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Enhancing Polymerase Chain Reaction Efficiency for Foot-and-mouth disease virus identification with Unfunctionalized Nanoparticles

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ABSTRACT

Polymerase chain reaction (PCR) is one of the important molecular biology techniques that helps amplify specific target DNA strands, which can be used in different downstream applications. However, PCR has many obstacles and challenges regarding its sensitivity and efficiency. To overcome those issues, we used metal oxide nanoparticles such as Zinc oxide (ZnO) nano-flower for efficient amplification of foot-and-mouth disease virus (FMDV) serotype O VP1 segment. Incorporating ZnO nanoflowers into the conventional PCR mixture has greatly enhanced the amplified product. The addition of 2 mM of ZnO was able to amplify a relatively low viral load such as 101 and increase the amplified products of higher viral load concentrations. ZnO nanoflowers were also tested with different clinical samples showing superiority over conventional PCR. Overall, the developed nano-PCR can greatly enhance FMDV diagnosis and identification.

INTRODUCTION:

The polymerase chain reaction (PCR) is a pivotal technique in molecular biology that allows for the precise and extremely sensitive amplification of targeted DNA sequences **(Mullis and Faloona 1987).** PCR, which was discovered by Kary Mullis in 1985 **(Saiki et al. 1985)**, has significantly transformed genetic analysis, diagnostics, and research applications by rapidly amplifying DNA by exponential replication **(Higuchi et al. 1992)**. Conventional PCR methods, although often used, may have restrictions in their ability to detect and amplify low levels of target molecules or

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when working with samples that have complicated compositions **(Valones et al. 2009).**

To address these constraints, researchers have investigated other approaches such as employing changed nucleotides, augmenting polymerases, and integrating nanoparticles into PCR reactions **(Pasparakis 2022)**. Nanoparticles, because of their distinctive physicochemical characteristics, have demonstrated the potential to augment the effectiveness and responsiveness of PCR **(Amadeh et al. 2021)**. Zinc oxide nanoparticles (ZnO NPs) have attracted interest because they can be used in living creatures, are easy to manufacture, and can enhance the efficiency of PCR experiments**(Gomez and Tigli 2013; Kołodziejczak -Radzimska and Jesionowski 2014).**

Zinc oxide nanoparticles (ZnO NPs) could interact with DNA and other biomolecules. This interaction has the capacity to enhance the stability of the DNA template and promote more effective hybridization of primers **(Zhang et al. 2004; Zhao et al. 2007; Cui and Jia 2017)**. This interaction has the capacity to enhance the effectiveness of amplification, particularly in difficult situations where the desired DNA is found in low amounts. Additionally, it has been observed that the inclusion of ZnO nanoparticles (NPs) can augment the heat conductivity of the polymerase chain reaction (PCR) mixture, resulting in a more consistent thermal cycling process and mitigating unintended amplification **(Upadhyay A. et al. 2020).**

Foot-and-mouth disease (FMD) is an extremely contagious viral disease that easily spreads among animals with split hooves, such as cattle, pigs, sheep, and goats **(Alexandersen et al. 2003).** The etiological agent of foot-and-mouth disease, referred to as FMDV, is classified within the Picornaviridae family. It is distinguished by its rapid dissemination and significant economic repercussions on the cattle sector **(Longjam et al. 2011**). Prompt and precise detection of FMDV is essential for establishing efficient management strategies and reducing extensive outbreaks **(Rodriguez and Gay 2011)**. Conventional diagnostic procedures, such as virus isolation and serological testing, may have longer turnaround times and not rapid provide molecular techniques **(Park et al. 2013).**

PCR-based methods have become essential tools for identifying and studying FMDV because of their exceptional sensitivity, specificity, and quick processing time **(Lim D.-R. et al. 2022).** Nevertheless, the identification of FMDV in clinical samples can present difficulties, particularly in cases where the virus is present in small quantities or when there are chemicals that impede its detection **(Romey et al. 2023).** Improving the sensitivity of PCR assays is crucial for enhancing the early detection and management of FMDV epidemics **(Lim D. et al. 2022).**

Recent research has demonstrated that the utilization of ZnO nanoparticles can greatly improve the conditions and outcomes of polymerase chain reaction **(PCR) (Upadhyay A. et al. 2020).** An exemplary demonstration of this phenomenon is the integration of a 1 mM concentration of ZnO nanoflowers into the PCR system, yielding outstanding outcomes. Zinc oxide nanoflowers demonstrate a high affinity for DNA, resulting in enhanced sensitivity and overall efficacy of the assay. Due to their compatibility with DNA molecules, they have the potential to safeguard DNA against harm, hence enhancing the effectiveness of PCR**(Upadhyay A. et al. 2020).**

The exceptional thermal conductivity of zinc oxide (ZnO) nanoparticles is being extensively employed to expedite the polymerase chain reaction (PCR)**(Zhu et al. 2022).** Research has shown that various types of ZnO nanomaterials, including tetrapod-like ZnO nanoparticles and single-walled carbon nanotubes, enhance the efficiency and accuracy of PCR **(Lin and Wu 2007)**. The impact of these nanomaterials on PCR is ascribed to their capacity to enhance heat conductivity, resulting in more effective thermal cycling and accelerated reaction times**(Yang et al. 2022a).**

This study investigates the utilization of unfunctionalized ZnO nanoparticles to improve the effectiveness of conventional one-step PCR. The explicit aim is to magnify a dis-

tinct segment of the VP1 gene for serotype O of FMDV. This specific gene segment has been selected as an instructive instance to showcase the potential use of ZnO nanoparticles in polymerase chain reaction (PCR). The primary goal is to validate the effectiveness of this strategy for various primers and serotypes. Utilizing non-functionalized nanoparticles offers significant benefits by streamlining the synthesis process and lowering costs, making this approach well-suited for regular laboratory applications.

MATERIALS and METHODS

ZnO nanoflower synthesis and characterization

The hydrothermal protocol was used to synthesize ZnO nanoflower following the protocol mentioned in the previous study **(Upadhyay A. et al. 2020).** In brief, under continuous magnetic stirring at room temperature, the nanoflowers were manufactured by dissolving 1.5 g of zinc acetate in a solution consisting of 20 ml ethanol and 10 ml deionized water. The solution was brought to a pH of 10 by adding ammonia water dropwise. The solution was then transferred to a Teflon-lined autoclave and incubated at 140 ^{o}C for 10 hours. The product in a Teflon-lined wall was washed using ethanol followed by de-ionized water. The product was then heat-dried at 60 ^{o}C and calcinated at 450 ^{o}C for 2 hours in a furnace. The final product was characterized using Dynamic light scattering (DLS) for average size distribution and charge analysis.

Viruses and clinical samples:

Archived samples that have been stored in -80 freeze and previously identified as FMDV were used. Different types of sample matrices were tested (Heart, tongue, and vesicular fluids). Viral isolate representing O serotype (O/Africa/EA-3) used for optimization and validation of Nano-PCR.

Viral RNA extraction:

The extraction of viral RNA was performed using a Patho Gene-spin DNA/RNA extraction kit following the instructions provided by the manufacturer. In summary, FMDV RNA was extracted from 300 µl of samples and then eluted in a final volume of 40µl of RNase-free water. The RNA extracts were either utilized immediately after extraction or stored at a temperature of -20◦C for subsequent analysis.

PCR amplification and Gel electrophoresis:

A specific primer pair for FMDV serotype O was used for the amplification of the variable region of the 1D gene. The primer sequence has been previously mentioned by **Bachanek-Bankowska et al. (2016)** (EUR 5`-GACATGTCCTCCTGCATCTGGTTGAT-3`) and (O_EA3 5`-CCTCCTTCAAYTACGGT-3`) amplifies 283 bp of the 1D gene of FMDV RNA. RT-PCR amplification of extracted RNA was done using Easy Script one-step RT-PCR super Mix kit (Code-AE411-02). The PCR reaction was performed in 20 µl reaction mixture containing 4 µl of extracted RNA,10 µl one-step master mix, 1 µl of the forward primer, 1 µl of the reverse primer, 0.4 of Enzyme mixture and 3.6 µl of nuclease-free water. The thermal profile used in RT-PCR reaction: at 45°c for 30 min, initial denaturation at 94°c for 6 minutes then denaturation at 94°c for 30 seconds, amplification occurs through primer annealing at 60°C for 30 seconds followed by extensional 72°C for 30 seconds, the cycle repeats for 35 times followed by a final extension at 72°C for 10 minutes. The amplification of the PCR products was examined using 1.5% agarose gel electrophoresis, and 100-bp DNA ladder (BH 100 bp DNA Ladder H3 RTU, BioHelix) was used to measure the amplicon size. The results were visualized using a gel documentation system.

Optimization of (ZnO NPs) PCR assay conditions:

Serial dilution of ZnO nanoparticle (ZnO NP) was used to optimize the nano-PCR test. Dilutions (0,0.125,0.25,0.5,0.75,2,4, and 8) were used with two different viral titres (10^4 and 10^3) to identify to best ZnO NP concentration. Concentration was added to the conventional PCR mix, 1.4 µl of ZnO NP in single reactions, and their volumes were deducted from water volume. PCR products were migrated in gel electrophoresis and results were

compared by measuring band intensity for each concentration.

Sensitivity of (ZnO NPs) Nano PCR assay:

Different dilutions (10^6 to 10^0) of the viral isolate were prepared and applied for RNA extraction. The extracted RNA was then added to the PCR reaction with and without optimized ZnO NP concentration for comparison of Nano -PCR sensitivity. PCR products were migrated, band presence was observed and band intensities were measured.

ZnO Nano-PCR test on clinical samples:

Eight clinical samples that represent different sample matrices were used to test optimized Nano-PCR. Extracted RNA from clinical samples was added to the PCR mixture with and without ZnO. PCR products were migrated in gel electrophoresis and specific band presence was observed.

Gel band intensity measurement:

PCR products were loaded to agarose gel in the same volume as the DNA ladder. After migration, The DNA ladder band concentrations were added to the Image Lab software (BioRad) for the molecular weight analysis. Rectangles with the same dimensional were drawn over the PCR product-specific band and the software calculated its concentration with the aid of provided ladder concentrations. The calculation results were then plotted in a bar chart for visualization.

RESULTS

ZnO nanoflower synthesis and characterization

ZnO nanoflower was successfully synthesized using the hydrothermal method. DLS measurements indicated well-dispersed particles with an average size of 256 nm w poly diversity index (PDI) of 0.4. Moreover, the ZnO nanoflower poses a negative charge with a zeta-potential of 28 mV indicating the formation of stable nanoparticles **Fig (1).**

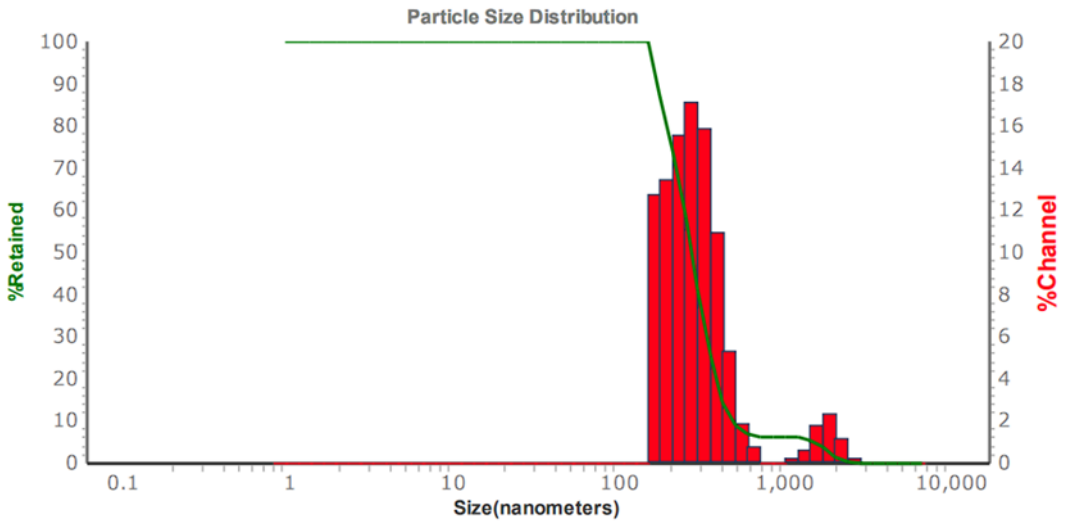

Figure (1): Size distribution of ZnO nanoflower using DLS.

Optimization of ZnO NPs concentration

The amplification of the FMDV serotype O segment (283 bp) was done with the addition of different concentrations of ZnO NPs. The conventional PCR was performed to amplify RNA extracted from two different viral loads (10^4 and 10^3) to subjectively identify the best and optimum ZnO NP concentration. **Fig. (2 and 3).** demonstrated a notable increase in the amplification of FMDV RNA when ZnO NPs are present, relative to the mixture without ZnO NP. From band intensity measurements, We can see comparable results within all tested concentrations, however, the optimum amplification happened in the presence of ZnO Np concentrations (0.75 and 2 mM) with viral load (10^4). ZnO NP concentration (2mM) showed a significant amplification with viral load (10^3). This signifies that the optimum concentration that can be used for nano-PCR is 2 mM of ZnO nanoflower.

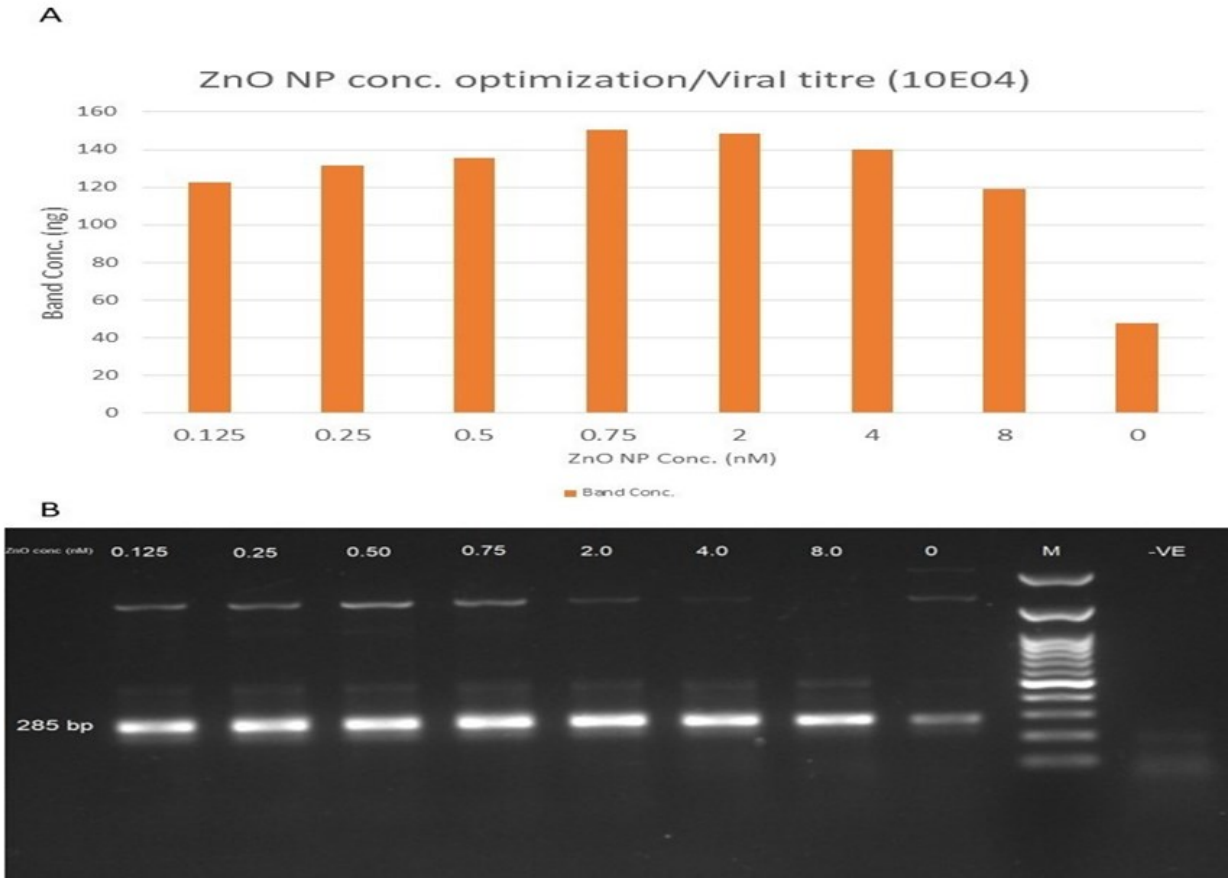

Figure (2): The effect of versions concentrations of zinc oxide nanoparticles (ZnO NPs) on Viral load (1E04) PCR amplification of a 283 bp specific segment.

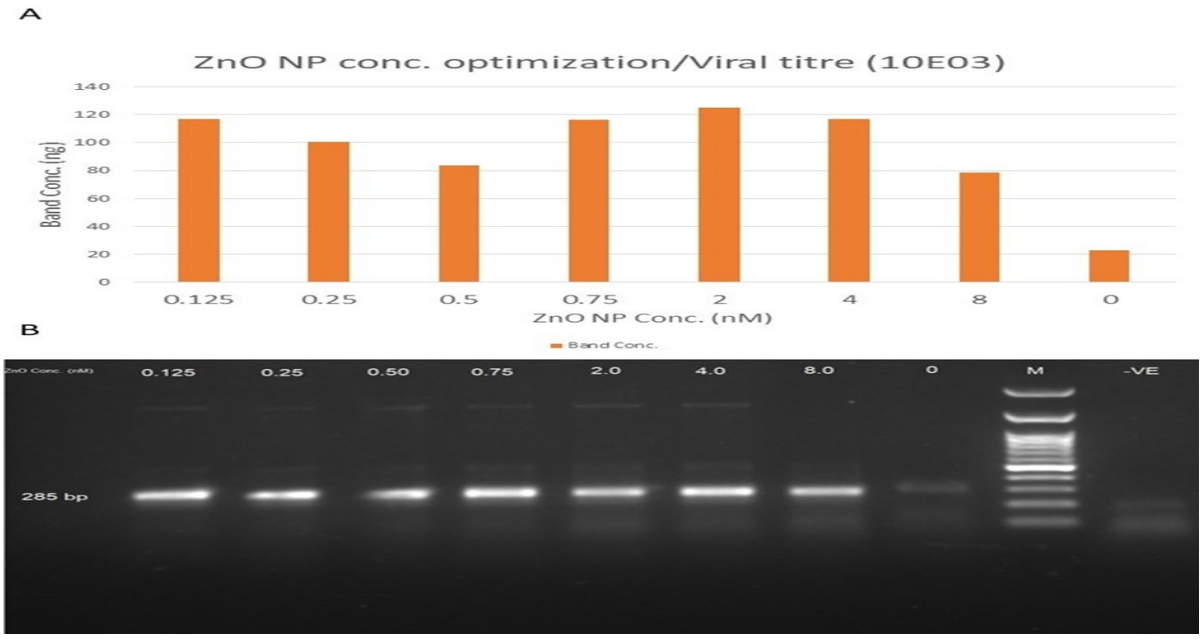

Figure (3): The effect of versions concentrations of zinc oxide nanoparticles (ZnO NPs) on Viral load (1E03) PCR amplification of a 283 bp specific segment

Viral load serial dilution showed an exponential decrease in band intensity of amplified PCR product. The higher viral load concentration (10^6 and 10^5) did not show a difference between PCR products amplified with and without the addition of ZnO NP. Nano-PCR showed a robust amplification in lower concentrations (10^4, 10^3, and 10^2) compared to PCR (without ZnO NP). Conventional PCR (without ZnO NP) showed no specific amplification in lower concentrations (10^1 and 10^0). In contrast, ZnO-contained PCR was able to amplify such lower concentrations but those bands were not measurable using Image Lab software **Fig (4).**

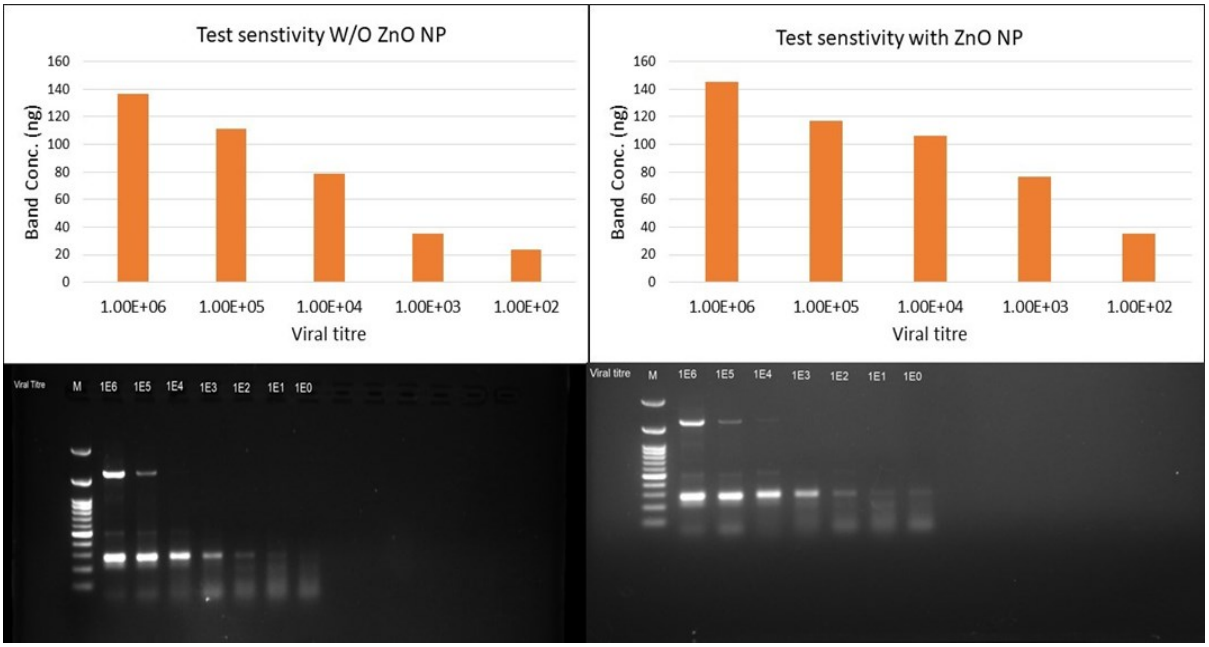

Figure (4): Comparison between the sensitivity of conventional and Nano-PCR

Nano-PCR evaluation on clinical sample

Nano-PCR was subjected to evaluation on clinical samples to unveil the interaction between ZnO NPs and the sample matrix content. Eight samples from different body fluids and tissues were used to evaluate the nano-PCR. Samples were compared to qPCR results. Nano -PCR was able to amplify (5 out of 8) samples, whereas conventional PCR was able to amplify (4 out of 8) samples. The different samples were extracted from the tongue sample. The three samples show negative in both PCRs were further tested for other serotypes and identified as Serotype A (data not shown.) **Fig (5), Table (1).**

Table 1. All sample types and qPCR, conventional, confirmed the sensitivities of the ZnO NPs nano PCR.

Sample No.	Sample type	qPCR	NP PCR	Conventional PCR
1	Heart	28	(+VE)	(+VE)
2	Vesical fluid	29	(+VE)	(+VE)
3	Tongue	28	(+VE)	(-VE)
4	Vesical fluid	27	(+VE)	(+VE)
5	Vesical fluid	28	(+VE)	(+VE)
6	Vesical fluid	24	(-VE)	(-VE)
7	Vesical fluid	27	(-VE)	(-VE)
8	Vesical fluid	26	(-VE)	(-VE)

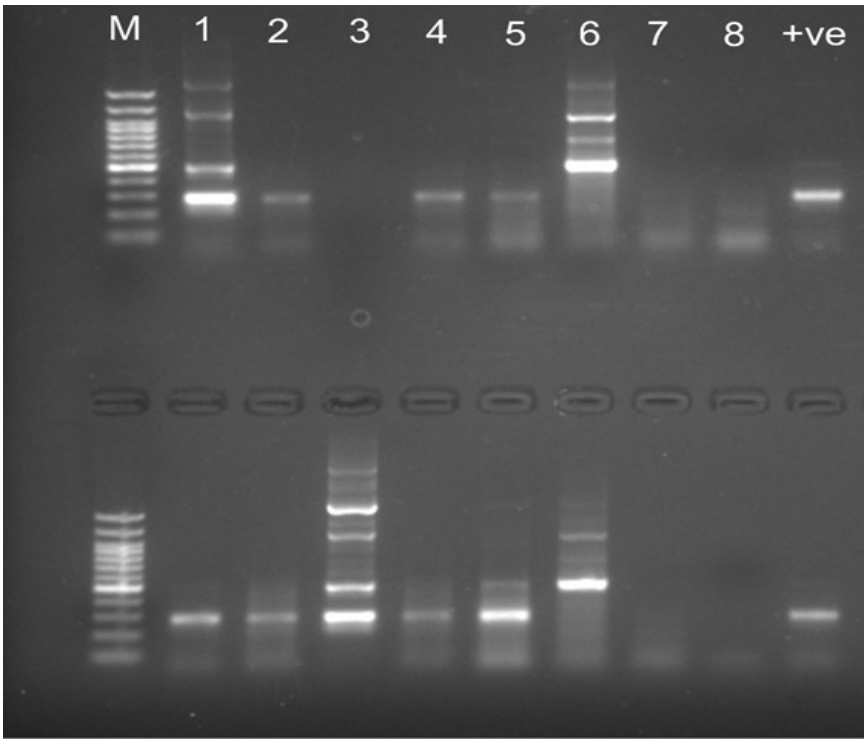

Figure (5): Conventional PCR amplification of 283 bp specific fragment (A) conventional PCR without ZnO NP (B) conventional PCR with ZnO NP.

DISCUSSION:

Foot-and-mouth disease (FMD) is a contagious viral disease that threatens the livelihoods and productivity of livestock farmers in endemic areas **(Hassan et al. 2022)**. It is caused by a virus from the Aphthovirus genus and family Picornaviridae, with seven different serotypes: A, O, C, SAT1, SAT2, SAT3, and Asia-1. FMD causes severe economic losses due to reduced animal productivity, trade restrictions, and control costs **(Hagag et al. 2023)**. FMD diagnosis is essential for effective control and prevention of the disease. There are different methods used for the diagnosis of FMDV in various samples collected from infected animals such as virus isolation (VI), virus neutralization test (VNT), complement fixation test (CFT), and ELISA for detection of antigens and antibodies of FMDV **(Admassu et al. 2015)**. Additionally, several molecular techniques are used to detect the genetic materials of the virus. These assays include real-time PCR (RT–qPCR), multiplex PCR, conventional PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP), reverse transcription recombinase polymerase amplification (RT-RPA), reverse transcription droplet digital PCR (RT-ddPCR), reverse transcription insulated isothermal PCR (RT-PCR) **(Mahmoud et al. 2018; Wong et al. 2020)**

Since then, PCR has become an important tool in the molecular diagnosis of FMDV, allowing for the amplification and detection of specific gene sequences with high sensitivity and specificity. Traditional PCR methods usually have some limitations related to temperature control, primer-dimer production, and test stability. In addition, FMDV's genetic diversity makes diagnosis difficult. As a result, improving diagnostic technologies is critical for strengthening laboratory capacity and coordinating national and regional initiatives **(Zewdie et al. 2023).**

Thus, nanotechnology has been used to improve PCR performance. Currently, many nanomaterials have been successfully used to enhance the specificity and efficiency of PCR, such as Au NPs, QDs, CNTs, graphene oxide (GO) and reduced GO (rGO), partial metal oxidation materials (e.g., titanium dioxide, zinc oxide), and other composite materials like macromolecule polymers doped with Au NPs, amino-modified semiconductor magnetic NPs **(Yang et al. 2022b).**

Zinc oxide (ZnO) nanoflowers have surfaced as a potentially useful nanomaterial to improve the sensitivity and performance of PCR tests. In the present study, we described the use of ZnO nanoflower-based PCR (nanoPCR) for the efficient diagnosis of FMDV through enhancing heat conductivity, amplification efficiency, stability, and specificity.

The production of zinc oxide nanoflowers is simple and can be easily applied in a standard lab utilizing ordinary available chemicals. While some labs may lack the necessary technology to test the quality of these nanoflowers, they can be commercially purchased and directly used in PCR experiments. Alternatively, they can be customized to meet specific experimental requirements, eliminating the need for extra testing in contexts with limited resources **(P.K. Upadhyay et al. 2020).**

ZnO nanoflowers exhibit a distinctive morphology resembling flowers, characterized by a high surface area to volume ratio and excellent thermal conductivity. These properties facilitate rapid and uniform heat transfer within the PCR reaction mixture, which is critical for achieving faster thermal cycling, reducing the overall PCR runtime, and improving PCR efficiency without compromising amplification specificity or sensitivity, Compared to other nanomaterials like gold nanoparticles, ZnO nanoflowers demonstrate superior thermal management capabilities, ensuring uniform temperature distribution across the reaction mixture **(Xu and Yao 2013; P.K. Upadhyay et al. 2020)**.

The large surface area of ZnO nanoflowers provides sufficient binding sites for DNA templates, primers, and DNA polymerase enzymes. This facilitates efficient interaction and binding of PCR components, thereby enhancing amplification efficiency. Studies have shown that ZnO nanoflowers can significantly

reduce nonspecific amplification and improve the specificity of PCR assays**.** This capability is crucial for accurate and reliable detection of target DNA sequences, particularly in complex biological samples. Previous studies have highlighted the effectiveness of ZnO nanoflowers in mitigating primer-dimer formation compared to conventional PCR methods and other nanomaterials. ZnO is known for its biocompatibility and stability under PCR conditions, ensuring minimal interference with enzymatic activities and PCR reagents. This characteristic is essential for maintaining assay reliability over multiple cycles and diverse sample types **(Li et al. 2020).**

In contrast, studies involving fewer stable nanomaterials have reported variability in PCR performance, underscoring the robustness of ZnO nanoflowers in diagnostic applications.In clinical diagnostics, the sensitivity and specificity of PCR assays are critical for accurate disease diagnosis and monitoring. ZnO nanoflower-based NanoPCR has been successfully applied in detecting various pathogens and genetic markers with enhanced sensitivity compared to traditional PCR methods. Recent advancements highlight the potential of ZnO nanoflowers to revolutionize molecular diagnostics by improving detection limits and assay reliability (**Li et al. 2020)**.

By incorporating ZnO NPs into PCR master mixes or reaction tubes, researchers can reduce the risk of false-positive results caused by contamination **(Sirelkhatim et al. 2015).**

CONCLUSION

ZnO nanoflower-based NanoPCR represents a significant advancement in PCR technology, offering enhanced efficiency, sensitivity, and specificity in detecting DNA targets. By leveraging the unique properties of ZnO nanoflowers—such as high thermal conductivity, improved binding surfaces, and stability—researchers can achieve more reliable and accurate PCR results. Comparative studies with other nanomaterials underscore the superior performance of ZnO nanoflowers in enhancing PCR assay robustness and diagnostic accuracy. However, further study of ZnO nanoflowers in comparison with other metal nanoparticles should be conducted.

Author contributions

Mostafa R. Zaher and Dalia M. El-Husseini designed the study, **Mostafa R. Zaher Dalia M. El-Husseini, Mohamed I. Abdallah,** and **Rabab T. Hassanien** performed experimental work and analyzed the data, **Naglaa M. Hagag and Dalia M. A ElMasry** supervised the experimental work, **Mostafa R. Zaher, Mohamed I. Abdallah,** and **Rabab T. Hassanien** generated manuscript first draft, **Naglaa M. Hagag** and **Momtaz A. Shahein** critically reviewed the manuscript.

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Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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