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SHORT COMMUNICATION

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The imperative for quality control programs in Monkeypox virus DNA testing by PCR: CIBERINFEC quality control

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Abstract

To evaluate molecular assays for Mpox diagnosis available in various clinical microbiology services in Spain through a quality control (QC) approach. A total of 14 centers from across Spain participated in the study. The Reference Laboratory dispatched eight serum samples and eight nucleic acid extracts to each participating center. Some samples were spiked with Mpox or Vaccinia virus to mimic positive samples for Mpox or other orthopox viruses. Participating centers provided information on the results obtained, as well as the laboratory methods used. Among the 14 participating centers seven different commercial assays were employed, with the most commonly used kit being LightMix Modular Orthopox/Monkeypox (Mpox) Virus (Roche®). Of the 12 centers conducting Mpox determinations, concordance ranged from 62.5% ($n = 1$) to 100% ($n = 11$) for eluates and from 75.0% ($n = 1$) to 100% ($n = 10$) for serum. Among the 10 centers performing Orthopoxvirus determinations, a 100% concordance was observed for eluates, while for serum, concordance ranged from 87.5% ($n = 6$) to 100% ($n = 4$). Repeatedly, 6 different centers reported a false negative in serum samples for Orthopoxvirus diagnosis, particularly in a sample with borderline $C_t = 39$. Conversely, one center, using the TaqMan™ Mpox Virus Microbe Detection Assay (Thermo Fisher), reported false positives in Mpox diagnosis for samples spiked with vaccinia virus due to cross-reactions. We observed a positive correlation of various diagnostic assays for Mpox used by the participating centers with the reference values. Our results highlight the significance of standardization, validation, and ongoing QC in the microbiological diagnosis of infectious diseases, which might be particularly relevant for emerging viruses.

KEYWORDS
Monkeypox, PCR, quality control

1 | INTRODUCTION

The 2022 Monkeypox (Mpox) outbreak was a global epidemic characterized by a significant increase in reported cases of Mpox infection.[1] Mpox is a zoonotic disease caused by an *Orthopoxvirus* closely related to Smallpox.[2] The outbreak affected multiple countries across different regions, resulting in over 80,000 confirmed cases in more than 100 countries.[3] The outbreak garnered attention due to its widespread nature and extensive human-to-human transmission.[4]

The outbreak prompted heightened research into Mpox epidemiology, transmission dynamics, diagnostics, and potential interventions. International health organizations and governments collaborated to provide support and resources for affected regions. The outbreak underscored the importance of global health surveillance, rapid response mechanisms, and research to better understand and mitigate the impact of emerging infectious diseases like Mpox.[5]

An accurate and timely identification of pathogens plays a pivotal role in disease management, epidemiological surveillance, and public health responses.[6] The advent of Polymerase Chain Reaction (PCR) technology has revolutionized the landscape of diagnostic testing, offering a rapid and sensitive approach for detecting viral DNA.[7] However, the reliability of PCR-based diagnostics hinges on the implementation of rigorous quality control (QC) programs.[8] These programs are paramount to minimize the risk of false-positive or false-negative results, providing clinicians, researchers, and policy-makers with accurate information to guide effective interventions. Clinical microbiology laboratories often need to respond quickly to emerging viral infections, and thus the availability of positive and negative controls and several reference laboratories is crucial.

This paper aims to evaluate molecular assays for Mpox diagnosis available in various clinical microbiology services in Spain through a QC program designed specifically for Mpox virus DNA testing to address the accuracy of results and confidence in diagnostic outcomes.

2 | MATERIALS AND METHODS

The Reference Laboratory situated at the Centro Nacional de Microbiología (CNM) prepared a panel of 16 samples, eight containing 200 µL of spiked DNA in a serum background and eight comprising 20 µL of nucleic acid extract, specifically DNA eluate. These samples were subsequently distributed to all participating centers, and the detailed composition of this panel is comprehensively presented in Table 1. To provide a succinct overview, within this panel, two serum samples were intentionally spiked with Mpox virus, and additionally three serum samples were spiked with vaccinia virus. Moreover, the composition included three DNA eluates spiked with Mpox and three DNA eluates spiked with vaccinia virus.

As Mpox, an isolate (347) obtained in Vero cells from the sample of a Spanish patient was used while for vaccinia virus WR strain was used at the reference center. Samples were inactivated by adding AVL, a viral lysis buffer from QIAamp Viral RNA Mini Kit and 10-fold dilutions were obtained. Nucleic acids extraction was performed following manufacturer's instructions. Samples were tested at CNM using a generic Orthpoxvirus qRT-PCR[9] and a Mpox specific PCR.[10] High, medium and/or low or limit concentrations were used. Serum samples were spiked with these nucleic acids.

In total, this study involved the participation of 14 distinct centers, strategically selected from diverse regions across the country, encompassing Castilla y Leon ($n = 1$), Baleares ($n = 1$), Canarias ($n = 1$), Galicia ($n = 1$), Andalucía ($n = 2$), Madrid ($n = 3$), and Cataluña ($n = 5$). The selection process involved a randomized approach, drawing from the pool of centers contributing to the Ciber de Enfermedades Infecciosas (CIBERINFEC), and particularly focusing on those equipped with the capability for Mpox testing and expressing a willingness to actively engage in the study.

Within the consortium of participating centers, the distribution of testing methodologies showcased diversity. A detailed description of extraction and amplification methods is shown in Table 2. Specifically, two centers were exclusively engaged in testing for Orthopoxvirus, four centers concentrated solely on Mpox testing, while the remaining centers undertook testing for both Mpox and Orthopoxvirus. Remarkably, a total of seven distinct commercial assay kits were employed across the participating centers to detect either Mpox or Orthopoxvirus. Among these, the LightMix Modular Mpox Virus kit (TIB MolBiol®, Roche) emerged as the most frequently utilized for Mpox detection, implemented by 6 centers. Meanwhile, two centers each employed the Mpox RealTime kit (Vircell) and an in-house protocol inspired by the work of Li et al.[10] Furthermore, individual centers adopted the Real Cycler Monk kit (Progenie) or the TaqMan™ Mpox Virus Assay (Thermo Fisher). In parallel, for Orthopoxvirus detection, the predominant choice was once again the LightMix Modular Orthopox Virus kit (TIB MolBiol®, Roche), which saw implementation in five centers. Another two centers utilized the Mpox RealTime kit (Vircell), with one center each adopting the RealStar® Orthopoxvirus kit (Altona) or an in-house protocol for Orthopoxvirus as detailed by Kulesh et al.[11]

TABLE 1 Composition of the reference panel.

n	Sample	Mpox C_t	Opox C_t	Virus	10-fold dilution
1 S	Serum	-	39	Vaccinia virus	−5
2 S	Serum	-	-		
3 S	Serum	27.34	29.01	Mpox	−1
4 S	Serum	-	25.36	Vaccinia virus	−1
5 S	Serum	-	-		
6 S	Serum	33.73	35,67	Mpox	−3
7 S	Serum	-	-		
8 S	Serum	-	32.58	Vaccinia virus	−3
1E	DNA	-	-		
2E	DNA	23.61	25.03	Mpox	−1
3E	DNA	-	20.75	Vaccinia virus	−1
4E	DNA	-	-		
5E	DNA	26.75	28.45	Mpox	−2
6E	DNA	32.61	34.98	Mpox	−4
7E	DNA	-	24.33	Vaccinia virus	−2
8E	DNA	-	31.49	Vaccinia virus	−4

Abbreviation: C_t, cycle threshold.

3 | RESULTS

In the assessment of Mpox virus detection, the agreement between serum samples' outcomes and those anticipated from the reference center exhibited variability. Across different centers, the concordance rates ranged from 75% (six out of eight samples) in one center, with the discrepancies stemming solely from false positive Mpox identifications in samples intentionally spiked with vaccinia virus, to a perfect 100% concordance in 10 centers. Another center achieved an 87.5% agreement (seven out of eight samples), where the discordance resulted from a mistaken positive call in a sample with a Cycle Threshold (C_t) value of 39 at the testing center.

Similarly, the evaluation of Mpox detection in DNA eluates yielded comparable trends. The concordance levels ranged from 62.5% (five out of eight samples) in a single center, once again attributable to false positive Mpox identifications in samples spiked exclusively with vaccinia virus, to a flawless 100% concordance in 11 centers.

During the examination of Orthopoxvirus detection, the alignment of results from serum samples with those projected by the reference center demonstrated variability across different centers. The concordance rates spanned from 87.5% (seven out of eight samples) in six centers to a complete 100% match in four centers. The instances of discordance were due to inaccurate negative outcomes

TABLE 2 Details of the extraction and amplification methods of the participating centres and the concordance outcomes.

			Serum		DNA	
	Extraction method	**Amplification method**	**MPOXV**	**OPOXV**	**MPOXV**	**OPOXV**
Center 1	MagCore® Viral Nucleic Acid Extraction Kit (RBC Bioscience Corp)	LigthMix Modular OPOXV + MPOXV (TIB Molbio, Roche)	100.00%	100.00%	100.00%	100.00%
Center 2	MagNA Pure 96 DNA and Viral NA Kit (Roche)	Li et al + Kulesh et al	100.00%	87.50%	100.00%	100.00%
Center 3	EZ1 DSP Virus Kit (Qiagen)	LigthMix Modular MPOXV (TIB Molbio, Roche)	100.00%		100.00%	
Center 4	MagCore® Viral Nucleic Acid Extraction Kit (RBC Bioscience Corp)	RealStar® Orthopoxvirus PCR kit (Altona Diagnostics)		100.00%		100.00%
Center 5	NucliSENS® easyMag® (bioMérieux)	LigthMix Modular OPOXV + MPOXV (TIB Molbio Roche)	100.00%	100.00%	100.00%	100.00%
Center 6	TanBead Nucleic Acid Extraction kit (Taiwan Advanced Nanotech)	Monkeypox RealTime kit (Vircell)	100.00%	87.50%	100.00%	100.00%
Center 7	EZ1 DSP Virus Kit (Qiagen)	LigthMix Modular OPOXV (TIB Molbio, Roche)		87.50%		100,00%
Center 8	MagCore® Viral Nucleic Acid Extraction Kit (RBC Bioscience Corp)	LigthMix Modular OPOXV + MPOXV (TIB Molbio, Roche)	100.00%	87.50%	100.00%	No sample
Center 9	MagCore® Viral Nucleic Acid Extraction Kit (RBC Bioscience Corp)	Monkeypox RealTime kit (Vircell)	100.00%	87.50%	100.00%	100.00%
Center 10	MagCore® Viral Nucleic Acid Extraction Kit (RBC Bioscience Corp)	LigthMix Modular MPOXV (TIB Molbio, Roche)	100.00%		100.00%	
Center 11	EMAG® (bioMérieux)	TaqMan™ Mpox Virus Assay (Thermo Fisher) + Li et al.	75.00%	87.50%	62.50%	100.00%
Center 12	EMAG® (bioMérieux)	Real Cycler Monk-CFX32 (Progenie)	100.00%		100.00%	
Center 13	NucliSENS® easyMag® (bioMérieux)	LigthMix Modular MPOXV (TIB Molbio, Roche)	87.50%		100.00%	
Center 14	MagNa Pure Total NA Isolation kit (Roche)	LigthMix Modular OPOXV + MPOXV (TIB Molbio, Roche)	100.00%	100.00%	100.00%	100.00%

reported by testing centers for a sample initially provided with a Ct value of 39 from the reference center. Conversely, when analyzing DNA eluates, a uniform 100% concordance was achieved across testing centers capable of conducting assessments (nine out of 10 centers). A comprehensive summary of these concordance outcomes is provided and visually presented in Table 2 for reference.

4 | DISCUSSION

The high number of reported Mpox cases globally during the 2022 outbreak emphasized the need for robust diagnostic strategies. The lack of uniformity in diagnostic methods across centers poses a significant concern for effective outbreak management and global health security. Implementing standardized diagnostic protocols is crucial to ensure accurate and comparable results across different laboratories, which is essential for effective patient management and epidemiological tracking during outbreaks. In the present study we evaluated the concordance across molecular assays used for the diagnosis of Mpox virus (Mpox) across various Microbiology services in Spain. Our findings provide valuable insights into the diagnostic landscape and shed light on the challenges and successes encountered by different centers in their efforts to detect Mpox.

The results from this study revealed both strengths and limitations in the diagnostic landscape: first, the diverse selection of commercial assay kits and in-house protocols used by the participating centers showcases the adaptability of diagnostic methods to local contexts, and demonstrated the diversity in testing methodologies employed by various clinical microbiology centers across Spain. Among the participating centers, a multitude of commercial assay kits and also some in-house protocols were utilized, highlighting the adaptability of diagnostic methods to regional contexts and available resources. Second, the findings from the concordance analysis of diagnostic results are pivotal. While some centers achieved high concordance rates, ranging from 87.5% to 100%, discrepancies were observed in others. False positives and negatives, mainly at high Ct values, as well as cross-reactions with related Orthopoxviruses, underscore the complexity of accurately diagnosing these infections. In particular, the detection of false positives in Mpox diagnosis due to cross-reactions with the TaqMan™ Mpox Virus Microbe Detection Assay (Thermo Fisher), highlights the necessity for thorough validation of diagnostic assays against closely related viruses and considering potential cross-reactivity. These discrepancies emphasize the importance of continuous QC, proficiency testing, and collaboration between diagnostic centers, especially when dealing with emerging pathogens.

External QC initiatives, as demonstrated in this study, play a critical role in addressing these challenges.[12] As in other QC programs,[8,13] the control samples distributed to the participating centers enabled the identification and rectification of diagnostic inaccuracies, leading to improved overall diagnostic accuracy. The positive correlation observed between the results of participating centers and the reference laboratory underscores the value of external quality assurance programs for harmonizing diagnostic practices.

In conclusion, this study reinforces the necessity of QC programs in molecular diagnostics, particularly in the context of emerging infectious diseases such as Mpox. The assessment of various diagnostic assays for Mpox diagnosis provided insights into the diversity of testing methodologies and the challenges associated with accurate detection. The positive correlation between QC results and reference center outcomes highlights the effectiveness of such programs in improving diagnostic accuracy. Ultimately, this research underscores the significance of standardization, validation, and ongoing QC in the microbiological diagnosis of infectious diseases, contributing to effective patient care, outbreak management, and public health responses.

AUTHOR CONTRIBUTIONS

Concept and design of the study: Miguel J. Martínez, Federico García, María Paz Sánchez-Seco Fariñas. *Data acquisition*: Adolfo de Salazar, Jessica Navero-Castillejos, Anabel Negredo, Juan Carlos Galán, Estrella Rojo Molinero, Eduardo Lagarejos, Carmen Muñoz-Almagro, Águeda Hernández Rodríguez, José Antonio Lepe, Andrés Antón Pagarolas, Sonia Pérez Castro, María Isabel Zamora Cintas, Marta Domínguez-Gil González, Jordi Niubó-Bosch, Almudena Gutiérrez Arroyo, Ana Vazquez. *Methodological support*: Adolfo de Salazar, Miguel J. Martínez, Jessica Navero-Castillejos, Federico García, María Paz Sánchez-Seco Fariñas. *Manuscript reviewing*: Jessica Navero-Castillejos, Anabel Negredo, Juan Carlos Galán, Estrella Rojo Molinero, Eduardo Lagarejos, Carmen Muñoz-Almagro, Águeda Hernández Rodríguez, José Antonio Lepe, Andrés Antón Pagarolas, Sonia Pérez Castro, María Isabel Zamora Cintas, Domínguez, Jordi Niubó-Bosch, Almudena Gutiérrez Arroyo, Ana Vazquez. *Data analysis and manuscript writing*: Adolfo de Salazar, Miguel J. Martínez, Federico García, María Paz Sánchez-Seco Fariñas.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available to investigators upon reasonable request.

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