American Journal of
Biomedical Science & Research

www.biomedgrid.com
ISSN: 2642-1747

Research Article

Simian and Human Smallpox: Proposal for a Simultaneous and Differential Molecular Diagnostic Method

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To Cite This article: *González-Arc F, Navarro-Venegas C*, Simian and Human Smallpox: Proposal for a Simultaneous and Differential Molecular Diagnostic Method. Am J Biomed Sci & Res. 2026 31(6) AJBSR.MS.ID.004097,* **DOI:** 10.34297/AJBSR.2026.31.004097

Received: July 25, 2026 **Published:** August 07, 2026

Abstract

Mpox (formerly known as monkeypox) is an emerging zoonosis caused by the Monkeypox Virus (MPXV), a double-stranded DNA virus belonging to the *Orthopoxvirus* genus. Although it has been endemic in Africa since 1970, its global incidence has risen significantly in recent decades—a phenomenon attributed to changes in human behavior, increased contact with animal reservoirs, and the loss of cross-immunity following the cessation of smallpox vaccination. While the disease is typically self-limiting, it can lead to severe complications in immunocompromised patients. In contrast, smallpox—caused by the Variola virus—affects only humans. Unlike mpox, it does not present with lymphadenopathy, though it shares a similar pattern of cutaneous progression. Although officially eradicated in 1980, smallpox remains relevant due to its potential use as a bioterrorism agent, necessitating the maintenance of viral strains in high-security laboratories and the development of molecular detection tools. The 2022–2023 mpox outbreak—the largest outside Africa—highlighted the urgent need to strengthen health surveillance in response to cases emerging without epidemiological links to endemic regions. Current treatment options include antivirals such as tecovirimat, cidofovir, and brincidofovir, although the latter two are limited by their toxicity. This work to propose a molecular diagnostic alternative based on the Polymerase Chain Reaction (PCR), through in *silico* primer design, the study seeks to develop a system for the simultaneous detection of both pathogens, thereby mitigating the epidemiological risk posed by the potential re-emergence of these Orthopoxviruses in the current population.

Background

Mpox Virus (MPXV)

It possesses a double-stranded DNA genome and belongs to the genus *Orthopoxvirus* and the family *Poxviridae*, classified into clade I (Congo Basin) and clade IIa (West Africa). The 2022 global outbreak, involving clade IIb, prompted the WHO to declare a Public Health Emergency of International Concern (PHEIC) in July 2022; this declaration was lifted in May 2023 following a decline in transmission. The PHEIC was reinstated in August 2024 due to a resurgence of cases in the Democratic Republic of the Congo (DRC) and other African countries associated with clade Ib—a variant linked to imported cases and local transmission outside Africa (including in Europe and the Americas) extending into late 2025 [1].

The incubation period ranges from 7 to 21 days. Clinical presentation includes fever, lymphadenopathy—a key distinguishing feature compared to other *Orthopoxvirus* infections—and a skin rash that progresses from the macular phase to the formation of scabs; transmissibility persists until the lesions have completely re-epithelialized [2].

Variola Virus (VARV)

Human smallpox is caused by the Variola virus, a double-stranded linear DNA pathogen with a genome of approximately

186 kb and 150 genes; it belongs to the genus *Orthopoxvirus* (family Poxviridae) and exhibits a characteristic brick-like morphology [3]. Two clinical variants are recognized: VARV major, which has a high fatality rate, and VARV minor, which follows a milder clinical course [4]. Officially eradicated in 1980, routine vaccination was suspended globally that same year [5]. Under WHO supervision, only the CDC in Atlanta (USA) and the VECTOR Institute in Russia are authorized to store and handle VARV strains [6].

VARV is considered one of the most critical agents for global public health due to its high transmissibility, lethality, and historically documented severe sequelae; there is ongoing concern regarding its potential accidental re-emergence or use as a bioterrorism agent [7].

According to the PAHO report from February 2026, between 2022 and January 2026, the Region of the Americas accounted for 73,641 (41%) of the 179,612 confirmed cases worldwide, surpassing the African (36.2%) and European (17.8%) regions. As of that date, eight countries in the region had reported 172 cases of mpox with no associated deaths [8]. At the national level, Chile reported 31 cases of mpox in January 2026, showing an upward trend since epidemiological week 42 of 2025 [8]. Although the case burden is lower than in other countries in the region, the sustained circulation of the virus reinforces the need to maintain epidemiological surveillance and molecular diagnostic systems to differentiate *Orthopoxvirus.* The national response is coordinated by MINSAL, which establishes epidemiological surveillance and control guidelines—supported by the SEREMIs regarding contact tracing—while the ISP serves as the national reference laboratory for diagnostic confirmation [9].

Prevention: Control strategies focus on interrupting the chain of transmission through epidemiological surveillance, timely molecular diagnosis, effective isolation, and contact tracing. Additionally, risk communication and prioritized vaccination for vulnerable groups constitute fundamental primary prevention measures [10].

Vaccination: Various vaccines based on the Vaccinia virus (VACV)—which were instrumental in the global eradication of human smallpox—have been developed, each with distinct biological properties depending on the generation [11]. Vaccines are classified into three generations based on their degree of purification. First-generation vaccines consist of pathogen suspensions with minimal purification. Second-generation vaccines incorporate progressive purification stages, ranging from the whole microorganism to the specific protective component. Third-generation vaccines contain defined or modified protective components designed to optimize the immune response, such as acellular vaccines or polysaccharides conjugated to carrier proteins [12]. First-generation vaccines (such as Dryvax™ or Aventis Pasteur Smallpox Vaccine™) were key to smallpox eradication; second-generation vaccines (such as ACAM2000™ and Lister) offer an improved safety profile; and third-generation vaccines (JYNNEOS™/MVA-BN) utilize non-replicating live viruses that are safer for the general population. All confer cross-immunity against MPXV, providing an estimated 85% protection [13].

Treatment. The FDA has authorized three antiviral drugs—originally developed for smallpox—that have demonstrated utility in managing complex cases of mpox [14,15].

a. Tecovirimat: Inhibits the viral envelope protein VP37, blocking the release of mature virions into the extracellular space and preventing cell-to-cell spread [16].

b. Cidofovir: A nucleotide monophosphate analogue that, following intracellular phosphorylation, competitively inhibits the incorporation of deoxycytidine triphosphate into viral DNA, thereby interrupting elongation and halting replication. Its primary limitation is dose-dependent nephrotoxicity; it requires intravenous hydration and probenecid and is contraindicated in cases of pre-existing renal dysfunction [16].

c. Brincidofovir: A lipid prodrug that is converted intracellularly into cidofovir, selectively inhibiting DNA synthesis in *Orthopoxvirus*. It exhibits lower nephrotoxicity, with adverse effects primarily involving the gastrointestinal tract and liver; consequently, monitoring of hepatic function is recommended during use [16].

Diagnostic Methods

There are four main methodologies for identifying MPXV: genetic, phenotypic, and immunological methods, as well as electron microscopy [17].

Genetic methods detect viral nucleic acids in clinical samples. PCR—specifically qPCR—constitutes the operational gold standard due to its high sensitivity and specificity, although it requires BSL-2 or higher biosafety conditions [18,19]. Genomic sequencing is the definitive gold standard for characterizing variants [20,21], though its cost and complexity limit its routine implementation in resource-limited settings [22].

Phenotypic methods rely on viral isolation and propagation in cell cultures, evidenced by cytopathic effects and plaque formation. They require strict biosafety measures, specialized personnel, and longer processing times [23,24]. Immunological methods have significant limitations due to cross-reactivity among *Orthopoxvirus* species. Detection of IgM and IgG indicates probable exposure—detectable from the fifth and eighth day post-rash onset, respectively—but cannot distinguish between infections caused by different *Orthopoxvirus* species or prior vaccination; consequently, their diagnostic value is lower than that of molecular methods [25,26].

Electron microscopy allows for the observation of MPXV's characteristic morphology but does not differentiate between *Orthopoxvirus* species, and its technical complexity limits its use for routine diagnosis [27,17]. The WHO recommends the use of

qPCR on skin lesion samples to confirm MPXV, utilizing validated platforms such as the Alinity m MPXV assay, cobas MPXV assay, and Xpert Mpox, which aim to improve timely diagnosis and decentralize testing [28]. In Chile, diagnostic confirmation of mpox relies on real-time PCR performed by the Institute of Public Health (ISP)—acting as the reference laboratory—in accordance with guidelines from the Ministry of Health (MINSAL) [9].

The selection of genomic targets is fundamental for specific and differential detection. The B6R gene—which encodes a viral envelope protein—has been validated as an MPXV-specific target with 100% specificity against 15 strains of various *Orthopoxvirus* species and bacterial agents, showing no cross-reactivity [29]; it was subsequently re-validated during the 2022 outbreak as a reference assay for clinical laboratories [30].

For VARV, gene nomenclature varies depending on the reference strain: the A38R gene was used as a specific VARV target [31], while the B9R and B10R genes [32] were also employed based on the VACV-COP reference genome nomenclature. Therefore, the final selection of molecular targets for VARV will be conducted through *in silico* comparative analysis of sequences available in Genbank®, identifying regions with sufficient interspecies variability to design multiplex primers [31,32].

Given that qPCR is the technique of choice for the confirmatory diagnosis of MPXV, this proposal does not aim to replace currently available diagnostic platforms but rather to outline a conventional molecular strategy capable of differential and multiplex detection. The key feature lies in the *In Silico* design of primers capable of amplifying selected regions of MPXV and VARV to generate amplicons of different sizes within a single reaction, thereby allowing for their differentiation via electrophoresis. This approach could serve as a methodological basis for molecular surveillance, education, or initial standardization, subject to subsequent experimental validation.

The objective of this work is to propose a conventional multiplex molecular diagnostic method for the simultaneous detection and differentiation of MPXV and VARV-through the *In Silico* design of specific primers based on a comparative analysis of their genomic sequences- as a complementary tool to support the surveillance of *Orthopoxvirus* species of public health importance.

The proposed methodology aims to address at least three aspects:

1. To identify conserved and differential genomic regions of MPXV and VARV through In Silico comparative analysis of sequences available in public databases, in order to select suitable molecular targets for primer design.
2. To design In *Silico* primers specific to MPXV and VARV that are compatible for use in a single conventional multiplex PCR reaction, taking into account criteria such as length, GC content, melting temperature, specificity, and expected amplicon size.
3. To propose a conventional multiplex PCR protocol for the simultaneous detection and molecular differentiation of MPXV and VARV, including general reaction conditions, theoretical controls, amplicon visualization via electrophoresis, and interpretation criteria.

Materials and Methods

This study can be conducted at any Faculty of Veterinary and Animal Sciences in any developing country, thanks to online bio-tools that remain free of charge. Simultaneous detection will be performed using conventional multiplex PCR, incorporating two pairs of specific primers into a single reaction to generate amplicons of different sizes, thereby allowing for visual differentiation via agarose gel electrophoresis. This work presents a methodological proposal based on **in silico** analysis; therefore, it does not involve the processing of clinical samples or the experimental execution of the PCR. At this stage, the study will focus on genomic sequence selection, comparative analysis, primer design, and the theoretical formulation of a conventional multiplex PCR protocol.

Item 1

a) To identify conserved and differential genomic regions

The study will utilize the Genbank® database to access reference genomic sequences for MPXV and VARV. Based on these sequences, an *In Silico* comparative analysis will be conducted to identify genomic regions that are conserved and differential between the two viruses. To this end, the B10R and B6R genes will be considered as candidate regions. The B10R gene will be evaluated because it has been described in molecular differentiation strategies within the context of *Orthopoxvirus* [31], while the B6R gene will be considered as a potential common target based on comparative analysis, given its use in molecular assays for the detection of MPXV and other *Orthopoxvirus* species [29]. The final inclusion of these regions will depend on the *in silico* comparative analysis of their conservation, variability, and specificity, with the aim of selecting suitable molecular targets for the subsequent design of primers compatible with conventional multiplex PCR.

Item 2

***b)* *In silico* design of specific primers for MPXV and VARV**

Based on the candidate genomic regions identified in the previous objective, *in silico* design of specific primers for the aforementioned viruses will be performed using bioinformatics tools for oligonucleotide design, such as Invitrogen's Oligo Perfect Design®. The resulting primers will be selected based on physicochemical and compatibility criteria suitable for conventional multiplex PCR:

For the comparative analysis, 40 official sequences for each virus available in Genbank® will be selected. This number is established as a defined methodological criterion, as the study

aims not to conduct an exhaustive phylogenetic analysis, but rather to evaluate the intraspecific conservation of candidate genes and identify differential regions useful for primer design. For MPXV, representative sequences from various clades and epidemiological contexts will be considered, including isolates associated with recent outbreaks and reference sequences. For VARV, given its eradication and the absence of current natural circulation, historical or reference sequences available in Genbank® will be used.

Sequences containing the complete candidate genes B10R and/or B6R, with clear taxonomic identification and associated basic metadata (such as country or year of isolation), will be included. Incomplete or duplicate sequences, or those with extensive ambiguous regions within the genes of interest, will be excluded. The selected sequences will be aligned using Clustal Omega to identify conserved regions within each virus and differential regions between MPXV and VARV.

Primer selection criteria [33]:

a. Primer length: 18–25 nucleotides.

b. GC content (%GC): 45–60%, with an optimal value close to 50%.

c. Melting temperature (Tm): Between 50–60°C.

d. Absence of secondary structures: Primers must not form dimers (self-dimers or hetero-dimers) or hairpin structures.

e. Specificity: They must recognize selected regions of the candidate genes B10R and/or B6R. In the case of B6R—since it is a common region—priority is given to segments exhibiting sufficient variability among the viruses. Specificity will be evaluated via in *silico* comparative analysis and similarity searches using BLASTn and/or Primer-BLAST.

f. Multiplex PCR compatibility: Primer pairs must have similar melting temperatures and generate amplification products of different sizes, allowing for their differentiation via electrophoresis.

g. Location on the template: Forward at the beginning of the fragment and Reverse on the complementary strand.

h. Size difference between amplicons: The MPXV and VARV amplification products must show a minimum difference of 150 bp between them so that they can be distinguished via electrophoresis.

Once candidate primers have been defined, their synthesis by a specialized company—such as Integrated DNA Technologies® (IDT®)—will be proposed for a subsequent experimental validation stage.

Item 3

To propose a multiplex conventional PCR protocol for the simultaneous and differential detection of *Monkeypox virus and Variola virus*, using primers designed in *silico*, with potential application in epidemiological surveillance.

The clinical samples considered for the application of this protocol would consist of swabs from skin lesions, scabs, or vesicular fluid from cases with clinical and/or epidemiological suspicion of *Orthopoxvirus* infection. Nationally, these samples would be referred through the healthcare network to the ISP (acting as the national reference laboratory), following guidelines established by MINSAL for the surveillance and confirmation of suspected cases [9]. The samples described here are those that would be used in a potential experimental validation phase of the proposed protocol.

The inclusion of four controls per PCR run is proposed. The positive control for MPXV and VARV will consist of synthetic DNA or plasmid constructs containing the target regions for each virus, respectively; given that handling actual VARV DNA requires BSL-4 facilities, the use of synthetic controls represents the standard methodological alternative for proposals of this type. The extraction negative control will consist of a clinical sample from an individual confirmed negative for *Orthopoxvirus*, processed identically to the test samples. Finally, the reagent control (NTC) will replace the DNA with nuclease-free water while retaining the other reaction components.

To obtain DNA from clinical samples, the use of a commercial extraction kit validated for skin samples—such as the QIAamp DSP DNA Mini Kit (Qiagen®)—is proposed, following the manufacturer's instructions. This method allows for the recovery of DNA of sufficient quality from skin lesion swabs and scabs, which constitute the sample type offering the highest diagnostic yield for *Orthopoxvirus*.

a) PCR reaction mixture. The mixture to be prepared in each PCR tube comprises DNA (derived from a suspect sample and a control or negative control), the designed primers, and a Master Mix solution (containing T*aq* polymerase, nucleotides (A, T, C, G), and Mg^{2+}). The PCR tube designated as the reagent control will contain nuclease-free water instead of DNA, along with all the other previously mentioned reagents.

b) PCR reaction. The protocol follows standard PCR procedures, generally comprising three steps: denaturation at 94°C for 30 seconds; annealing at a temperature determined by primer design—initially (Tm-5) °C—for 30 seconds; and polymerization at 72°C for 1 minute. This cycle is repeated 35 times, followed by a final extension step at 72°C for 10 minutes.

The visualization of the amplified product Is proposed via 2% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer. Electrophoresis may be performed at 90 Volts for 40 minutes, using a molecular size marker. Subsequently, the agarose gel may be stained in a GelRed® solution for 30 minutes at room temperature.

Finally, the use of an ultraviolet (UV) transilluminator is proposed to obtain a photographic record.

The positivity criterion is based on the visualization of bands on the agarose gel relative to the molecular weight marker used. A sample will be considered positive for MPXV if a band is observed at the position corresponding to the specific amplicon for that virus, and positive for VARV if a band is visualized at the position corresponding to that virus's amplicon. In cases of co-detection, the simultaneous presence of both bands is expected, each at its characteristic position. The negative control must not show amplification bands. The exact sizes of each amplicon will be determined based on the primers designed in specific objective 2, ensuring a minimum difference of 150 bp between the two products to guarantee proper resolution on a 2% agarose gel.

Results and Discussion

Strictly speaking, applying the described methodology –and biotools online- would make it possible to resolve each of the issues raised and to address—unequivocally—this and any other research question, in both veterinary and human clinical settings. Undoubtedly, Kary Mullis's brilliant insight has a definitive role in medicine; it is not without reason that medicine is said to be divided into the eras before and after PCR.

Conclusions

The type of work proposed is feasible in any of our developing countries. Updating the state of a research topic is made possible by certain molecular techniques—or variants thereof—described by the giants on whose shoulders we stand.

Acknowledgments

To Dr. Aron Mosnaim of the Wolf Foundation (Illinois, USA) for his constant support through partial funding of Project JWF-CH-005, and to the students of FAVET for the interest they have shown over the years.

Conflicts of Interest

None.

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