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ORIGINAL ARTICLE

Application of gold nanoparticle-assisted PCR for equine herpesvirus 1 diagnosis in field samples

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Abstract Equine herpesvirus 1 (EHV-1) is one of the most significant pathogens that affects equine species worldwide, causing sporadic abortion, neonatal deaths, chorioretinopathy, as well as neurological and upper respiratory tract diseases. Currently, conventional PCR targeting different genes is used widely for the molecular detection of EHV-1, but the low viral titer in some clinical samples can lead to false negative results. In this study, we aimed to assess gold nanoparticle (GNP)-assisted PCR as an inexpensive, highly efficient, and sensitive method for the detection of EHV-1, and to compare its results with conventional PCR and real-time quantitative PCR (qPCR). Out of 83 field samples, 28.9%, 26.5%, and 15.6% were EHV-1-positive by qPCR, GNP-assisted PCR and conventional PCR, respectively. All three techniques specifically target the viral glycoprotein B gene. The optimized GNP-assisted PCR showed no cross-reactivity with EHV-1-negative samples (diagnosed by qPCR). GNP-assisted PCR is a powerful new tool for EHV-1 detection and surveillance, because of its simplicity, sensitivity and specificity. It can be used as an alternative to qPCR in laboratories that cannot afford the expense of a qPCR system.

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Introduction

The Arabian horse is one of the most popular, oldest and purest breeds worldwide. It is essential to investigate the existence and prevalence of equine herpesviruses (EHVs) that could infect this valuable horse breed. Equine herpesvirus 1 (EHV-1) is considered to be the most clinically and economically significant equine viral pathogen, as it affects reproduction and performance in races [1]. EHV-1 causes sporadic abortion, neonatal deaths, chorioretinopathy, as well as neurological and upper respiratory tract diseases. It is highly contagious, infects all domestic equine species, and can be transmitted easily through inhalation or ingestion of infected droplets from contaminated surfaces [2]. Inhalation of the virus causes damage to epithelial cells of the upper respiratory tract due to viral replication. The virus then disseminates through a CD8+ T lymphocyte-associated viremia into the endothelial cells of internal organs including the uterus, where it can cause abortion, or into the central nervous system (CNS), causing equine herpesvirus myeloencephalopathy (EHM) [3]. The infection can be latent or active. Established latent EHV-1 infection in the trigeminal ganglions may be reactivated through exposure of the horse to an appropriate stimulus, causing similar symptoms to the primary infection, without recurrence of viremia [4, 5].

The EHV-1 genome is a linear double-stranded DNA molecule of 150 kb that encodes 76 genes. Some of these genes are essential as they encode proteins that are crucial for virus replication and structural components, while the rest encode accessory proteins, which are not critical but contribute to viral survival in the infected host [6]. The glycoprotein genes are distributed throughout the EHV-1 genome. The envelope glycoprotein B (*gB*) plays a significant role in the virus entry process and its gene is the

most common target for polymerase chain reaction (PCR) diagnosis of, and differentiation between, EHV strains [7]. EHV-1 infection is usually difficult to diagnose based on clinical findings alone, even when a complete history is available and detailed regular clinical examinations are performed on equine farms [8]. This is because the virus induces symptoms similar to other viral and bacterial agents, for example, fever, coughing, nasal discharge, and variable enlargement of lymph nodes.

The gold standard laboratory diagnostic assay for EHV-1 is the virus isolation test [9], but its sensitivity varies depending on the cell lines used, as well as on storage and transportation conditions, and may result in false negatives. After this test, the isolated virus also needs to be confirmed by other techniques, even after indication of the cytopathic effect. Other widely used laboratory diagnostic methods are serological assays such as the virus neutralization test and enzyme-linked immunosorbent assay (ELISA). These are considered to be less useful tests as they detect pre-existing antibodies resulting from vaccination or previous infection with EHV-1, EHV-4, or both. Nucleic acid amplification-based (i.e. PCR-based) detection techniques for EHV-1 are the most reliable, because they neither require the presence of the infectious virus in the sample, nor are they affected by previous vaccination or infection. PCR techniques including conventional, nested, and real-time quantitative PCR (qPCR) are commonly used for molecular detection of, and differentiation among, EHVs [10, 11].

Because of the high specificity, sensitivity and accuracy of qPCR, many laboratories around the world use it for EHV-1 detection and quantification, especially for samples with low viral titer. However, the qPCR system and its reagents are expensive and the design of the primers and probes is laborious; thus, this technique may not be affordable in laboratories in developing countries. Conventional PCR requires inexpensive reagents and less laborious primer design, but it suffers from sensitivity and specificity drawbacks that affect the final outcome. Since the invention of PCR, different additives such as dithiothreitol, glycerol, and bovine serum albumin have been used to enhance the PCR efficiency [12, 13]. During the last decade, as the nanotechnology field has prospered and nanoparticles have been applied to the therapy and diagnosis of different diseases, gold nanoparticles have been added to PCRs to measure their effect on reaction efficiency [14]. Gold nanoparticle (GNP)-assisted PCR has been proven to have higher sensitivity in the diagnosis of various bacterial and viral agents than the conventional method [15]. There are two main hypotheses that explain the observed enhancement in PCR outcomes when GNP solution is added. The first suggests that the enhancement occurs because of interaction and adsorption of different PCR components onto the surface of the GNPs, and the other suggests that it may be due to heat transfer, as a result of the high conductivity of GNPs [16, 17]. Recently, it was shown that GNP-assisted PCR is 100–1000-fold more sensitive than conventional PCR [18, 19].

In this study, we evaluated the efficiency and sensitivity of GNP-assisted PCR for the diagnosis of EHV-1 in clinical samples. We compared the results with qPCR and conventional PCR assays.

Materials and methods

Samples and DNA extraction

In total, 83 clinically suspected samples were collected during the period July 2015 to July 2016, from different farms in Cairo and Giza, Egypt. Samples included 10 blood and 31 nasopharyngeal swabs from respiratory disorder cases, and 30 lung and 12 liver tissue samples from aborted fetuses. The samples were collected in aseptic conditions and transferred on ice to the Biotechnology Department, Animal Health Research Institute, Dokki, Giza. They were subjected immediately to viral genomic DNA extraction using a GeneJET Genomic DNA Purification Kit (Thermo Scientific™, USA), according to the manufacturer's instructions for each sample type. The viral DNA was extracted from whole blood without separating the white blood cells. The lung and liver tissues were weighed to 30 mg and directly added to the lysis buffer included in the extraction kit. EHV-1 inactivated vaccine (Duvaxyn®, Australia) was used as a positive control for validation of the assays. The extracted DNA from each sample was eluted in 50 µl of elution buffer and stored at −20 °C until it was subjected to PCR.

qPCR assay

The extracted DNA from the vaccine and collected samples were firstly subjected to EHV-1 detection using EHV-1 dtec-qPCR test Kit (GPS™, Spain). Amplification was carried out in a 10 µl reaction volume containing 2 µl of 5× Mixstable qPCR master mix, 0.5 µl of EHV-1 dtec-qPCR mix, 5 µl of DNase/RNase free water, and 2.5 µl of the template (either positive control, negative control or sample). The reaction was performed in an Applied Biosystems® StepOne™ qPCR System using the following conditions: initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s and extension/data collection at 60 °C for 60 s.

To determine the detection limit of the assay, three replicates of a 10-fold serial dilution, from 2×10^6 to 2×10^0 copies of positive control (supplied with the kit),

were applied to the PCR mix to establish a standard curve. Then, the DNA extracted from the vaccine and samples were tested and their cycle thresholds (Ct) compared to the standard curve.

Conventional PCR assay

Primers, that were specifically designed for a conserved region of the *gB* gene, were used to amplify a PCR product of 188 bp (Table 1) [20]. The primer set was checked for its specificity using the NCBI nucleotide database; it showed 100% identity for *gB* of EHV-1 and no cross-matching with genomic DNA of EHV-2, EHV-3, EHV-4, or EHV-5. Both conventional and GNP-assisted PCRs were performed using a GoTaq®G2 Flexi DNA Polymerase kit (Promega, USA). For conventional PCR, the 15 µl reaction mix consisted of 5× Taq DNA polymerase buffer (3.3 µl), Taq DNA polymerase (1.25 units), 10 mmol of dNTPs (0.6 µl), 10 pmol of primer mix (0.67 µM) and DNA template (1 µl); then the reaction was adjusted to 15 µl with PCR-grade water. The prepared mixture was then placed into a Bio-Rad MJ Mini™ thermocycler. The thermal profile used for PCR was: initial denaturation at 94 °C for 5 min; followed by 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; and a final extension at 72 °C for 5 min. The reaction mixtures were stored at 4 °C. The conventional PCR annealing temperature and primer concentration were previously optimized for ideal target amplification [18]. Equal volumes of the PCR products (6 µl) were migrated on 1.5% ethidium bromide-stained agarose gels, then photographed and analyzed using the BioRad GelDoc XR documentation system. The electrophoresis was run at a constant 85 V for ~45 min.

GNP-assisted PCR assay

GNPs (15 ± 3 nm) were synthesized using the Turkevich–Frens method [21] and characterized by transmission electron microscopy and UV-visible spectrophotometry to determine the mean size, dispersity, morphology and concentration (Online Resource 1). The synthesized GNPs were then added to the conventional PCR mixture at an optimized concentration (1 nM), as previously described [18]. The same thermal profile and thermocycler were used for the GNP-assisted PCR technique as in the conventional PCR (section "Conventional PCR assay").

Table 1 Primer set specific for *gB* gene of EHV-1

Name	Primer location	Sequence (5′–3′)
FC3	*gB* 2699–2718	ATACGATCACATCCAATCCC
R1	*gB* 2886–2867	GCGTTATAGCTATCACGTCC

Results

qPCR

qPCR detection limit

The detection limit of qPCR was determined by measuring 10-fold serial dilutions of EHV-1 positive control DNA of known copy number. The serial dilution from 2×10^6 to 2×10^0 copies was introduced into the qPCR mixture. The Ct detected for each copy number was used to establish a standard curve. The detection limit for the qPCR assay was 2×10^0 copies and the standard curve showed an inverse linear relationship between the Ct value and the DNA copy number with a slope of −3.51 and an R^2 value of 0.994 (Online Resource 1).

EHV-1 detection in clinical samples by qPCR

The qPCR technique was applied to detect and quantify viral DNA in the collected clinical samples. Samples were divided into four qPCR runs of 20 samples each, except for the last run which included 23 samples. The detected Ct value for each sample was compared to the standard curve for the determination of DNA copy numbers. Negative and positive controls were used in each run to ensure successful amplification. Ct values ≤35 were considered positive, while Ct values from 36 to 40 were considered suspect (requiring a retest using a different technique), and Ct values >40 were considered negative [22]. Out of 83 samples, 24 were determined to be positive for EHV-1 by qPCR. The EHV-1 positive samples included: 9 of 31 (26.6%) nasal swabs, 3 of 10 (30%) blood samples, 7 of 30 (23.3%) lung samples, and 5 of 12 (41.6%) liver samples (Table 2).

EHV-1 detection in field samples by conventional and GNP-assisted PCR

The 83 clinically suspected samples were divided into two groups according to the results obtained from qPCR (i.e., negative and positive groups) and both groups were subjected to diagnosis by conventional and GNP-assisted PCR. The negative group was used to determine the specificity of both techniques to diagnose EHV-1, while the positive group was used to determine the sensitivity and efficiency of both techniques for EHV-1 diagnosis. The samples found to be negative by qPCR all showed negative results when subjected to diagnosis by conventional or GNP-assisted PCR. Positive samples by qPCR were subjected simultaneously to conventional and GNP-assisted PCR in the same conditions, to ensure accurate evaluation of the results. These samples were numbered from 1 to 24 and equal volumes of PCR product (6 µl) from both techniques

Table 2 Sample type and qPCR, conventional and GNP-assisted PCR results

Sample no.	Sample type	qPCR Ct	GNP-assisted PCR	Conventional PCR
1.	Lung	21.4	+ve	+ve
2.	Nasal swab	26.1	+ve	-ve
3.	Liver	17.9	+ve	+ve
4.	Lung	23.0	+ve	+ve
5.	Liver	19.1	+ve	+ve
6.	Nasal swab	30.2	+ve	-ve
7.	Lung	24.5	+ve	-ve
8.	Nasal swab	35.9	-ve	-ve
9.	Lung	22.1	+ve	+ve
10.	Nasal swab	28.6	+ve	-ve
11.	Lung	21.4	+ve	+ve
12.	Liver	19.8	+ve	+ve
13.	Lung	23.1	+ve	+ve
14.	Liver	18.3	+ve	+ve
15.	Lung	20.8	+ve	+ve
16.	Nasal swab	31.1	+ve	-ve
17.	Nasal swab	26.3	+ve	-ve
18.	Liver	18.9	+ve	+ve
19.	Blood	25.3	+ve	-ve
20.	Blood	20.4	+ve	+ve
21.	Nasal swab	29.2	+ve	-ve
22.	Nasal swab	37.6	-ve	-ve
23.	Blood	18.7	+ve	+ve
24.	Nasal swab	27.1	+ve	-ve

Notes: (+ve) indicates a positive result, while (-ve) indicates a negative result. All organ samples were from abortion cases, all nasal swabs and blood were from respiratory disorder cases

were migrated on 1.5% agarose gels (Fig. 1). Conventional PCR detected EHV-1 DNA in only 13 samples, while GNP-assisted PCR detected EHV-1 DNA in 22 of 24 samples (Fig. 1). Among all the positive samples identified by both techniques, the band intensity appeared to be brighter for the PCR product resulting from the GNP-assisted PCR than that from the conventional PCR. Both techniques detected EHV-1 in the five positive liver samples due to the high viral DNA copy number in those samples. Conventional PCR was not able to detect any of the positive nasal swab samples, while GNP-assisted PCR detected 7 of the 9 positive nasal swab samples. For lung and blood samples, conventional PCR detected 6 of 7 and 2 of 3 samples, respectively, while GNP-assisted PCR detected all the positive blood and lung samples (Table 2).

Discussion

EHV-1 causes serious disease and economic loss in all sectors of the equine industry throughout the world. A fast and reliable technique is required for EHV-1 diagnosis because of the low viral titer in some clinical samples, which may results in false negatives when conventional laboratory techniques are used, allowing further transmission of the virus [1]. Currently, TaqMan qPCR is a gold standard diagnostic assay for EHV-1 as it is highly sensitive and specific compared with conventional methods such as virus isolation, ELISA and conventional PCR [23]. However, an expensive qPCR thermocycler and its reagents are required and are thus not available in all laboratories. As an alternative, gold nanoparticles may be added to conventional PCR to increase the sensitivity.

Recent studies revealed that GNPs can improve the thermal conductivity of PCR mixtures, which consequently promotes template unwinding and dissociation of mismatched primers from the DNA template (either the original sample DNA or PCR product), leading to an increase in the PCR yield and a decrease in non-specific product formation [24]. GNPs also adsorb different PCR reactants such as Taq polymerase, primers and PCR product, therefore affecting the amount of active polymerase in the reaction mixture, decreasing the melting temperatures (Tm) for complementary and mismatched primers (while increasing the Tm

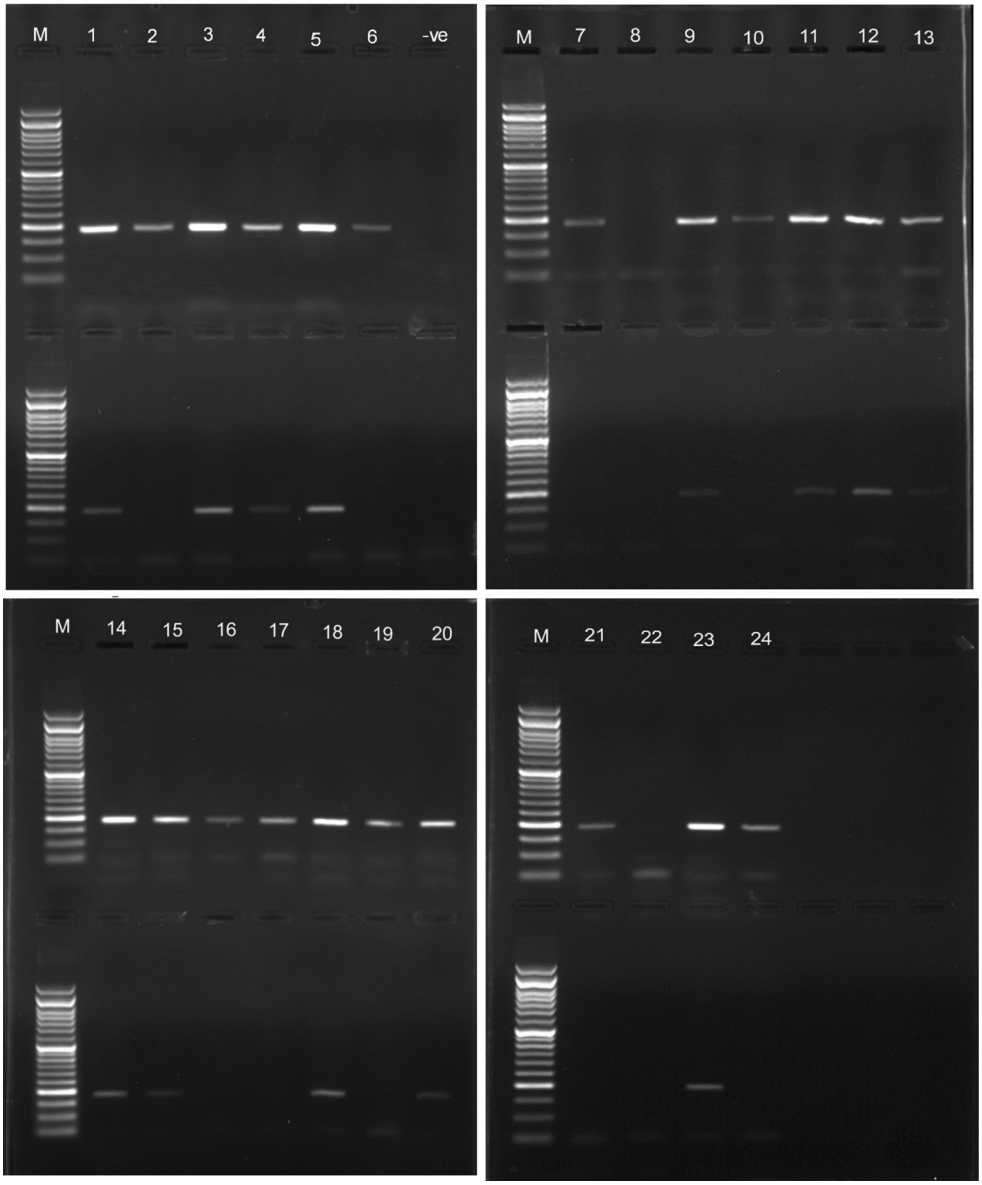

Fig. 1 GNP-assisted PCR results (upper row of each agarose gel) vs. conventional PCR results (lower row of each agarose gel) for the 24 samples found to be positive by qPCR

difference between them in the annealing step), and facilitating the dissociation of PCR products in the denaturation step. Consequently, these events regulate the PCR, enhance its specificity, and increase the amplified product quantity [14, 24, 25]. Previously, we optimized the GNP-assisted PCR assay for the detection of EHV-1 and found that it is a highly-sensitive technique with a detection limit of 10^2 DNA copies, compared with the detection limit of conventional PCR (10^4 copy). This result gives GNP-assisted PCR superiority as a very reliable tool, when accurate and sensitive detection is required [18].

To our knowledge, the present study shows the first application of the highly-sensitive GNP-assisted PCR for detecting the EHV-1 *gB* gene in clinically suspected samples. Among all the submitted samples (n = 83), the percentage of samples detected as positive by GNP-assisted PCR was 22/24 (91.7%), while it was 13/24 (54.2%) by conventional PCR. No negative samples detected by GNP-assisted PCR were positive by conventional PCR, but nine negative samples by conventional PCR were positive by GNP-assisted PCR. Amplification with GNPs resulted in highly efficient detection of EHV-1 compared with conventional PCR, with specificity, sensitivity and accuracy of 100%, 92.3% and 97.6%, respectively, in comparison to 100%, 68.6% and 88.3%, respectively for the conventional PCR. The sensitivity of the conventional technique was

thus enhanced by 23.7%. These numerical results assume that the qPCR had 100% specificity, sensitivity and accuracy in EHV-1 detection. TaqMan-based qPCR for detection of EHV-1 from clinical samples exhibited several advantages over conventional PCR, including the fact that it could detect as few as 2×10^0 DNA copies. In related studies, EHV-1 diagnosis by qPCR achieved highly specific viral detection, with a detection limit of one DNA copy. qPCR also proved to have 10–100-fold higher sensitivity than gel-based nested PCR [26].

Nine more samples (from 83 tested) were detected as positive for the *gB* gene of EHV-1 by GNP-assisted PCR than by conventional PCR. This may be due to the low viral titer in those samples, which consequently could not be detected by conventional PCR. Only two nasal swabs (number 8 and 22) were not detectable by either conventional or GNP-assisted PCR but were positive by qPCR. However, they gave Ct values of 37.6 and 35.9, respectively, in qPCR, which were borderline values that required follow-up. Their high Ct values reflect very low DNA copy numbers. This further confirms that the results of GNP-assisted PCR were highly comparable to those of qPCR, in terms of EHV-1 detection.

The GNP-assisted PCR proved to be easily performed, economical with respect to the instrument and the reagents required, reliable, fast, and less laborious than other diagnostic techniques. The results of GNP-assisted PCR were in agreement with other studies that used nano-PCR for the detection of viral nucleic acids in clinical samples. For example, Yuan et al. found that among 35 porcine epidemic diarrhea virus samples, 23 were positive by nano-PCR, while only 18 were positive by conventional PCR [19]. Nano-PCR developed for porcine bocavirus detection showed 12.4% and 16.6% more sample detection for clinically suspected samples and normal samples, respectively, than conventional PCR [15]. Nano-PCR developed for detection of Chinese mink enteritis virus showed 18.3% higher sample detection than conventional PCR [27]. The variabilities in the percentages of how many more samples can be detected using nano-PCR than conventional PCR depends upon the viral titer in the samples, which will vary depending on the disease and type of samples collected (for example, specific organs).

Conclusions

It is important to improve the efficiency of EHV-1 diagnostic tools that are accessible in developing countries to overcome problems with low viral titer and allow rapid containment of infected animals and fast vaccination of uninfected animals to prevent the spread of EHV-1. This could be achieved by using the highly-sensitive optimized GNP-assisted PCR, as it can detect as few as 100 viral DNA copies with a sensitivity enhanced by 23.7% compared with conventional PCR assay. This method could be used for regular checkups in stables and examination before equine transportation. GNP-assisted PCR is a powerful new tool for EHV-1 detection, surveillance and investigation of vaccine effectiveness because of its simplicity, sensitivity and specificity. In addition, it can be used as an alternative to qPCR in laboratories that cannot afford the expenses of a qPCR system.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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