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Mpox infection investigation using multiplexed syndromic diagnostics: Evaluation of an AusDiagnostics multiplexed tandem PCR (MT-PCR) syndromic panel

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ABSTRACT

Background: Detection of mpox virus during investigation of viral vesicular rash illness is required to identify mpox infection.
Objectives: This study evaluated the performance of a research-use-only (RUO) AusDiagnostics MT-PCR syndromic assay containing an mpox virus target.
Methods: The analytical specificity and limit of detection (LoD) of the AusDiagnostics MT-PCR mpox assay was verified using control material. Clinical performance was evaluated using anonymised residual nucleic acids extracted from swab specimens previously tested for mpox virus using a laboratory developed test (LDT). Residual nucleic acids were derived from consecutive sample panels collected during two periods in the 2022 mpox outbreak.
Results: The AusDiagnostics MT-PCR assay demonstrated an LoD of 35 input copies of mpox virus and correctly detected all relevant members of a specificity panel ($n = 34$). 175 residual nucleic acids were included in the study with a prevalence of mpox of 40.0% (95%CI 32.7–47.6). The AusDiagnostics MT-PCR mpox assay demonstrated an accuracy of 98.9% (95%CI 93.8–99.9), sensitivity of 94.2% (95%CI 85.2 – 98.1) and specificity of 100% (95%CI 95.6 -100), when compared to the LDT qPCR assay. The AusDiagnostics MT-PCR mpox assay detected additional vesicular rash pathogens in 26.8% samples. Co-detection with other vesicular rash pathogens was described in 12.8% of mpox virus detected samples
Conclusions: Performance of the RUO AusDiagnostics MT-PCR mpox assay was comparable to an LDT qPCR for the detection of mpox virus in nucleic acids extracted from swab specimens. The RUO AusDiagnostics MT-PCR mpox assay facilitated the simultaneous detection of additional infective etiologies of vesicular rash syndromes.

1. Background

Mpox is a zoonotic viral infection caused by the mpox virus from within the genus *Orthopoxvirus* [1]. Endemic transmission of mpox occurs within specific geographic areas and infections identified outside of these regions were until recently only associated with travel related acquisition [2].

Mpox infection emerged as a cause of viral rash illness outside of endemic areas in May 2022 [3], with transmission observed to occur within sexual networks of gay or bisexual and other men who have sex with men [4]. Diagnosis of mpox during the current outbreak has been aided by the detection of viral Deoxyribonucleic acid (DNA) within clinic samples using nucleic acid amplification test's (NAAT).

The incidence of infection is currently in decline, following a peak in the UK during July 2022 [5]. However, mpox virus should now be considered as one of a number of aetiological agents attributable to viral vesicular rash symptoms, suggesting than its inclusion within multiplexed syndromic panels may be warranted.

AusDiagnostics Multiplexed Tandem Polymerase Chain Reaction (MT-PCR) assays enable highly multiplexed detection of pathogens [6], enabling the investigation of respiratory tract [7] and central nervous system syndromes [8].

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Table 1
Characteristics of patient samples included in the study.

	Panel 1	Panel 2
Characteristics of patient samples		
Number of samples	88	87
Anatomical site swabbed (if available)		
Penis swab	8.0%	3.4%
Perianal swab	2.3%	
Skin swab site stated	13.6%	
Skin swab no site stated	15.9%	23.0%
Swab no site stated	10.2%	64.4%
Throat swab	22.7%	2.3%
Vesicle no site stated	27.3%	4.6%
Rectal swab		1.1%
Unspecified swab		1.1%
Routine Test results		
Mpox detected by qPCR	51.1%	22.9%
Orthopox detected by qPCR only	–	1.2%
Mpox qPCR indeterminate	3.4%	–
Enterovirus detected	1.1%	–
Herpes simplex virus type 1 detected	1.1%	4.6%
Herpes simplex virus type 2 detected	1.1%	3.4%
Varicella-zoster virus detected	9%	4.6%

This table describes i) patient characteristics ii) Anatomical site of swabbing and iii) outcome of routine testing (if performed) of two sample panels included in the study.

2. Objectives

In this study, we evaluated the performance of a research-use-only (RUO) AusDiagnostics MT-PCR assay that included a mpox virus target as part of a wider panel of pathogens attributable to vesicular rash illness and central nervous system infections. We describe the performance of this assay in comparison to quantitative real-time PCR (qPCR) and detail the prevalence of additional pathogens responsible for vesicular rash syndromes present within “early outbreak” and “contemporaneous” sample panels.

3. Study design

3.1. Clinical samples and patient population

The samples used in this study consisted of residual nucleic acids extracted from vesicle swabs submitted from patents requiring mpox virus DNA detection as part of routine clinical care (see Table 1). Two opportunistic panels of sequentially processed residual nucleic acids were used for this evaluation; Panel 1 (early outbreak) extracts were derived from Sigma Virocult swabs (Cat No. MW9515) submitted as part of mpox workup during the period 15th May 2022 to 24th June 2022. During this study period all patients were routinely tested for Mpox virus at the Rare and imported pathogens laboratory (RIPL) of the UK Health Security Agency.

Panel 2 (contemporaneous) extracts were derived from swabs collected using Roche cobas® PCR Media Dual Swab kits submitted for mpox virus detection in the period 30th August 2022 to 5th September 2022. During this study period all patients were routinely tested for mpox virus using a qPCR laboratory developed test (MPOX LDT). For both panel 1 and panel 2 samples the results of mpox qPCR, age, sex, anatomical site of swabbing (if recorded) and any additional relevant viral detection were recorded and the samples then anonymised, prior to testing with the AusDiagnostics MT-PCR mpox assay.

Ethical approval was not required for this diagnostic evaluation study.

3.2. Sample processing

Panel 1 swabs were processed depending upon the number of swabs received. If duplicate swabs were received, one swab was referred for mpox testing to RIPL and the second processed for routine vesicular rash testing and used in this study. In this case 50 µL of viral transport media from the swab was combined with 200 µL of buffer AVL prior to extraction. If only a single swab was received, 125 µL of viral transport media was retrieved and combined with 125 μl of Roche off board lysis buffer (Cat no. 06374913001). Panel 2 extracts were prepared by directly using 200 µL of Roche PCR media as the input for nucleic acid extraction.

Nucleic acids were extracted using the Qiagen Viral RNA kit and Qiagen EZ-1 advanced Excel with a 200 µL sample input volume and 50 µL elution volume and included 5 μL Qiagen Internal Control DNA (High conc.) (Cat No: 211392) alongside the carrier RNA.

3.3. qPCR methods

The Mpox LDT was adapted from two previously published assays [9,10]. The G2R WA probe [10] was modified to include a 5-FAM label and 3-BHQ-1 and the E9L-NVAR probe [9] to include a 5-Cy5 label and 3-BHQ-2. All oligonucleotides where supplied by Eurofins genomics. An extraction control was included as described in the Qiagen QuantiFast Pathogen PCR +IC Kit (Cat no: 211,354). qPCR was performed as per the Kit instructions with a final reaction volume of 25 μL which included 10 μL of extracted nucleic acid material.

A positive control plasmid quantified by digital PCR [11] and then normalised to approximately 100 copies per µL was included, together with a negative control, in each run. qPCR was performed on an ABI 7500 fast system (Thermofisher scientific) with the following cycling conditions: 95 °C for 5 min, then 40 cycles of 95 °C denaturation for 10 s and 60 °C annealing and extension for 10 s, with the fluorescence signal acquired in the FAM, VIC and Cy5 channels after each annealing and extension stage. All samples in this study were tested with the Mpox LDT. The Mpox LDT was reported as Mpox Detected if amplification occurred in both the G2R WA and E9L-NVAR assays. Samples were reported as Mpox Indeterminate if amplification was observed to occur in only one of the two Mpox LDT qPCR assays.

The RUO AusDiagnostics MT-PCR mpox assay was performed as part of a wider multiplexed panel that included a total of 15 pathogen targets (human herpesvirus 1, human herpesvirus 2, varicella zoster virus, Epstein-Barr virus, cytomegalovirus, human herpesvirus 6, human herpesvirus 7, *Treponema pallidum*, enterovirus, parechovirus, adenovirus groups F&G, adenovirus groups B,C & E, BK virus, JC virus, mpox virus,

Table 2
Results of AusDiagnostics RUO MT-PCR testing of patient samples included in the study.

AusDiagnostics RUO MT-PCR panel results				
	Panel 1		Panel 2	
	Prevalence in all samples (n = 88)	Prevalence in mpox detected samples (n = 46)	Prevalence in all samples (n = 87)	Prevalence in mpox detected samples (n = 20)
Enterovirus	3.4%	6.5%	1.1%	5%
Herpes simplex virus type 1	5.7%	4.3%	13.8%	5%
Herpes simplex virus type 2	1.1%	2.2%	6.9%	5%
Treponema pallidum	1.1%	–	4.6%	–
Varicella-zoster virus	10.2%	–	9.2%	5%

This table describe the Prevalence of pathogens attributable to vesicular rash illness detected using the AusDiagnostics RUO MT-PCR panel, stratified by the presence of mpox conifection.

human DNA adequacy control and a spike internal assay control). The assay required 10 μL of input extracted nucleic acid material. The Mpox virus assay targeted the F3L gene. Due to the RUO nature of the assay testing was performed using a single lot of reagents.

Analytical specificity was confirmed using a panel of Zeptometrix NatMEp-Bio (n = 14) and QCMD controls 101S QAV994105 (n = 10) 19S QAV994105 (n = 10), reflecting pathogens expected to be present in skin swabs and Cerebrospinal fluid specimens. Limit of detection was performed by analysis of a serial dilution of Vircell mpox total control material (Cat No.MBTC023-R) in Sigma Virocult swabs (Cat No. MW9515) viral transport media. The serial dilution was performed in triplicate. Positive percent agreement (PPA), negative percent agreement (NPA) and overall accuracy were calculated for mpox virus detection for the AusDiagnostics MT-PCR mpox assay.

The proportion of pathogens detected across the two panels of samples was described, along with the prevalence of co-detection of additional pathogens responsible for vesicular rash syndromes in mpox cases.

4. Results

4.1. Analytical performance of the AusDiagnostics MT-PCR assay

The tandem-Plex assay correctly detected all members of the Zeptometrix (NatMEp-Bio n = 14) and QCMD controls 101S (n = 10) 19S (n = 10) analytical specificity panels. Serial dilution of Vircell Mpox total control material determined the assay to possess a limit of detection of 35 copies of Mpox virus genomic target in extracted nucleic acid material.

4.2. Patient samples

A total of 175 swab samples, were tested during the study, with mpox virus DNA detected in 51.1% and 21.9%, of panel 1 and panel 2 samples, respectively. Information regarding the site of swabbing was unavailable for over half of the samples collected as part of panel 2, reflecting that samples were collected using anonymised sexual health identifiers.

4.3. Clinical performance of the AusDiagnostics MT-PCR assay

The AusDiagnostics MT-PCR mpox assay demonstrated an accuracy of 98.9% (CI 93.8–99.9), with, sensitivity of 94.2% (CI 85.2 – 98.1) and specificity of 100% (CI 95.6 –100). Four mpox samples were either indeterminate (n = 2) or detected (n = 2) by the MPOX LDT and not detected by the AusDiagnostics MT-PCR mpox assay. The computed Ct values obtained from mpox detected samples using the AusDiagnostics MT-PCR Mpox assay ranged from 9.58 to 35.07 with a median of 17.2, these results were comparable to G2R WA (range 12.54 to 37.77, median 22.01) and E9L-NVAR (range 15.44 to 37.31, median 22.97) components of the Mpox LDT qPCR.

In addition to mpox virus, the AusDiagnostics MT-PCR assay enables samples to be screened for 5 further pathogens routinely causative of vesicular rash and at least one of these pathogens was detected in 26.8% samples within the study (Table 2) (18 in panel 1, 29 in panel 2). Notably, 13.7% of samples (Panel 1 n = 10 and Panel 2 n = 14) were infected with a pathogen attributable to vesicular rash with no diagnostic testing performed for it at clinical attendance.

Co-detection of mpox virus with other vesicular rash pathogens was uncommon and detected in 12.8% of mpox virus detected samples, with enterovirus the most commonly detected pathogen present in 5.7% of all mpox detected samples.

5. Discussion

This study observed an accuracy of 98.9% (93.8–99.9%) for the detection of mpox virus when assessing the performance of the AusDiagnostics RUO MT-PCR panel designed for investigation of vesicular rashes. Mpox infection was identified as the prevalent cause of viral rash within our study samples, detectable at a higher prevalence early in the outbreak and declining two months later.

The high overall prevalence of mpox virus detected within study samples, reflects the geographic location of our laboratory which has served patients that have been disproportionately affected by the mpox outbreak within the United Kingdom [12]. Although test positivity rates documented in this study may not reflect the prevalence observed elsewhere [13], our findings may be of interest to those providing mpox diagnostics.

This study highlighted that although mpox virus was detected in a significant proportion of those patients screened during the outbreak, other pathogens attributable to vesicular rash syndrome, remained prevalent and were frequently not diagnosed, highlighting a benefit of the multiplexed panel under evaluation.

Mpox virus co-infection with other causes of viral rash illness (Enterovirus, HSV-1, HSV-2 and VZV) was uncommon in this study, possibly reflecting the older age of patient cohort in comparison to other studies examining co-detection [14]. Coinfection was frequently not detected by routine approaches due to an absence of samples collected for testing, we hypothesise that this is attributable to acquisition bias when testing patients for mpox during the outbreak.

We note that our sample size was small and not powered to explore statistical differences in performance between the two mpox NAAT's, this limitation was as a consequence of the availability of the RUO AusDiagnostics MT-PCR assay for this clinical validation. In addition, the samples used within the study were selected as opportunistic validation panels and therefore it is not possible to broadly describe pathogen prevalence in vesicular rash illness through the course of the 2022 mpox outbreak.

The data presented in this validation study demonstrates that mpox diagnostics can be readily incorporated within multiplexed syndromic panels [15], for use in the management of infections occurring in endemic or outbreak affected regions. Such syndromic panels may offer benefit to clinical virology laboratories, in that accreditation may be achieved for a single diagnostic as opposed to multiple single pathogen assays. In the context of mpox, such an approach may provide sustained capacity, enabling the investigation of emergent infection if required in the future.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

M.J. Pond: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **J. Al-Mufti:** Conceptualization, Data curation, Investigation, Writing – review & editing. **P. Madona:** Conceptualization, Investigation, Writing – review & editing. **M.A. Crone:** Conceptualization, Methodology, Writing – review & editing. **K.G. Laing:** Conceptualization, Methodology, Writing – review & editing. **R.S. Hale:** Writing – review & editing. **D. Muir:** Conceptualization, Methodology, Writing – review & editing. **P. Randell:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing.

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