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Molecular and immunological diagnosis of Monkeypox virus in the clinical laboratory

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SUMMARY The 2022 monkeypox outbreak outside Africa is ongoing. Cases have been reported in Hong Kong and Chongqing, China. In order to better prevent and control the potential spread of monkeypox virus in China, the development of sensitive and reliable detection commercial kits is imminent. This correspondence reviews the existing laboratory assays and related technologies for nucleic acid (PCR) and serological assays for the diagnosis of monkeypox virus to provide reference for the management and decision-making departments. Due to the serological cross-reactivity of orthopoxviruses, PCR is the laboratory test of choice to confirm monkeypox virus infection. We recommend a dual-target PCR approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence.

Keywords Monkeypox, diagnosis, PCR, *Orthopoxvirus*

The monkeypox outbreak has been recognized as a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) on 23 July 2022 (*1*). Data as of 02 December 2022, it has currently caused 82, 062 cases and 65 deaths and has spread to more than 100 countries, 98.8% in locations that have not historically reported monkeypox (*2*). Before monkeypox, WHO classified only six outbreaks as PHEIC, including the most recent pandemic COVID-19 (the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)) that caused 644 million infections and 6.64 million deaths. Monkeypox can be transmitted to humans or certain animals. Human-to-human transmission of monkeypox is primarily through close contact with contaminated objects, such as skin lesions, body fluids, respiratory droplets and beddings. Interestingly, in the 2022 monkeypox outbreak, most cases were among men who had sex with men (MSM), and the disease displayed characteristics of a sexually transmitted infection (STI), with symptoms often confined to the anogenital region (*3*). Currently, the WHO estimates the global pandemic risk of monkeypox as "moderate", but as "high" in Europe. Given the current China "dynamic zero COVID-19" strategy, the risk of monkeypox spreading in China is still low (*4*). However, the risk of monkeypox epidemics should not be underestimated.

Monkeypox virus belongs to the genus *Orthopoxvirus* and the family Poxviridae, the same as infamous variola virus (smallpox). The monkeypox virus genome is approximately 197 kb of double-stranded DNA containing 190 closely packed genes (*5*). There are two clades of monkeypox virus, the Congo Basin (Central African) clade and the West African clade, and they exhibit approximately 0.5% genome sequence differences and circulate in different regions of Africa (*6*). In humans and cynomolgus monkeys, the Congo Basin clade is more virulent than the West African clade, with estimated human case fatality rates of 10.6% and 3.6% respectively (*7*). The current outbreak is caused by a less virulent West African clade of monkeypox virus.

Because the clinical presentation of this outbreak may have been atypical, accurate differential virological diagnosis should be performed in patients with STI-associated rashes, even the rashes are localized and not (yet) spread. Laboratory diagnosis is particularly important in such a circumstance. As with SARS-CoV-2, timely and valid laboratory testing is a key approach that can be deployed quickly enough to provide prompt feedback on any public health interventions. So far, more than 30 Chinese *in vitro* diagnosis (IVD) companies have obtained the European Union conformite europeenne (CE) certification for monkeypox virus diagnostic products. This article aims to analyze the characteristics and clinical application scenarios of detection methods.

1. Diagnosis for monkeypox virus nucleic acid

Monkeypox diagnosis can be confirmed by the detection

of virus DNA through polymerase chain reaction (PCR) and next-generation sequencing in a clinical specimen or the detection of monkeypox virus through the CRISPR-Cas12a-based approach (*8*). However, CRISPR-like technologies currently have no commercial diagnostic products for other pathogens, even for the widespread COVID-19, they are mostly still in the research stage. The real-time PCR is the preferred method for routine diagnosis of monkeypox virus with low cost and high speed.

The real-time PCR assay was used to target the conserved regions of the DNA polymerase gene *E9L*, *B7R*, DNA-dependent RNA polymerase subunit rop18, extracellular envelope protein gene *B6R* and *F3L* gene (Table 1) (*9-12*). This PCR assay, if poorly designed, may have false-negative results (below limit of detection) or reduced analytical sensitivity. The monkeypox virus is not a new virus and there are several published assays available, and this is a very different scenario from that of COVID-19 in early 2020 (*13*). Poxviruses have a lower mutation rate than RNA viruses due to the 3'-5' exonuclease proofreading activity of the viral DNA polymerase in Poxvirus's DNA genome replication (*14*). The monkeypox virus genome is typical of orthopoxviruses, in which the orthopoxvirus species homology genes are located in the center of the linear genome, and genes reflecting differences in tissue tropism, host range, and virulence

Table 1. published PCR assays to detect *Orthopoxvirus* and monkeypox virus

Targeted lineage	Gene target	primers and probes	Sequence (5'-3') and fluorescence labeling	Reference
Orthopoxvirus	I7L	F	TAATACTTCGATTGCTCATCCAGG	(*27*)
		R	ACTTCTCACAAATGGATTTGAAAATC	
		P	FAM-TCCTTTACGTGATAAATCAT-NFQ MGB	
	rpo18	F	CTGTAGTTATAAACGTTCCGTGTG	(*28*)
		R	TTATCATACGCATTACCATTTCGA	
		P	FAM-ATCGCTAAATGATACAGTACCCGAA T* CTCTACT p	
	E9L-NVAR	F	TCAACTGAAAAGGCCATCTATGA	(*11,29,30*)
		R	GAGTATAGAGCACTATTTCTAAATCCCA	
		P	TET-CCATGCAATATACGTACAAGATAGTAGCCAAC-QSY7	
Monkeypox	F3L	F	CTCATTGATTTTTCGCGGGATA	(*19,31*)
		R	GACGATACTCCTCCTCGTTGGT	
		P	FAM-CATCAGAATCTGTAGGCCGT-MGBNFQ	
	F3L	F	CATCTATTATAGCATCAGCATCAGA	(*32*)
		R	GATACTCCTCCTCGTTGGTCTAC	
		P	JOE-TGTAGGCCGTGTATCAGCATCCATT-BHQ1	
	N3R	F	AACAACCGTCCTACAATTAAACAACA	(*31*)
		R	CGCTATCGAACCATTTTTGTAGTCT	
		P	FAM-TATAACGGCGAAGAATATACT-MGBNFQ	
	B6R	F	ATTGGTCATTATTTTTGTCACAGGAACA	(*11,29,30*)
		R	AATGGCGTTGACAATTATGGGTG	
		P	MGB/DarkQuencher-AGAGATTAGAAATA-FAM	
	B7R	F	ACGTGTTAAACAATGGGTGATG	(*33*)
		R	AACATTTCCATGAATCGTAGTCC	
		P	TAMRA-TGAATGAATGCGATACTGTATGTGTGGG-BHQ2	
	G2R_G	F	GGAAAATGTAAAGACAACGAATACAG	(*9,28*)
		R	GCTATCACATAATCTGGAAGCGTA	
		P	FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-BHQ1	
Monkeypox West African-specific	G2R_WA	F	CACACCGTCTCTTCCACAGA	(*9,28*)
		R	GATACAGGTTAATTTCCACATCG	
		P	FAM-AACCCGTCGTAACCAGCAATACATTT-BHQ1	
Monkeypox Congo Basin-specific	C3L	F	TGTCTACCTGGATACAGAAAGCAA	(*9*)
		R	GGCATCTCCGTTTAATACATTGAT	
		P	FAM-CCCATATATGCTAAATGTACCGGTACCGGA-BHQ1	
Variola virus	B12R	F	ATGTTCAAGCTGTTAATATCAATCTCG	(*32*)
		R	TTTGCCACTGAACCATTCTATCAT	
		P	FAM-CTGTCGGAGCCACAGTTTCGAGACG-BHQ1	
	A38R	F	TCTGTACTATGTGTTAAAAGATTCTACAA	(*33*)
		R	AATGTATCTGTTATAGTCAGCATACCC	
		P	FAM-CGTTGATGGACACCACGTTTGTTATTA-BHQ1	
Cowpox virus	D11L	F	AAAACTCTCCACTTTCCATCTTCT	(*33*)
		R	GCATTCAGATACGGATACTGATTC	
		P	JOE-CCACAATCAGGATCTGTAAAGCGAGC-BHQ1	
Vaccinia virus	B10R	F	GGCAATGGATTCAGGGATATAC	(*33*)
		R	ATTTATGAATAATCCGCCAGTTAC	
		P	Cy5-CAATGTGTCCGCTGTTTCCGTTAATAAT-BHQ3	
Varicella-zoster virus	ORF38	F	AAACCGCACATGATAACGC	(*32*)
		R	GATTAGGACCATCCCCCG	
		P	TAMRA-ACAATGAGTAGTGGCTTTATGGCGAG-BHQ2	
Human DNA	RNase P	F	AGATTTGGACCTGCGAGCG	(*29,30*)
		R	GAGCGGCTGTCTCCACAAGT	
		P	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	

F: forward; R: reverse; P: probe; T* marks the position of the quencher.

are located in the terminal regions (*15*). The genome terminus contains an inverted terminal repeat of the same sequence but in opposite directions, and the genome is very low in guanine and cytosine (33%) (*16*). To help the scientific community quickly obtain monkeypox genomic information, the China National Center for Bioinformation (CNCB)-National Genomics Data Center (NGDC) has established an open-access information resource on monkeypox virus (MPXV): the Monkeypox Virus Resource (MPoxVR; *https://ngdc.cncb.ac.cn/gwh/poxvirus/*). MPoxVR has created BLAST, genome annotation, variant identification, and variant annotation, and these four commonly used online tools can be freely accessed (*17*). Another research team illustrated the frequency of variation at different positions in the monkeypox virus genome in the nucleotide (nt) context (*18*).

The Chinese technical plan for monkeypox prevention and control (2022 version) provides primers and probes for the monkeypox virus target gene F3L (*19*), which can be used for the detection of monkeypox virus by laboratory's self-established detection methods. Positive results obtained by PCR testing for orthopoxvirus also require confirmation of monkeypox virus by PCR and/or sequencing (*20*). We recommend a dual-target approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence (*21*). More than 30 monkeypox virus nucleic acid kits (developed by Sansure Bioretch, Shanghai ZJ Bio-Tech, Guangzhou Wondfo, Beijing Innovita, Hangzhou Dean Biotechnology, and Hotgen Biotech *etc*.) have obtained European Union CE certification, meaning that these kits can be marketed in European Union countries and countries that recognize European Union CE certification. But none of these products have been approved by the Chinese or US FDA. Roche Diagnostics has announced that it has developed three multiplex PCR solutions specific for orthopoxviruses and monkeypox virus clades to help researchers make rapid diagnoses. To standardize the technical review of monkeypox virus nucleic acid kits, the China Medical Device Evaluation Center organized and compiled the key points for the development of monkeypox virus detection methods (*https://www.cmde.org.cn/*). The China National Institute of Metrology has successfully developed two pseudoviruses, B6R wild type and F3L mutant, which are reference materials of monkeypox virus. The reference materials can be used as measurement standard for the development and performance evaluation of monkeypox virus detection kits, and provides traceability.

In addition, the type and quality of the specimen, and the type of laboratory testing are important for the confirmation of monkeypox infection. Samples from skin lesions (*22,23*) – fluid from vesicles and pustules, and dry crusts are the best diagnostic samples for monkeypox. During sample collection, a swab will be swabbed vigorously over the rash lesions and on more than one lesion. If monkeypox is suspected, professionals should collect appropriate samples and transport them safely to the appropriate laboratory. Packaging and shipping of the specimens should follow national and international requirements. Patient blood samples should not be routinely collected because PCR blood tests are usually inconclusive due to the short duration of viremia relative to symptoms onset. Prolonged shedding of viral DNA from the upper respiratory tract after resolution of skin lesions challenges current infection prevention and control guidelines (*24*).

Monkeypox virus culture should be carried out in a Biosafety Level 3 (BSL-3) laboratory, and monkeypox related inactivation materials should be operated in a Biosafety Level 2 (BSL-2) laboratory. Therefore, sample tubes containing guanidine salts (guanidine isothiocyanate or guanidine hydrochloride, *etc*.) or surfactants can be used to ensure viral inactivation for biosafety clinical detection. Staff are advised to be vaccinated if possible.

2. Monkeypox virus antigens and antibodies detection

The mainstream immunoassay method for the rapid detection of monkeypox virus antigens and antibodies is the colloidal gold method. Beijing Hotgen Biotech announced that the antigen detection kit of monkeypox virus (colloidal gold method) has been certified by the UK MHRA. Types of samples for monkeypox antigen detection are skin lesion swabs (swabs of lesion tops, exudate of rash or growth) and lesion crusts. In 2005, investigators found that anti- monkeypox virus IgM test performed between day 5 and day 77 post rash onset was 94.8% sensitive and 94.5% specific in both vaccinated and unvaccinated patients. In patients unvaccinated or monkeypox virus naïve, assay of anti- monkeypox virus IgG from day 14 onwards was 100% sensitive and 88.5% specific (*25*). Orthopox-specific IgM can be detected by in-depth serological assays in cynomolgus macaques detected during the 6-day incubation period before the rash development (*26*). The anti- monkeypox virus IgG in these animals produced 23 distinct recognizable *Orthopoxvirus* proteins. A diagnosis of monkeypox virus infection is possible if IgM and IgG antibodies are detected in the serum of an unvaccinated individual with a history of rash and severe illness. Antigen and antibody assays cannot make a monkeypox confirmation because of the serological cross-reactivity of orthopoxviruses, but serologic testing may be feasible in areas where monkeypox virus is endemic. Currently, testing is only recommended if a patient has a rash consistent with monkeypox. The diagnosis of monkeypox cannot rely solely on plasma/serum antibody test, and a serological detection can be used to further investigate past infection for epidemiological tracing.

3. Conclusion

Due to the serological cross-reactivity of orthopoxviruses, antigen and antibody detection methods cannot make the monkeypox-specific confirmation. PCR is the laboratory test of choice because of its high accuracy and sensitivity. At present, there is no monkeypox PCR or serological test kit approved for marketing in China. Due to the severe situation of the global monkeypox epidemic worldwide, it is necessary to accelerate the pace of product development. We recommend a dual-target PCR approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence.

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