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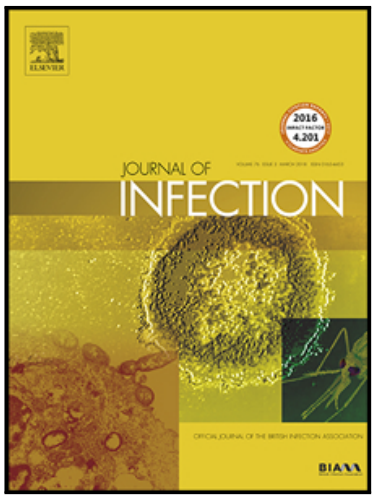

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Development of monoclonal antibody-based antigens detection assays for Orthopoxvirus and Monkeypox virus: Reply to Yutong Sui et al.

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Dear Editor:

We read with great interest the paper by Yutong Sui et al., which developed a CRISPR-Cas12a-based detection assay for monkeypox virus (MPXV)[1]. Nucleic acid detection and antigen detection are two important methods for the diagnosis of the infection of the viruses, the two methods complement each other. The CRISPR-based assay developed by Sui et al. provides a visual and fast alternative for the diagnosis of the monkeypox virus, this assay usually requires a nucleic acid enrichment process, and in this study, samples from authentic MPXV were not used for testing. Rapid antigen detection tests are highly valued in the detection of potentially infectious patients, as they can provide prompt results in a cost-effective manner. As a complement, we develop monoclonal antibody-based antigens detection assays for the rapid detection of Orthopoxvirus and MPXV.

MPXVis a zoonotic virus that was first discovered in monkeys in 1958 and in humans in 1970[2], belongs to the orthopoxvirus, which also includes smallpox, cowpox, camelpox, and vaccinia viruses. Since May 2022, human cases of MPXV infection have been reported persistently in non-MPXV-endemic regions of Europe and North America and MPXV-endemic regions of Central and West Africa. As the

number of human cases of MPXV infection continues to rise, the World Health Organization (WHO) declared on July 23, 2022, that monkeypox outbreaks in several countries and regions have amounted to a "Public Health Emergency of International Concern" (PHEIC), raising the MPXV epidemic to the same level as the COVID-19 pandemic. As of October 21, 2022, 109 countries and regions have reported cases of MPXV, bringing the total number of confirmed cases to 75,348[3]. The infection of MPXV can have a significant impact on multiple organ systems of the host, including the skin and mucosal barriers, lymphatic, lung, and gastrointestinal tract, the skin of an infected person can be severely exfoliated, and airway inflammation and bronchoppneumonitis resulting from infection can limit air intake and reduce the willingness and/or ability to ingest food and water[4]. Of note, severe MPXV infection can result in death, with a mortality rate of 10.6% the for clade I virus and 3.6% the for clade II virus[5]. The proportion of asymptomatic MPXV infection is more than 13%, and the symptoms are often "atypical", which is easy to be misdiagnosed as other diseases, such as venereal diseases and COVID-19, therefore, the hidden transmission risk of MPXV is great[6]. Timely diagnosis of MPXV infection can prevent the wide spread of this virus and facilitate prompt treatment and recovery of infected persons. Although some monoclonal antibodies (mAbs) against poxviruses have been reported previously[7-9], there are few studies on the specific diagnosis against the antigen of MPXV. In this study, three mouse mAbs against the A29L of MPXV were developed, using enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA), orthopoxvirus and MPXV can be sensitively and rapidly detected by two independent antibody pairs. These antibody-based A29L detection assays against MPXV only take 10-15 minutes and no specific equipment in a clinical laboratory is required.

We synthesized the expression sequence of A29L of clade II MPXV (Genbank number YP_010377135.1) and expressed and purified the A29L protein using E. coli prokaryotic expression system. Several MPXV A29L protein-specific mAbs were generated by immunization of 6-8-week-old BAL b/c mice with purified A29L using the murine hybridoma technique. The binding activity of all antibodies was preliminarily identified using the supernatant of hybridoma cells, and three of them were found to reveal good binding reactivity to A29L antigen, designated 3A1, 8F8 and 2D1, respectively. The three mAbs were produced in large quantities (Fig 1A), and the variable region sequences of the antibodies were obtained by polymerase chain reaction using specific primers[10] (Fig 1B). Then, we tested the binding reactivity of the three antibodies against MPXV A29L, camelpox virus A27L, and the corresponding membrane proteins of taterapox virus using the ELISA. 3A1 and 9F8 showed decent binding activity to the three distinct poxvirus antigens, the minimum reaction limits of 3A1 to the antigens of monkeypox, camelpox and taterapox were 256.00 pg/mL, 1280.00 pg/ ml and 1280.00 pg/ ml, respectively (Fig C-E), the minimum reaction limits of 9F8 to the antigens of monkeypox, camelpox and taterapox were 1280.00, 1280.00 and 1280.00 pg/mL, respectively (Fig F-H). 2D1 antibody showed good binding activity only to MPXV A29L, with a minimum reaction limit of 256.00 pg/mL (Fig I), and weak binding activity against camelpox

and taterapox antigen proteins, with the minimum reaction limits of 4 μg/mL (Fig J and K). Next, we performed double antibody pairs sandwich-ELISA experiments base on the above three antibodies and found that one pair was sensitive for the detection of the antigens from all orpoxviruses, and the other pair specifically detected MPXV antigen only. Using 9F8 as capture antibody and 3A1 as detection antibody can detect all three orthopoxvirus antigens in a low concentration, the minimum detection limits for the antigens of monkeypox, camelpox and taterapox were 128.00, 640.00 and 3200.00 pg/mL, respectively (Figure L-N). Using 3A1 as the capture antibody and 2D1 as the detection antibody, we can detect MPXV A29L in a low concentration, with the minimum detection limit of 128.00 pg/mL (Fig O), this antibodies pair failed to detect antigens from camelpox and taterapox viruses (Fig P and Q). Only vaccinia virus (Tiantan strain) has been successfully cultured in our laboratory, and authentic MPXV and related clinical infection samples have not been obtained. In a further experiment, we tested the detection activity of the above two antibody pairs against the authentic vaccinia virus and found that when using the pair of 9F8-3A1, the vaccinia virus could be detected at the level of 280 PFU (Fig R), in contrast, when used the pair of 3A1-2D1, vaccinia virus could not be detected. This data was consistent with the result that pair of 9F8-3A1 could detect all poxvirus antigens, whereas pair of 3A1-2D1 could only specifically detect MPXV antigen in the ELISA assay (Fig S). Finally, we used LFIA technology to develop a rapid detection kit for poxvirus based on 9F8-3A1 antibody pairing, which was still reactive to the vaccinia virus at a concentration of 250 PFU (Fig T), and a specific rapid detection kit for monkeypox virus based on 3A1-2D1 antibody pairing, which is still reactive to A29L antigen at a concentration of 10 ng/mL (Fig U). These two kits can get the detection result within 10-15 minutes, without professional equipment. The antibodies developed in this study can be applied to the rapid detection of orthopoxvirus and the rapid detection of MPXV specifically. MPXV is still prevalent worldwide and the epidemic area is still expanding at present, the findings in this study may provide a powerful tool for the prevention and control of MPXV epidemic. In the further study, we will try to contact the organizations where authentic MPXV was isolated and clinical samples from MPXV infection have been obtained, and test the reactivities of our diagnostic kits against these kinds of samples.

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Competing interests

The authors declare no competing interests.

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Figures and Figure legends

Fig. 1 | Purifications, CDR sequences, antigen binding activities and double antibody sandwich antigen or virus detection activities of three monoclonal antibodies against A29L protein of MPXV. (**A**) SDS-PAGE analysis of purified 3A1, 9F8 and 2D1 antibodies

which specific to A29L of MPXV. (**B**) CDR sequences of 3A1, 9F8 and 2D1 antibodies. (**C**) Reactivity of 3A1 with MPXV A29L protein in ELISA. (**D**) Reactivity of 3A1 with camelpox A27L protein in ELISA. (**E**) Reactivity of 3A1 with taterapox IMV protein in ELISA. (**F**) Reactivity of 9F8 with MPXV A29L protein in ELISA. (**G**) Reactivity of 9F8 with camelpox A27L protein in ELISA. (**H**) Reactivity of 9F8 with taterapox IMV protein in ELISA. (**I**) Reactivity of 2D1 with MPXV A29L protein in ELISA. (**J**) Reactivity of 2D1 with camelpox A27L protein in ELISA. (**K**) Reactivity of 2D1 with taterapox IMV protein in ELISA. (**L**) Detection of MPXV A29L protein using the 9F8-3A1 sandwich ELISA. (**M**) Detection of camelpox A27L protein using the 9F8-3A1 sandwich ELISA. (**N**) Detection of taterapox IMV protein using the 9F8-3A1 sandwich ELISA. (**O**) Detection of MPXV A29L protein using the 3A1-2D1 sandwich ELISA. (**P**) Detection of camelpox A27L protein using the 3A1-2D1 sandwich ELISA. (**Q**) Detection of taterapox IMV protein using the 3A1-2D1 sandwich ELISA. (**R**) Detection of authentic vaccinia virus using the 9F8-3A1 sandwich ELISA. (**S**) Detection of vaccinia virus using the 3A1-2D1 sandwich ELISA. (**T**) The rapid detection assay of a gradient concentration of vaccinia virus, in the format of LFIA, using LFIA test strip and two highly specific monoclonal antibodies against the A29L protein (9F8 for coating and 3A1 for conjugating). (**U**) The rapid detection assay of a gradient concentration of MPXV A29L protein, in the format of a LFIA, using LFIA test strip and two highly specific monoclonal antibodies against the A29L protein (3A1 for coating and 2D1 for conjugating). NC-Ab, a negative antibody which specific to SARS-CoV-2 generated by our laboratory. NC-Ag, a negative antigen (a receptor binding domain protein of SARS-CoV-2). NC-Virus, an influenza virus strain.