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REVIEW PAPER

Molecular detection of monkeypox and related viruses: challenges and opportunities

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Abstract

The recent widespread emergence of monkeypox (mpox), a rare and endemic zoonotic disease by monkeypox virus (MPXV), has made global headlines. While transmissibility ($R_0 \approx 0.58$) and fatality rate (0–3%) are low, as it causes prolonged morbidity, the World Health Organization has declared monkeypox as a public health emergency of international concern. Thus, effective containment and disease management require quick and efficient detection of MPXV. In this bioinformatic overview, we summarize the numerous molecular tests available for MPXV, and discuss the diversity of genes and primers used in the polymerase chain reaction-based detection. Over 90 primer/probe sets are used for the detection of poxviruses. While hemagglutinin and A-type inclusion protein are the most common target genes, tumor necrosis factor receptor and complement binding protein genes are frequently used for distinguishing Clade I and Clade II of MPXV. Problems and possibilities in the detection of MPXV have been discussed.

Keywords Diagnostics · Emerging infectious diseases · Epidemiology and public health · Molecular evolution · Orthopoxvirus · Viral genome surveillance

Introduction

The emergence of new pathogenic viruses is a constant threat to humanity as each year novel viruses are evolving [1]. Unprecedented global changes—population growth, increased trade and travel, and climate change—in the recent decades have made the threat of re-emergence of viruses even more likely [2]. The recent emergence of monkeypox (renamed as mpox by the World Health Organization on 28 Nov 2022) disease by monkeypox virus (MPXV) has made global headlines. Between 13 and 21 May 2022 over 92 monkeypox cases were reported from 12 countries [3]. Although there was a major outbreak of monkeypox in the United States in 2003 via prairie dogs infected from an imported Gambian pouched rat [4, 5], the latest unrelated outbreaks in multiple countries have caused serious concerns since the coronavirus disease (COVID) pandemic and pressed for the need of disease surveillance and viral detection.

Monkeypox is a rare zoonotic disease endemic to the African continent—mainly in the northern and central Democratic Republic of the Congo (DRC). Although the disease has been reported only sporadically in neighbouring

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Republic of the Congo (ROC) regions, it has emerged several times in west and central Africa between 1970 and 2017 [6]. The MPXV was first identified in 1958 in crab-eating macaque monkeys (*Macaca fascicularis*), but multiple animal species including rodents can easily transmit the virus [7]. The MPXV is related to variola virus (VARV) that causes smallpox, both belonging to the genus *Orthopoxvirus*.

Compared to smallpox, monkeypox has a much lower infectivity or human to human transmission with basic reproduction number (R_0) < 1 [8, 9], perhaps ≈0.576 [10], and an attack rate (the fraction of at-risk population that contracts the disease in a specified time interval) of ≈50% [11]. While antiviral drug ST-246, a potent *Orthopoxvirus* egress inhibitor, can protect non-human primates from VARV or MPXV [12], smallpox vaccine (for example, Dryvax) seems 85% effective in humans against monkeypox [13]. The MPXV has two distinct clades [14]. The Clade I causes illness similar to smallpox and has a case fatality rate of up to 10% in unvaccinated populations. The Clade II causes less severe and less inter-human transmissible disease [15, 16].

The disease severity of monkeypox, compared to smallpox, is considerably less with milder rash. However, due to its lengthy incubation period of up to 21 days and prolonged morbidity of two to four weeks, the World Health Organization has declared monkeypox as a public health emergency of international concern. Thus, effective containment and disease management require quick and efficient detection of MPXV. In this bioinformatic overview, we summarize the numerous molecular tests available for MPXV, discuss the diverse genes and primers used in the polymerase chain reaction (PCR)-based detection, and highlight the challenges in detecting MPXV and discriminating different clades and poxviruses.

Methods of detection

There are numerous ways to detect MPXV at the molecular level using appropriate patient specimens. While any type of body fluid might be used, blood in particular, was not found to contain high level of virus. On the other hand, lesions exudate on a swab or crust are considered as the best and least invasive patient samples [17].

Detection of viral particles

Live virus can be grown from patient lesion samples using chorioallantoic membrane or other cell-based viral culture methods. However, apart from the necessity of a fresh/live sample, this approach takes several days. In addition, it requires further characterization for the identification of viral particles. The electron microscopy is used for the conventional physical characterization. However, even if the negative staining reveals a brick-shaped viral particle, as a general form, it cannot be used to distinguish MPXV from other orthopoxviruses [17]. As our emphasis is on molecular detection, this section is not elaborated in depth.

Detection of antigens/antibodies

The easiest way to detect MPXV is to test for the virus-specific antigens in biopsy or other samples. One such method is called specific peptide-based rapid antigen test (RAT) [18]. The peptide-based antigens depend on specific peptides which bind to targets designed using protein–protein interactions. Highly conserved regions of interacting proteins of antigen are used to detect the virus. While RAT might be easy and quick, it might not be specific to MPXV due to high molecular similarities among numerous orthopoxviruses [17]. Despite this, RAT is being utilized for the specific detection of MPXV [19]. Ignoring specificity issues if any, one advantage of RAT is that there might be 100 s or 1000 s of copies of antigens on a virion which greatly increase the possibility of detection compared to a single copy of nucleic acid target for PCR [18]. While RAT might be useful for quick and largescale screening/diagnostics as in COVID-19, it is found to have considerable drawback due to its high false negative rate when the virus load is low [20, 21].

Alternatively, the virus-specific antibodies can also be detected in patient samples. The presence of anti-*Orthopoxvirus* immunoglobulin M (IgM) antibodies indicate a recent exposure to *Orthopoxvirus* or it might also be due to smallpox vaccination. However, this can be used as a diagnostic test for *Orthopoxvirus* infection if prior smallpox vaccination was not so recent, perhaps up to six months [17, 22]. On the other hand, the presence of anti-*Orthopoxvirus* IgG antibodies indicate a previous (and not so recent) exposure to *Orthopoxvirus* or smallpox vaccination. Known positive and negative serum samples are always used as assay controls [4].

A number of specific methods such as complement fixation test, hemagglutination inhibition assay, enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test, western blot, and electrochemiluminescence (ECL) assay can be used for the detection of antigens or antibodies [4, 7, 22].

The main limitation of antibody-based detection methods is that it is invasive—requires the collection of patient blood/serum samples. In addition, it might also require a cold storage facility for samples/reagents. Further, the assay is not specific to MPXV as it only assesses a previous exposure to any *Orthopoxvirus* [4] and is affected by prior smallpox vaccination. However, one particular advantage of serological tests is that the polyclonal nature of the immune

response permits broader/robust detection compared to the species-specific PCR method that might fail due to mutations in the short genome targets [4].

Mass-spectrometry-based detection

Qualitative and quantitative proteomics using liquid chromatography tandem mass spectrometry (LC–MS/MS) were also used to differentially identify MPXV proteins from other orthopoxviruses. Manes et al. [23] identified numerous MPXV-specific proteins such as J2L (tumor necrosis factor receptor homologue), J3L and D7L (ankyrin repeat containing proteins), and F6R (NF-κB inhibitor), and vaccinia virus-specific proteins such as C22L (tumor necrosis factor receptor homologue), C10L (ankyrin repeat containing protein), and C1L (complement-binding host defence modulator). Similar quantitative proteomics strategy also identified cowpox virus-specific proteins such as host range factor CP77 and secreted chemokine binding protein CPXV-GRI D1, and showed the overrepresentation of A-type inclusion protein in vaccinia virus [24].

Eshoo et al. [25] developed a detection method combining PCR and electrospray ionization-mass spectrometry (PCR/ESI–MS) for the identification of all *Orthopoxvirus* members based on DNA and RNA helicase and polymerase genes. The method was able to resolve viruses at sub-species level. The PCR/ESI–MS technique was also used to detect MPXV in spiked human blood and aerosol-infected cynomolgus macaque samples, as well as to identify MPXV and vaccinia virus spiked into macaque blood sample at various concentration [26]. The authors claimed that the technique was able to identify all *Orthopoxvirus* members in a single assay along with quantitative identification of MPXV DNA in clinical specimens, thus eliminating the need for sequencing.

Detection of virus-specific nucleic acid targets

There are numerous variants of PCR-based methods for the detection of *Orthopoxvirus*/MPXV genomic targets (Table 1 and S1). Over 90 primer/probe sets (see Methods in the Supplemental information) are used to target as many as 38 poxvirus genes (Table S1). Ropp et al. [27] used conventional PCR to amplify near-full-length hemagglutinin (HA, also known as B2R) gene and endonuclease digest electropherograms to distinguish 10 species of orthopoxviruses, including North American orthopoxviruses and MPXV. However, given the close similarities among sequences, primers were not exclusive to species (Table 1 and S1, Fig. S1 and S2) and thus cross-hybridized to multiple members of the genus. Further, the use of multiple endonuclease cleavage profiles overly complicated the species identification. In addition, no attempts were made to distinguish different clades/variants of MPXV.

Nonetheless, with at least 29 unique primer sets (Table S1), HA gene was the most common target used for the detection of orthopoxviruses [7, 13, 27–33]. Yet, due to its high sequence similarity among different *Orthopoxvirus* members (Fig. S2), HA gene was not used for the specific detection of MPXV or its clades/variants. For example, an rt-PCR primer and probe set used by Edghill-Smith et al. [13] can detect over a dozen orthopoxviruses. Interestingly, with a unique primer set design, HA gene can be used for the specific detection of MPXV from other orthopoxviruses. For example, the MPXV and cowpox virus have a six-nucleotide deletion at around 431, whereas only cowpox virus has additional six-nucleotide deletions at 323 and 533 (Fig. S2).

The A-type inclusion (ATI, also known as A27L) protein/gene was the second most common target [34–37]. The MPXV ATI gene has 72 to 95.3% identity with other *Orthopoxvirus* species such as variola, vaccinia, cowpox, ectromelia, and camelpox viruses. By designing a primer to the region containing 8-bp deletion in MPXV, Neubauer et al. [36] differentiated 19 strains of MPXV from five *Orthopoxvirus* species. While Saijo et al. [37] used rt-PCR, Iizuka et al. [34] used loop-mediated isothermal amplification (LAMP) method to detect MPXV (Table 1). Both the sets of primers were designed such that the non-coding region downstream of ATI gene was also included. Further, given the large difference in this stretch of genome (Fig. S1) due to multiple long indels, the primers/methods could uniquely detect Clade I and Clade II of MPXV.

The reverse transcription polymerase chain reaction (rt-PCR) is a standard method for the universal detection of poxviruses [11]. Numerous variants of the method—for example—multiplexed PCR, assay based on dried PCR reagent, etc. have been tried and tested. Combination of primers and/or probes were also used. For example, only variola virus was specifically detected by a FAM (6-carboxyfluorescein)-labelled probe while camelpox, cowpox, monkeypox, and vaccinia viruses were detected by a TET (6-carboxytetramethylrhodamine)-labelled probe in a single PCR reaction [29]. Davi et al. [38] used recombinase polymerase amplification (RPA) assay targeting the G2R (=J2R) gene for the specific and rapid detection of MPXV within 3 to 10 min. The RPA-assay was claimed to be highly sensitive with a limit of detection of 16 DNA molecules/μl.

The tumor necrosis factor receptor (TNFR, also known as J2R) gene in particular seems to be a good target for the specific detection of MPXV and its two clades—Clade I and Clade II. For example, Li et al. [16] used two sets of rt-PCR primers and probes. While the first set of primers/probes would potentially detect MPXV over other PXVs (Fig. 1A), a second set of primers/probes seemed specific

Table 1 List of commonly used genes, primers, and probes in the detection of MPXV

Gene	Forward and reverse primers [and probes]	Len	Assay	References
A4L (CP)	GTCAACGCTGGAAGGAGTG CCAGCAGACAGCCTATCC [CTCCTGTACTAAAACCACGWCAACAAACT]	217	rt-PCR	[52]
A27L (ATI)	AATACAAGGAGGATCT CTTAACTTTTTCTTTCTC	1549	PCR	[35]
	GAGAGAATCTCTTGATAT ATTCTAGATTGTAATC	601	PCR	[36]
	GAGATTAGCAGACTCCAA GATTCAATTTCCAGTTTGTAC [GCAGTCGTTCAACTGTATTTCAAGATCTGAGAT/ CTAGATTGTAATCTCTGTAGCATTTCCACGGC]	163	rt-PCR	[37]
	CCGTTACCGTTTTTACAATCGTTAATCAATGCTGATATGGAAAAGAGA ATAGGCTAAAGACTAGAATCAGGGATTCTGATTCATCCTTTGAGAAG	–	LAMP	[34]
	TACAGTTGAACGACTGCG AGTTCAGTTTTATATGCCGAAT	221		
	GATGTCTATCAAGATCCATGATTCT TCTTGAACGATCGCTAGAGA	115		
	TGGAGTCTGCTAATCTCTGTAAGATTAGAGAACTAGAGAATAAGTTGACC TGAGTGAATGCCGTGGAAATGCGCAGTCGTTCAACTGTA	–		
	CACAAGAAGTTGATGCACTG CAGCATTGATTTCATTATTACGT	235		
	CGCTCTCGATGCAGTC CAGAGATTACAATCTAGAATCTCAG	128		
A29L (14kd protein)	CCAGAGATATCATAGCCGCTCTT GAAACTCTCAAACAACGRCTAACT [TAAATAGAACGTCATCATT]	156/157	rt-PCR	[53]
B2R (HA)	ATGACACAATTACCAATAC CTAGACTTTGTTCTCTG	942	ORF	[27]
	CTGATAATGTAGAAGAC TTGTATTTACGTGGGTG	406	PCR	
	GATGATGCAACTCTATCATGTA GTATAATTATCAAAATACAAGACGTC [AGTGCTTGGTATAAGGAG]	131	rt-PCR	[13]
B6R (EEV)	ATTGGTCATTATTTTTGTCACAGGAACA AATGGCGTTGACAATTATGGGTG [AGAGATTAGAAATA]	83	rt-PCR	[48]
B7R	ACGTGTTAAACAATGGGTGATG AACATTTCCATGAATCGTAGTCC [TGAATGAATGCGATACTGTATGTGTGGG]	99	rt-PCR	[54]
C3L/D14L	TGGGAGCATTGTAACTTATAGTTGCCCTCCTGAACACATGACA ATCCTCGTATCCGTTATGTCTTCCCACCTATTTGCGAATCTGTT	-	LAMP	[34]
	TGGGTGGATTGGACCATT ATGGTATGGAATCCTGAGG	199		
	GATATTCGTTGATTGGTAACTCTGG GTTGGATATAGATGGAGGTGATTGG	117		
	TGTCTACCTGGATACAGAAAGCAA GGCATCTCCGTTTAATACATTGAT [CCCATATATGCTAAATGTACCGGTACCGGA]	100	rt-PCR	[16]
E9L (DNA polymerase)	TCAACTGAAAAGGCCATCTATGA GAGTATAGAGCACTATTTCTAAATCCCA [CCATGCAATATACGTACAAGATAGTAGCCAAC]	101	rt-PCR	[48]
F3L	CTCATTGATTTTTCGCGGGATA GACGATACTCCTCCTCGTTGGT [CATCAGAATCTGTAGGCCGT]	107	rt-PCR	[7]

Table 1 (continued)

Gene	Forward and reverse primers [and probes]	Len	Assay	References
	CATCTATTATAGCATCAGCATCAGA GATACTCCTCCTCGTTGGTCTAC [TGTAGGCCGTGTATCAGCATCCATT]	79	rt-PCR	[55]
J2R (TNFR)	CACACCGTCTCTTCCACAGA GATACAGGTTAATTTCCACATCG [AACCCGTCGTAACCAGCAATACATTT]	82/85	rt-PCR	[16]
	GGAAAATGTAAAGACAACGAATACAG GCTATCACATAATCTGGAAGCGTA [AAGCCGTAATCTATGTTGTCTATCGTGTCC]	90		
	AATAAACGGAAGAGATATAGCACCACATGCAC GTGAGATGTAAAGGTATCCGAACCACACG [ACAGAAGCCGTAATCTATGTTGTCTATCGQTFCCTCCGGGAACTTA]	181	RPA	[38]
N3R	AACAACCGTCCTACAATTAAACAACA CGCTATCGAACCATTTTTGTAGTCT [TATAACGGCGAAGAATATACT]	139	rt-PCR	[7]

LAMP loop-mediated isothermal amplification, *ORF* open reading frame, *RPA* recombinase polymerase amplification, *rt-PCR* real-time polymerase chain reaction

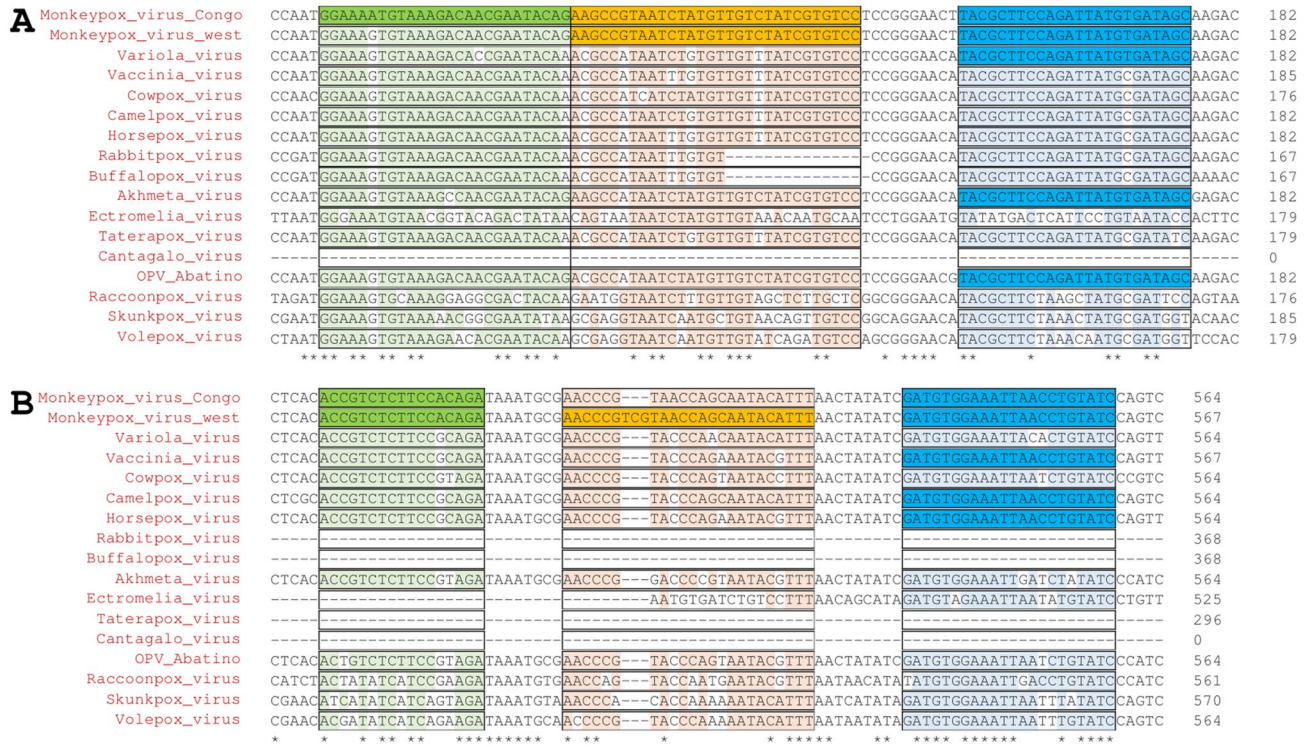

Fig. 1 Detection of MPXV using TNFR (J2R) gene. Li et al. [16] proposed two sets of primers and probes for the specific rt-PCR detection of MPXV and its two clades—Clade I and Clade II. **A** While the primer GGAAAATGTAAAGACAACGAATACAG seems specific for Clade I, it might bind non-specifically to Clade II MPXV and other PXVs. However, due to multiple mismatches with probe, other PXV sequences might unlikely to be detected. On the other hand, **B** the probe AACCCGTCGTAACCAGCAATACATTT seems to be specific for Clade II MPXV due to a three-nucleotide insertion in the sequence. Forward (green) and reverse (blue) primers and probes (orange) are shaded

for Clade II of MPXV due to a three-nucleotide insertion in the sequence (Fig. 1B).

C3L/D14L gene was another target used in either LAMP [34] or rt-PCR method [16]. It seems that C3L/D14L is completely missing in the Clade II of MPXV. However, as many orthopoxvirus (OPV) members such as vaccinia virus (VACV) and VARV have a highly similar C3L/D14L sequence, it would be very misleading to use C3L/D14L as a distinguishing target for the detection of the Clade I of MPXV.

The Pan American Health Organization (PAHO) interim guidance on laboratory testing for monkeypox virus mentions that while commercial PCR kits for detecting OPV and MPXV in particular are under development, no validated PCR kits are available in the market at present. PAHO/WHO currently follow two protocols for OPV and MPXV detection [39]. The first one involves subjecting the samples (lesion swabs, vesicular fluids, and crusts) from suspected cases to OPV rt-PCR and the positive samples are subjected to MPXV-specific rt-PCR. In the second one, the samples are directly subjected to MPXV-specific rt-PCR followed by differentiation of Clade I and Clade II. The PAHO/WHO uses primers/probes specific for G2R (=J2R) and C3L/D14L genes that have several issues as discussed in detail in this review.

Detection of MPXV: challenges and opportunities

The 196,858-bp MPXV genome has at least 190 open reading frames of ≥ 60 amino acid residues each [40]. However, it seems that just 11 genes have been used as likely targets (Table 1). The choice of target for the specific detection of MPXV is important but tricky. The sequence in the central region of the MPXV genome, which encodes essential enzymes and structural proteins, is 96.3% identical to VARV. On the contrary, MPXV and VARV are said to have considerable differences in the regions encoding virulence and host-range factors near the ends of the genome [41].

While highly similar sequences lead to nonspecific primer/probe binding and lead to ambiguous detection of OPVs, highly divergent or variable sequence regions pose considerable challenge to detection. As in other viruses [42], comparison among OPVs such as VACV and VARV revealed that indels of 3–25 bp are common events in poxviruses [43]. The A27L (ATI) gene is one such hypervariable target containing variations including truncations, deletions, insertions, and base changes in the two clades of MPXV [35]. The C3L/D14L gene is completely missing in Clade II [16]. While these targets are often used to differentiate Clade I and Clade II of MPXV [37], given the inherent issues in the molecular detection, a negative result should not be taken as a confirmation. For, poxviruses can undergo rapid changes as VACV was shown to acquire 7–10% increase in genome size via K3L gene amplification [44] and there are considerable differences among MPXV clades [45].

Further, like in all viruses, the evolution of the genome due to ongoing point-mutations is leading to additional challenges in detection. Compared to RNA viruses such as Poliovirus-1 that has a very high rate of 0.01 substitutions per site per year, the MPXV has a far lower rate of 7×10^{-7} [46]. Yet, different MPXV/OPV sequences contain or can acquire a considerable number of substitutions (Fig. 1, S2, and S3), which may result in undesirable outcomes. For example, primers with one or more mismatches can bind to templates and effect PCR amplification by < 1.5 to > 7 cycle threshold [47]. The substitutions are also problematic in restriction fragment length polymorphism-based detection [27]. Thus, it is advisable to use multiple targets and primer/probe sets for the unambiguous detection of MPXV [7, 48].

While rt-PCR is the state-of-the-art method, it is a costly, highly sophisticated and laborious/multistep process, needing fine instrumentation and technical expertise. The development of LAMP and RPA overcomes some of these issues, even as MPXV detection urgently requires a validated test kit [39].

Conclusions and future outlook

Since its recent emergence, at least 84,639 cases of MPXV and 80 deaths have been reported from 110 countries [49, 50]. At present it is not a serious concern per se. However, given that MPXV has multiple natural hosts—mainly rodents—that find expanding conducive geographical range, pet trade, intercontinental travel, etc. all point to the possibility that monkeypox infections could continue to intensify or re-emerge in the future [51]. Although smallpox vaccination or infection has given some immunity, MPXV is a persistent threat. Thus, for effective disease surveillance, easy, quick, and effective viral detection is required. As explored in this work, over 90 primer/probe sets are used for the detection of poxviruses. HA and ATI are the common target genes, whereas TNFR and C3L are frequently used for distinguishing Clade I and Clade II of MPXV. There are multiple issues in the primer/probe sets and/or targets used in the detection of MPXV that require considerable attention while developing a validated test kit [39].

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Data availability The data used in this work were collected/curated from the literature/NCBI. Relevant data are given in the supplemental information section.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval The work is in compliance with ethical standards. No ethical clearance was necessary.

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