different primer concentrations ranging from 0.33 to 1.67 μM and compared to a negative control (−ve) and 50 bp marker.

concentrations ranging from 0.2 to 2 nM. The PCR yield is directly proportional to the intensity of PCR product band on the agarose gel. It was found that the optimal GNPs concentration in the PCR reaction was 1 nM, which had a 3-fold higher band intensity compared to a reaction without the addition of any GNPs (Fig. 3). The intensity of the band increases as the concentration of the GNPs increases up to 1 nM, then it starts to decline with higher GNPs concentrations such as 1.5 and 2 nM. Although the interaction between GNPs and the PCR reactants is not fully clarified yet, this enhancement may be due to: (1) the GNPs act like SSBP that is present in the DNA replication system, which increases the specificity; (2) the GNPs regulate PCR through their interaction with the polymerase enzyme; (3) the GNPs enhance PCR specificity through the adjustment of T_m in the annealing step; (4) the GNPs increase the product yield through allowing the efficient dissociation of the PCR products in the denaturation step. Hence, there is more DNA template for more PCR products.[28] It was found that an excess concentration of GNPs in the reaction could decrease its efficiency or even cause a total inhibition of the reaction.[19] In related studies, it was revealed that the total surface area of the GNPs is the main cause in the inhibition rather that their size.[29,30] The inhibition effect can occur with high concentration of GNPs where DNA polymerase can be strongly but not completely adsorbed on the negatively charged surface of GNPs, with their polar groups modulating the amount of active polymerases in the reaction. By this concept, GNPs will lower the PCR efficiency. This effect could be overcame by using higher concentrations of Taq polymerase or competitive proteins, such as BSA and thrombin, where both compete with Taq polymerase to bind on the GNPs
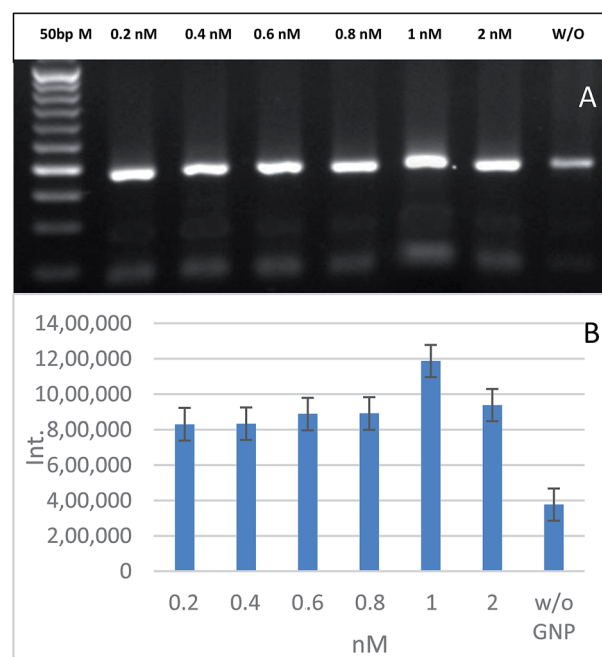

Fig. 3 The effects of different GNPs concentrations on PCR amplification of 188 bp specific fragment for EHV-1 compared to a reaction without (W/O) the addition of GNPs and 50 bp marker (A). Relative quantification of the PCR yield through measuring the band intensity (B).

surface through a Vroman-like effect.[31,32] These effects of GNPs on the PCR technique basically depend on the surface interaction with different reaction components, such as Taq polymerase, primers and products, where they are kinetically adsorbed and dissociated from the GNPs surface.[33] For more clarification on the effect of GNPs on PCR, more studies were performed on different PCR reactants.

3.4 Effect of GNPs on primer concentration

After the optimization of GNPs concentration in the PCR mix, we tested different concentrations of PCR primers, ranging from 0.167–1.67 μM, to highlight the effect of GNPs on the PCR buffer and primers. Fig. 4 shows that the optimal primer concentration to increase the PCR yield and specificity is 1 μM. Oligonucleotide primers, as short negatively charged DNA strands, interact with the GNPs surface due to adsorption–desorption kinetics as they replace the negatively charged citrate ions on the surface of GNPs. These single stranded DNA (ssDNA) sequences attach much more strongly than double stranded DNA (dsDNA) sequences to GNPs because of the easier bases exposure to the GNPs surface. The PCR primers attach to GNPs until they associate with their complementary sequences on the DNA template.[34] This adsorption causes a decrease of the melting temperatures (T_m) for both complementary and mismatched primers and increases the T_m difference between them. This may explain the improved specificity and yield of GNPs-assisted PCR. PCR buffer usually contains NaCl and MgCl$_2$, which are considered to cause GNPs aggregation, unless a certain amount of ssDNA is added into the reaction.[35] GNPs effects differ from one assay to another according to the different binding affinities of the nucleotides towards the GNPs

surface, where adenine shows a higher binding affinity for GNPs surfaces than thymine, while guanine and cytosine show a similar but less affinity to the GNPs surface.

Fig. 5 The effects of different Taq polymerase concentrations on GNPs-assisted PCR amplification of 188 bp specific fragment for EHV-1 compared to 50 bp marker (A). Relative quantification of the PCR yield through measuring the band intensity (B).
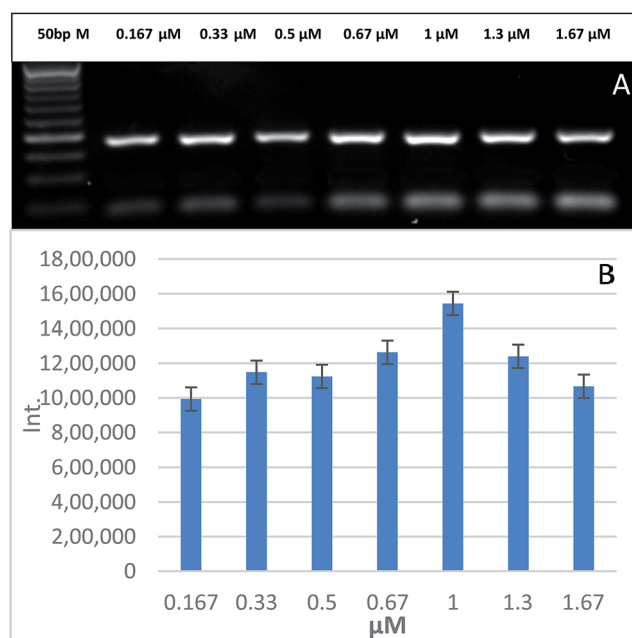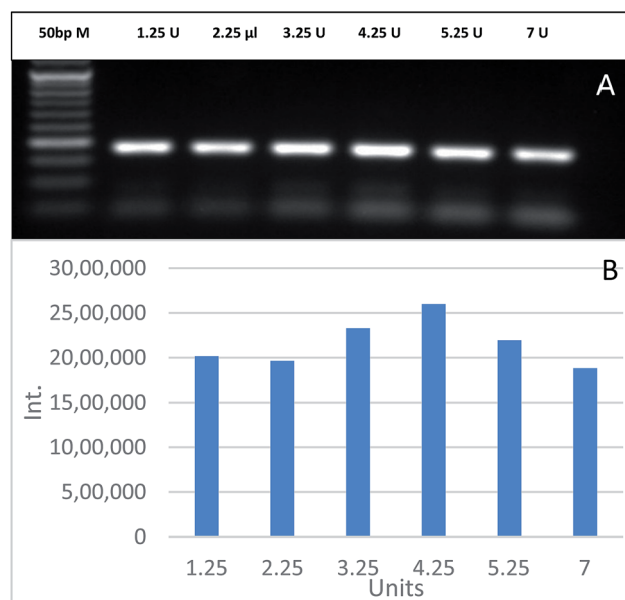

Fig. 4 The effect of different primer concentrations on GNPs-assisted PCR amplification of 188 bp specific fragment for EHV-1 compared to 50 bp marker (A). Relative quantification of the PCR yield through measuring the band intensity (B).
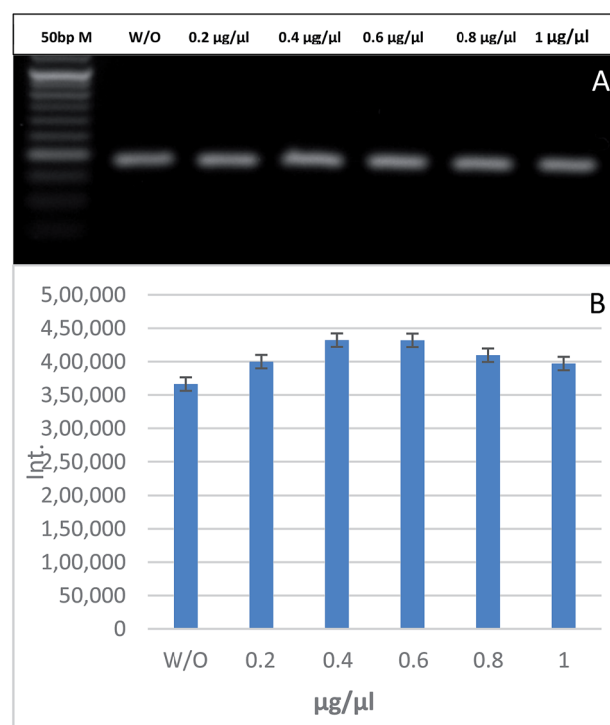

Fig. 6 The effects of different BSA concentrations on GNPs-assisted PCR amplification of 188 bp specific fragment for EHV-1 compared to 50 bp marker (A). Relative quantification of the PCR yield through measuring the band intensity (B).

3.5 Effect of GNPs on Taq polymerase

To evaluate the effect of GNPs on Taq polymerase, PCR was carried out using 1 nM of GNPs and different Taq polymerase concentrations ranging from 1.25 to 7 units. Fig. 5 shows that the optimal Taq polymerase concentration is 4.25 units. PCR yield increases as the units of Taq polymerase increases up to 4.25 units, then it declines with higher concentrations. This effect is due to the fact that an excessive concentration of Taq polymerase leads to an increase in the artefacts associated with its intrinsic exonuclease activity, resulting in producing unwanted DNA fragments or complete reaction inhibition.[36] The gradual increase in PCR yield up to 4.25 units was due to the compensation of the added Taq polymerase units for the modulated amount of Taq polymerase by GNPs. PCR efficiency enhancement by GNPs mostly depends on the type of polymerase used in the reaction.[37]

3.6 Effect of GNPs on PCR containing BSA

PCR was carried out using 1 nM of GNPs and different BSA concentrations. Fig. 6 shows that there is no significant change in the band intensity of the PCR products between the GNPs-assisted PCR reaction without BSA and with different concentrations of BSA ranging from 0.2 to 1 $\mu g \, \mu L^{-1}$. It is well known that BSA can improve the efficiency of conventional PCR.[14] It was hypothesized that BSA could be used to modulate the adsorption of Taq polymerase and act as a competitor for the GNPs surface to increase the efficiency of GNPs-assisted PCR. In this study, we found that the addition of BSA to GNPs-assisted PCR had very little effect on the PCR yield. This effect could be explained as the BSA is a protein with a low isoelectric point

(pI = 4.7) compared to Taq polymerase (pI = 6), while the typical PCR mix has a pH $\sim$ 8.3, which means that BSA retains more negative charges than Taq polymerase, leading to stronger repulsion with the negatively charged GNPs and DNA. Thus, less efficient adsorption on the surface of GNPs.[35]

3.7 Sensitivity of GNPs-assisted PCR vs. conventional PCR

To examine the GNPs-assisted PCR sensitivity, 10-fold serial dilutions of the template EHV-1 DNA were added to the PCR mixture containing 1 nM of GNPs. Fig. 7 shows a comparison between the sensitivity of GNPs-assisted PCR (A) and the sensitivity of conventional PCR (B), where a serial dilution of DNA template from 10^7 to 10^1 was used. The detection limit of the GNPs-assisted PCR is 10^2, which means that it can detect 100 copies of the target DNA, while the detection limit of the conventional PCR is 10^4, as determined through the experimental repetition. If the UV-trans illuminator is used to visualize the gel, the 10^4 dilution band cannot be clearly seen as a positive band and it needs the high resolution CCD camera of the gel documentation system to be recognized.

4. Conclusion

Every reactant added into the PCR mixture, including primers, DNA polymerase enzyme and BSA concentrations as well as DNA template and primer sequences, and also the size and surface modification of GNPs all affect the PCR efficiency. Consequently, it is important to evaluate these factors on a case-by-case basis as it will provide different results. In this study, the PCR technique was dramatically enhanced after the
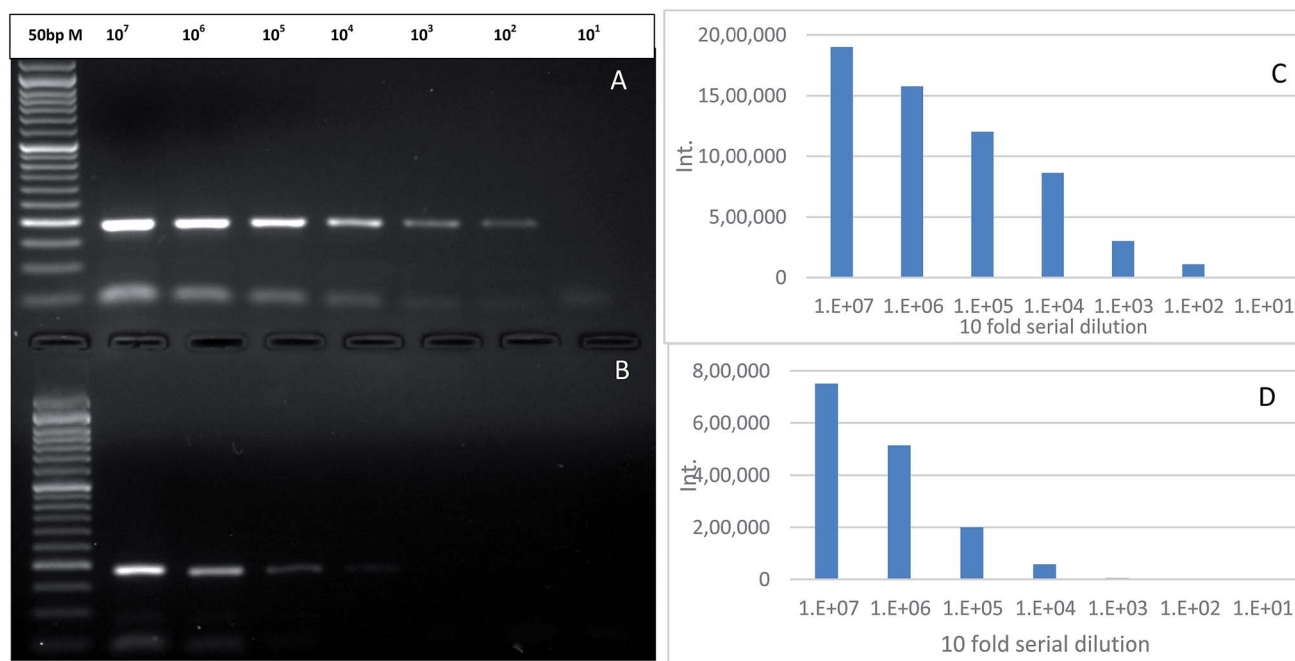

Fig. 7 Sensitivity of GNPs-assisted PCR (A) versus conventional PCR (B). Serial dilution of 10-fold of EHV-1 DNA was used. All experiments underwent a 3 times repetition and similar results were achieved. Relative quantification of the PCR yield through measuring the band intensity, for the serial dilution of the GNPs-assisted PCR (C) and the serial dilution of the conventional PCR (D).

optimization of the GNPs, PCR primers and Taq polymerase concentrations to 1 nM, 1 μM and 4.25 U, respectively. It was found that BSA had very little effect on the GNPs-assisted PCR technique. Moreover, the higher sensitivity of the GNPs-assisted PCR technique, with a detection limit of 10^2 DNA copies, allowed the detection of lower viral titer and therefore detection of the virus at an early stage of the infection. It also eliminates the necessity to carry out the two round PCR (nested PCR). We found that the developed GNPs-assisted PCR technique could be used as a highly efficient, sensitive and specific diagnostic technique for EHV-1 rather than conventional PCR.

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