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Serological methods for the detection of antibodies against Monkeypox virus applicable for laboratories with different biosafety levels

Marica Grossegesse[1], Daniel Stern[1], Natalie Hofmann[1], Claudia Kohl[1], Janine Michel[1], and Andreas Nitsche[1]

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June 20, 2023

Abstract

The monkeypox virus (MPXV) outbreak in 2022 has renewed interest in the detection of antibodies against orthopox viruses (OPXV) and MPXV, as serological methods can aid diagnostics and are key to epidemiological studies. Here three complementary serological methods are described with different strengths to aid the development and evaluation of in-house assays: An immunofluorescence assay (IFA) for specific detection of IgG and IgM, an enzyme-linked immunosorbent assay (ELISA) for higher sample throughput to aid epidemiological studies and a neutralization test (NT) to detect virus neutralising antibodies. As implementation of MPXV-specific diagnostics is often hampered by the requirement for a dedicated biosafety level 3 laboratory (BSL-3), the focus is on biosafety aspects to facilitate safe testing also under BSL-2 conditions. To this aim, IFA and NT titres of sera are compared after MPXV or cowpox virus infection or vaccinia virus immunization by using both homologous and heterologous antigens. Because of a large degree of cross-reactivity, vaccinia virus could be used as a less virulent alternative to MPXV, as VACV can be handled under BSL-2 conditions. Furthermore, an inactivation method to remove potentially infectious virus particles from viraemic sera without compromising antibody detection was developed. Although the existing immunological cross-reactivity between OPXV prevents species differentiation by general serological methods, it also provides an opportunity for the interchangeable usage of different OPXV species in serological assays, enabling MPXV serology outside of BSL-3 facilities.

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Running title: Methods for MPXV antibody detection

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The monkeypox virus (MPXV) outbreak in 2022 has renewed interest in the detection of antibodies against orthopox viruses (OPXV) and MPXV, as serological methods can aid diagnostics and are key to epidemi-

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Key words:

monkeypox virus, orthopox virus, antibody detection, serology, virus inactivation

Introduction

Monkeypox viruses (MPXV) belong to the family of *Poxviridae* , subfamily *Chordopoxvirinae* , and the genus of Orthopoxviruses (OPXV). OPXV comprise mostly animal-borne viruses with zoonotic potential such as cowpox virus (CPXV), vaccinia virus (VACV) and camelpox virus, amongst others [1]. However, the smallpox-causing Variola virus (VARV) also belongs to the OPXV genus, and infection with MPXV can potentially result in a smallpox-like disease. Until 2022 MPXV has historically been endemic in Central and Western Africa. However, between May 2022 and the writing of this manuscript in May 2023, WHO reports more than 87,000 laboratory-confirmed MPXV infections (Mpox) with about 140 fatalities reported internationally from 111 countries, especially from Europe and the Americas, with the Mpox outbreak being sustained by human-to-human transmission via close contact [2]. After entry through a mucous membrane or the skin, MPXV can replicate in the local endothelial tissue and spread via the blood stream to infect other tissues and organs. Following a second viraemia which enables further virus dissemination, the characteristic lesions appear on the skin [3, 4]. Typical symptoms of Mpox include, amongst others, the characteristic skin lesions, fever, headache, lethargy or exhaustion as well as more systemic respiratory and gastrointestinal symptoms [5]. While Mpox is typically self-limiting, some patients experience complications such as encephalitis and require hospitalization [6]. Whilst the case fatality rate during global outbreaks has remained relatively low (about 0.03 %), in previously endemic areas in Central and Western Africa, reported case fatality rates ranged from 3 to 6 % [7] which, combined with other factors, resulted in MPXV being classified as a risk group 3 biological agent. In contrast, CPXV is endemic in Europe with sporadic human cases being directly linked to contact with infected animals, with no proven human-to-human transmission. CPXV infection in humans results in localised lesions mostly on the fingers, hands and face, with complications mostly reported in immunocompromised individuals [8, 9]. The IMVANEX vaccine contains an attenuated version of VACV (modified vaccinia virus Ankara), and both CPXV and VACV are classified as risk group 2 biological agents.

Anti-OPXV antibodies have been shown to be cross-reactive against a broad range of different OPXV species, including VACV, CPXV and MPXV [10]. Antibodies against MPXV can be detected in serum approximately one to two weeks after the onset of symptoms [4]. Although detection of viral DNA is the gold standard to confirm an MPXV infection, detection of antibodies against MPXV by serological assays can aid diagnostics under certain circumstances. Furthermore, sero-epidemiological studies solely rely on the detection of antibodies in the post-acute phase to determine the overall burden of disease in an at-risk population or the presence and endurance of protective antibodies induced by immunization campaigns [11, 12].

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For the detection of antibodies against MPXV, different laboratory tests can be used: IgM and IgG antibodies can be detected by immunofluorescence assay (IFA) [13, 14] or enzyme-linked immunosorbent assay (ELISA) [11, 15-17], while neutralising antibodies are commonly detected by neutralization tests (NT) [18-20]. Here, both the IFA and the ELISA detect antibodies binding to immobilised viral antigen. For the IFA, the viral antigens are present in infected and fixed cells, usually on multi-well slides to enable the testing of various serum dilutions for simultaneous antibody titration. Non-infected cells can also be included on the same slide to test for specificity, as well as a positive serum on infected cells to act as a positive control. Bound antibodies are detected by adding fluorophore-coupled human IgG- or IgM-specific secondary antibodies, and signals are read out on fluorescence microscopes. For OPXV ELISAs, different antigens have been employed, ranging from purified viral particles to lysed infected cells or recombinant viral proteins. Due to the antigen immobilization on microtitre ELISA plates, a greater number of sera can be tested simultaneously, leading to a higher throughput. Bound antibodies are usually detected by horseradish-peroxidase (HRP) or alkaline phosphatase (AP) labelled antibodies, leading to colorimetric chemical reactions which can be quantified by ELISA microplate readers. Finally, virus neutralising antibodies, which block virus uptake and replication in target cells, are quantified by NTs. Here, the assay read-out are plaques caused by the lysis of infected cells in a confluent cell monolayer which can be quantified as plaque-forming units (PFU). Alternatively, the assay can be simplified by determining cytopathic effects per well to determine tissue-culture infectious doses ($TCID_{50}$). Currently, there are only very few commercially available ELISA kits for the detection of IgG and IgM antibodies against OPXV/MPXV. Furthermore, several commercial antibody rapid tests are available, but to date it is not known how these tests perform in comparison to in-house methods. Finally, there is no commercial assay for the detection of neutralising antibodies against MPXV, which is why these assays are generally established as in-house assays by specialist laboratories. Setting up such assays comes with different challenges and requirements with regard to equipment, reagents, facilities (biosafety level) and expertise needed. Furthermore, each of the assays has its merits and drawbacks and hence should be chosen according to its intended use.

In addition to technical limitations, one of the biggest hurdles when performing diagnostics for MPXV is the need for a dedicated BSL-3 facility when replication-competent virus must be handled, e.g., for antigen preparation or to perform NTs. Hence, safer protocols for MPXV serology, which still deliver meaningful results, enhance the overall biosafety while enabling broader applicability of diagnostic methods. Both are needed in the light of the ongoing MPXV circulation worldwide. Here different serological methods are described, the protocols provided and, moreover, options to perform safe antibody detection against MPXV under BSL-2 conditions are shown.

Materials and Methods

Samples

Five serum samples from PCR-confirmed MPXV-infected individuals and six serum samples from confirmed (by PCR or serology) CPXV-infected individuals were collected in the course of the routine diagnostics in the Consultant Laboratory for Poxviruses at the Robert Koch Institute. Five serum samples from vaccinated volunteers (IMVANEX) were collected four weeks after the first vaccine dose (n=1) or two (n=3) or three (n=1) weeks after the second vaccine dose. For establishment of the ELISA a panel of 28 sera (25 sera from routine MPXV diagnostics and three pre-immune sera from volunteers prior to IMVANEX vaccination) was employed. All sera were titrated by IFA to determine end-point titres for both IgM and IgG antibodies as described below. Sera were selected to cover a broad range of reactivities, including sera with low (below 1:80), medium (1:320) and high (1:1280 and above) titres. Ethical clearance was obtained from the Berliner Ärztekammer (BÄK Eth-44/22).

Neutralization test

Micro-neutralization tests (NT) were set up with three different OPXV strains: MPXV clade IIb (in-house isolate), VACV Lister-Elstree Bavarian Nordic (LELS-2003-007, Bavarian Nordic GmbH) and CPXV strain HumGri07/1 (in-house isolate) [16]. For this purpose, Vero E6 cells (#85020206, European Collection of

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Authenticated Cell Cultures (ECACC)) in DMEM (Gibco) with 10 % FCS (Sigma) were infected at MOI 0.1. After four to seven days — depending on the morphological cell status — the infected cells were lysed by three freeze-thaw cycles and the virus suspension was vortexed for 10 sec. Cell debris was pelleted for 10 min at 1300xg, and from the supernatant a stock virus solution with a target titre of 1000 $TCID_{50}$/mL was prepared in cell culture medium and stored in aliquots at -80 degC. The stock titre was confirmed by triplicate titration. For detection of neutralising antibodies, the patient sera were diluted in medium (DMEM) to six two-fold dilution steps, resulting in dilutions of 1:10 to 1:320. 500 µL of virus stock were added to 500 µL of each respective serum dilution and mixed by pipetting up and down. Following incubation at RT for one hour, 100 µL of each virus/serum dilution were added per well in eight replicates to 96-well plates containing Vero E6 cells seeded the previous day (1.5×10^4 cells/well in 100 µL of medium) and incubated at 37 °C, 5 % CO_2, for 7 days. In each experiment, the virus stock used was titrated as a control. For this purpose, 1 mL of the virus dilution was mixed with 1 mL of medium and incubated at RT for 1 h. Decimal dilutions were prepared in medium, resulting in dilutions of $1:10^1$ to $1:10^9$, then 100 µL of each virus dilution were added per well in eight replicates to a 96-well plate pre-seeded with Vero E6 cells (1.5×10^4cells/well in 100 µL of medium). As a negative control, 100 µL of medium/well was added to eight wells. After 7 days the cells were inspected by microscopy for CPE. The titre of the virus stock was calculated according to the following formula: $TCID_{50}$/mL = 10^((n/8+0.5))/0.5 where n = number of wells with CPE. The titre of the patient sera was calculated according to the following formula: Titre (1:x) =10×2^((n/8+0.5)) where n = number of wells without CPE. In case that not all wells in the last dilution step showed a CPE, the titre was indicated as "[?]".

Immunofluorescence assay

The in-house IFA uses OPXV-infected cells as target antigens and can detect IgM or IgG antibodies but does not distinguish neutralising antibodies. For the preparation of about 30 slides, $3x10^6$ suspended HEp2 cells (ECACC) per mL in 15 mL of medium were pelleted for 5 min at 216xg. The pellets were resuspended in 1 mL of virus-containing medium and infected with VACV Lister-Elstree at MOI of 0.5 or CPXV HumGri07/1 at an MOI of 0.9 for 1 h at 37 degC, 5 % CO_2. After mixing with 10 mL of medium, the cells were pelleted for 5 min at 216xg, resuspended in 3 mL of medium, and 30 µL each were applied to 12-well cavity slides (PTFE(Teflon)-coated, e.g., VWR #631-9423). A negative control with uninfected cells was included on each slide. The slides were incubated for 24 h at 37 °C, 5 % CO_2, the supernatant was removed and the slides were air-dried. Subsequently, the cells were fixed in acetone for 60–90 min at room temperature, air-dried and stored at -20 °C until usage. Quality control of the slides was performed with known OPXV-positive controls for IgM and IgG. As standard procedure, all serum samples were inactivated for 30 min at 56 °C. For IgM detection, the serum was additionally pre-treated with Mastsorb Absorbens (Mast Diagnostika #651003) for 5 min at room temperature following supplier recommendations. Briefly, 75 µL of Mastsorb were mixed with 60 µL of PBS and 15 µL of inactivated serum were added. After 30 min of incubation at room temperature, the treated serum was centrifuged for 5 min at 2000×g and the supernatant used for IgM detection. Four consecutive dilutions of the serum, e.g., 1:20, 1:80, 1:320 and 1:1280, were prepared in buffer (PBS with 2 % BSA) and 20 µL of each dilution or control were added into the wells of a thawed slide. The slide was incubated for 1 h at 37 °C in a humidity chamber, washed with PBS and allowed to dry. The secondary antibody (goat anti-human IgM (H+L)/FITC and anti-IgG (Fc-γ specific)/FITC; Invitrogen) was diluted 1:50 in PBS with 2 % BSA. Subsequently, 10 µL of the secondary antibody dilution was mixed with 10 µL of 0.1 % Evans Blue (Sigma) in water and added per well. After 1 h of incubation at 37 °C the slide was washed with PBS, dried and covered with mounting medium (e.g., ROTI®Mount FluorCare from Carl Roth). Evaluation of the staining was done by fluorescence microscopy.

ELISA

The in-house ELISA was performed as described before [11] with slight changes to the protocol to harmonise the assay with other serological in-house assays. As antigen, UV-inactivated RIPA lysate from VACV New York City Department of Health Laboratories (ATCC, catalogue # VR-1536®) infected HEp2 cells was coated at a concentration of 4 µg/mL in 100 µL of 50 mM carbonate buffer (pH 9.6) per well to the surface

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of one half of a MaxiSorp ELISA plate (Nunc). Similarly, lysed non-infected HEp2 cells were coated on the other half of each plate to serve as a non-specific negative control. Coating was done at 4 °C overnight. The next day, the plates were washed four times by using an automated ELISA washer (Hydrospeed, Tecan) with 300 µL of washing buffer (PBS with 0.1 % Tween 20) per well before the plates were blocked for 1 hour at room temperature using 200 µL per well of casein blocking buffer (200 mM Tris pH 7.3, 2.5 % casein, Sigma-Aldrich, # C-5890, 0.1 % Tween 20, 0.02 % 5-Bromo-5-Nitro-1,3-Dioxan, Bronidox). Subsequently, divergent protocols were developed for the detection of either IgG or IgM antibodies. For IgG detection, sera were diluted either 1:100 and 1:1000 or in a 1:4 dilution series ranging from 1:100 to 1:6400 in casein blocking buffer and incubated for 1 h at 37 °C. For each serum dilution, 100 µL were incubated on both VACV-infected as well as non-infected HEp2 cell lysate. After a washing step was performed as described before, 100 µL of HRP-labelled goat anti-human IgG antibody (Fc-γ-specific, Jackson ImmunResearch, obtained from Dianova, # 109-035-008) diluted 1:2500 (final dilution 1:5000 due to storage in glycerol at a 1:1 dilution) were added per well and incubated for 1 h at 37 °C. For IgM detection, sera were pre-treated with Mastsorb (Mast Group) similar to the IFA protocol and diluted in LowCross-Buffer (Candor Bioscience) to minimise non-specific background binding (see supporting figure 1). Detection of bound IgM was done by incubation with HRP-labelled goat anti-human IgM antibodies (5µ-specific, Jackson ImmunResearch, obtained from Dianova, # 109-035-043) diluted 1:2500 (final dilution 1:5000 due to storage in glycerol at a 1:1 dilution). After a final wash with eight washing steps, 100 µL of TMB substrate (Seramun Slow TMB substrate, Seramun Diagnostica GmbH) were added per well for 15 min at room temperature before the reaction was stopped by adding 100 µL of 0.25 M H_2SO_4 per well. The absorption was read with an ELISA microplate reader (Tecan Infinite M200) at 450 nm referenced to 620 nm. For each serum and dilution, signals for binding to non-infected HEp2 lysate were subtracted from signals for binding to VACV-infected HEp2 lysates to account for unspecific binding, leading to Delta results (VACV minus HEp2). On each plate, a standard curve was included using a 1:4 dilution series of Vaccinia Immune Globulin (obtained through BEI Resources, NIAID, NIH: Polyclonal Anti-Vaccinia Virus (immune globulin G, Human), NR-2632) starting at a 1:500 dilution and ranging to a 1:128,000 dilution. Delta results were quantified by interpolation to the standard curve by using a four-parameter sigmoidal fit over log-transformed VIG concentrations using the statistical software R (version 4.1.2) and the drLumi package (version 0.1.2) [21]. A mean concentration was calculated from all dilutions that could be interpolated from the standard curve while dilutions above or below the limit of quantification were excluded.

Inactivation of VACV and MPXV

For inactivation experiments, published protocols for virus inactivation are used as a starting point [22, 23]. Here, detergents were added to blood products to inactivate enveloped viruses by removal of the viral membrane in combination with heat inactivation. To this aim, VACV VR-1536 (ATCC #VR-1536) or MPXV clade IIb (in-house isolate) were diluted 1:4 in 20 % human serum albumin (HSA, PAN Biotech) to simulate a viraemic serum sample. Inactivation reagent was prepared as a stock solution in PBS and diluted 1:3 in simulated viraemic serum to a final concentration of 0.3 % tri-n-butyl-phosphate (TnBP, Merck)/0.3 % polysorbate 80 (Tween-80, Sigma-Aldrich)/1 % octoxynol-9 (Triton X-100, Sigma-Aldrich) or 1.5 % Tween-20/1.5 % Triton X-100 and incubated either at room temperature, 30 min at 56 °C or 1 h at 60 °C. As a control an equal amount of buffer (PBS) was added to the simulated serum sample. Efficacy of the inactivation treatment was tested by the reduction of infectious doses in cell culture. To do this, the treated samples were filtered through DetergentOUT GBS10-5000 spin columns (G-Biosciences) according to the manufacturers' recommendations to remove interfering detergents. Subsequently, the flow-through was diluted in cell culture medium (DMEM, with 10 % FCS), and 100 µL/well of dilutions ranging from 10^0 to 10^{-7} were added to 96-well plates pre-seeded with Vero E6 cells. After 7 days of incubation at 37 °C, 5 % CO_2, the cells were analysed for CPE by light microscopy, and the wells with CPE were counted to calculate the $TCID_{50}$/mL. Additionally, for verification of inactivation, three replicates of simulated viraemic VACV serum treated with 1.5 % Tween-20/1.5 % Triton X-100 were then heated for 30 min at 56 °C and were passaged three times in cell culture on Vero E6 cells. For this purpose, the supernatants of the dilutions 10^0 to 10^{-5} of the first titration step were pooled and the corresponding cells trypsinised and added to the supernatant.

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1 mL of supernatant/cell suspension was added to prepared 6-well plates with non-confluent Vero E6 cells and incubated for seven days. After incubation, the cells were trypsinised and pooled with the corresponding supernatants and 0.5 mL were added to fresh cells in a 6-well plate. This procedure was repeated once again for a total of three rounds of passaging. Finally, the cells were lysed by three freeze/thaw cycles and the DNA was extracted with the Qiagen DNA Blood kit according to the manufacturers' recommendations. The amount of poxvirus DNA was determined using a pan-OPXV real-time-PCR targeting the rpo gene as described elsewhere [24].

Results

For the detection of antibodies against OPXV in low sample numbers, IFA is the assay of choice and was the first assay adapted to detect antibodies against MPXV in serum samples. To verify the antibody cross-reactivity, cells infected with different OPXV (MPXV, CPXV and VACV) were used to detect IgG and IgM antibodies by IFA. Moreover, in addition to sera from MPXV-infected individuals, sera were analysed from individuals with CPXV infection or from individuals that were recently vaccinated against poxviruses by using the IMVANEX vaccine. Using the different OPXV IFAs, IgG antibodies binding to MPXV, CPXV and VACV could be detected with comparable titres in all samples (Figure 1A). Although some differences could be observed for some sera when tested on MPXV-, CPXV- or VACV-infected cells, no clear trend could be seen for homologue serum/antigen sets when IgG and IgM were considered, as most sera gave similar results irrespective of the virus strain used as antigen. The only exception was for IgG where slightly higher titres could be seen after MPXV infection on MPXV IFA slides. However, differences were minor (usually a single 1:4 dilution step of the serum in the IFA). The same pattern was observed for IgM antibodies (Figure 1B).

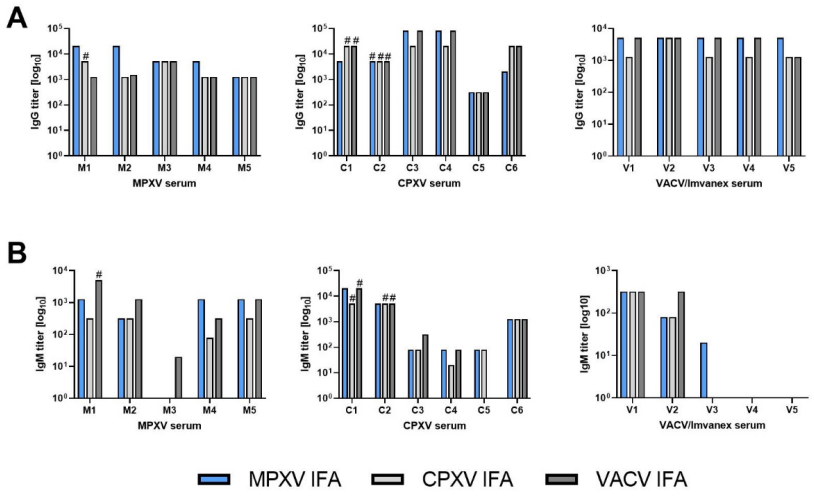

Figure 1. Analysis of IgG (A) and IgM (B) titres by IFA. Sera from MPXV-infected (M1-M5) or CPXV-infected (C1-C6) patients or individuals vaccinated against poxviruses (V1-V5) were analysed by using IFAs with different viruses (MPXV, VACV and CPXV). # = the titre is higher than the highest serum dilution analysed ([?]). Different serum dilutions were analysed for some IFAs; therefore the highest measurable titre can vary by sample or test.

In addition to IFA, NTs are used in the lab at the Robert Koch Institute to detect neutralising antibodies against poxviruses. This assay – like IFA – is suited only for smaller numbers of samples. NTs were performed using different poxviruses (MPXV, CPXV and VACV) and the same set of serum samples were analysed that were used in the IFA cross-reactivity evaluation. For samples from MPXV-infected or IMVANEX-vaccinated individuals, neutralising antibodies could be detected when using all OPXVs (MPXV, VACV and CPXV; Figure 2) in the NT. Larger differences in titre against the different OPXVs were observed in the NTs as

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compared to the IFAs. These differences were most pronounced when using samples from MPXV-infected persons, where the lowest titres were observed when using CPXV and the highest titres when using VACV in the NTs (Figure 2). Neutralising antibodies in five out of six sera from CPXV-infected individuals were not detected by all OPXV NTs; however, if detected, their titre was low and close to the limit of detection of the test. In a single CPXV sample with a higher titre, neutralising antibodies were also detected with all three OPXV NTs.

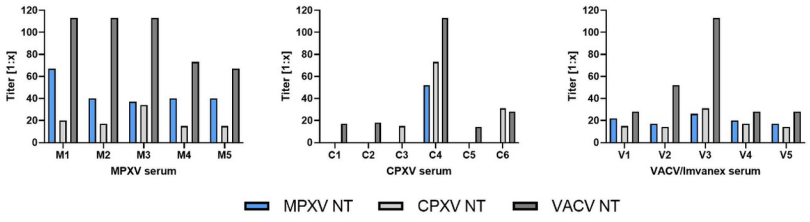

Figure 2: Micro-neutralization tests using different OPXVs. Five sera from MPXV-infected (M1-M5) and six sera from CPXV-infected persons (C1-C6) plus five sera from IMVANEX-vaccinated individuals (V1-V5) were analysed by using $TCID_{50}$ assays with different viruses.

Since IFA and NT are both low-throughput methods to detect binding or neutralising antibodies, they are not suited to screen larger sample numbers, e.g., in an outbreak situation; therefore, for this purpose an ELISA was updated that had previously been established in the lab [11]. The ELISA uses VACV-infected HEp2 cells as the specific antigen and non-infected HEp2-cells as the negative control. Hereby, unspecific or background binding can be eliminated effectively by subtracting non-specific HEp2 signals from VACV-specific signals. To render the ELISA also useful for the detection of acute infections, IgM analysis was newly established. Initial experiments for IgM detection using serum dilutions in the casein blocking buffer (also used for serum dilution prior to IgG detection) led to high unspecific binding. Hence, different blocking buffers and commercially available diluents were tested (supporting figure 1). As LowCross buffer was most efficient in reducing high background signals while retaining specific binding of IgM-positive sera, it was used as the serum diluent in the final assay protocol.

To test the agreement between the established IFA and the modified or newly established IgG and IgM detection by ELISA, the quantified ELISA results for 28 sera were compared with the corresponding IFA titres (Figure 3). For IgG detection, a high level of correlation was found between IFA titres and ELISA results with higher titres, leading to higher ELISA results. This was also true for IgM detection, although differentiation between lower titres was not possible.

Figure 3: Comparison of IgG (A) and IgM (B) titres by IFA with results obtained by ELISA. IFA titres were determined by titration against CPXV- or VACV-infected HEp2 cells.

After demonstrating that IFA and ELISA, moreover, using less pathogenic OPXVs like VACV as antigens, can be used to detect (cross-reactive) antibodies against MPXV, the next aim was to further enhance the method's biosafety with a robust inactivation method for the serum samples. A literature review resulted in two potential protocols using either 0.3 %Triton/0.3 %Tween/1 %TNBP (protocol 1, p1) [23] or 0.5 %Triton/0.5 %Tween/1h60 °C (protocol 2, p2) [22]. For comparison, standard heat inactivation was used for 30 min at 56 °C. First the protocols were tested with VACV, demonstrating that both protocols resulted in a depletion of infectious poxvirus particles (Figure 4A). However, the protocol p2 (Figure 4A) led to a better inactivation compared to protocol p1. Heat inactivation alone was least efficient in inactivating infectious virus particles (Figure 4A, heat). Since protocol p2 worked well the next aim was to reduce the heat inactivation time and reduce the incubation temperature to allow inactivation while still retaining the antibody-binding ability. For this purpose, p2 was tested with classical heat inactivation conditions (30 min 56 °C, protocol 3, p3) which led to a depletion comparable with the p2 protocol (Figure 4, p2 and p3). Next,

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three replicates of VACV inactivated with p3 were passaged three times in cell culture and checked for viral DNA by real-time-PCR in the cells and the supernatant. This resulted in verifying that in the 10^{-1} dilution of the $TCID_{50}$ there are few infectious particles left, while in the 10^{-2} dilution no infectious particles could be detected in all three replicates. Taken together, using the p3 protocol, a depletion in infectious virus particles of about 5 log-steps could be shown. The inactivation potential of the p3 protocol was then also verified with MPXV (Figure 3B).

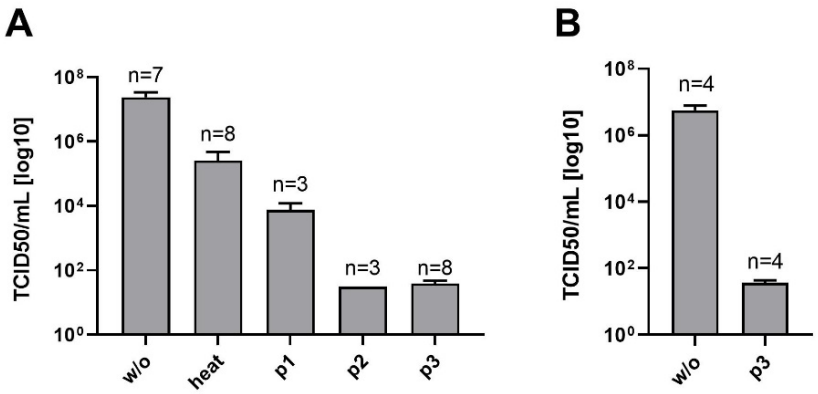

Figure 4: Inactivation of VACV and MPXV with different methods. (A) VACV was spiked into 20 % HSA and inactivated either with heat (30 min 56 °C), protocol p1=0.3 %Triton/0.3 %Tween/1 %TNBP, p2=0.5 %Triton/0.5 %Tween/1h60 °C or p3=0.5 %Triton/0.5 %Tween/30min56 °C. The number of infectious particles was then measured by $TCID_{50}$. (B) MPXV was spiked into 20 % HSA and inactivated with p3, and the number of infectious particles was measured by $TCID_{50}$. As a reference non-inactivated virus spiked into 20 % HSA was used (w/o). n = number of replicates. The titre of p2 and p3 are maximum titres since residual toxicity of the inactivation reagents prohibited an evaluation of the lowest dilution step.

Finally, serum was tested after inactivation with the p3 protocol in the IFA and the ELISA. For comparison of native and inactivated IFA titres, five sera from IMVANEX-vaccinated individuals were used which showed an expected and easy-to-interpret cytoplasmic fluorescence of the cells (plasma fluorescence, PF). Additionally, a panel of five sera was taken that had been collected in the course of routine diagnostics and whose fluorescence signals were more challenging to interpret. These sera showed fluorescence of inclusion bodies (IBs; n=4) or fluorescence of cytoplasm and IBs. Analysing these ten sera by IFA showed that the p3 inactivation step did not alter the results (Table 1).

Table 1: Comparison of IFA titres with standard heat and heat/detergent inactivation.

Sample
PF1
PF2
PF2
PF4
PF5
IB1
IB2
IB3
IB4
IB5

[a]30min 56 °C [b]0.5 % Triton/0.5 % Tween/30 min 56 °C (p3) [c]PF = fluorescence of cytoplasm, IB = fluorescence of inclusio

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To further determine whether the serum inactivation protocol detrimentally impacts the ELISA, a small panel of nine sera, both native and inactivated, was tested with p3 in two dilutions (1:100 and 1:1000 for native sera and 1:33 and 1:333 for inactivated sera) by ELISA. Results for both native and inactivated sera were congruent (supporting figure 2), indicating that inactivation slightly lowers the overall antibody reactivity by a factor of 3. However, identical results could be obtained by adjusting the dilution factor, thus proving that a safe protocol for antibody detection from viraemic sera could be established.

Discussion

To aid the establishment of MPXV serology in other laboratories, in the present work methods are described for the detection of OPXV IgG and IgM antibodies as well as neutralising antibodies. To this aim, a small panel of sera from patients after MPXV or CPXV infection or VACV/IMVANEX vaccination was used and IgG and IgM detection was tested by IFA and ELISA and the presence of neutralising antibodies by NT. Furthermore, MPXV, CPXV and VACV were employed as sources of antigens in the IFA and the NT test. The aim was to test discrepancies or agreements between different assays and different antigens for sera with different infection or immunization backgrounds. It could be confirmed that IFAs and NTs based on different OPXV species can be used for the detection of antibodies in sera from individuals who had an infection with MPXV or CPXV or were vaccinated against poxviruses, due to the well-known antibody cross-reactivity between different OPXV species [1, 10].

Some sera (M3 and V3 to V5, Figure 1B) were not detected by all OPXV IFAs as a result of their very low titre ([?]20) at or below the limit of detection of the IFA. Additionally, after MPXV infection some sera showed higher titres on MPXV-infected slides, indicating higher reactivity against homologue virus strains as previously described [25].

The greatest differences in reactivity to the different OPXV were observed in the NT. These titre differences could result from the biological variation of the NTs since different OPXV species were used and the tests were not optimised for each virus individually. Since cell culture supernatant as well as virus particles from cells were used, the virus stocks contained a mixture of the two poxvirus particle forms: the extracellular enveloped virions (EEV) and the intracellular mature virions (IMV). For EEV and IMV, different antibodies are needed for neutralization since the epitopes on the virion surface differ. Hence, the ratio of EEVs to IMVs could have been different in the different OPXV stock preparations, leading to larger titre discrepancies. Furthermore, although the amount of virus particles used was set to be comparable between the different OPXV (1000 $TCID_{50}$/mL), fewer virus particles were detected in the back-titration of the VACV stock (supporting table S1). This could explain the fact that the observed neutralising antibody titres were highest in the VACV NT and not in the NT corresponding to the "source virus" of the antibodies contained in the different samples. However, except for this minor technically induced variation, the NT results corresponded to the expected results: CPXV sera showed the highest titres in the CPXV NT and MPXV sera in MPXV NT. Finally, the larger differences between the titres observed in the different OPXV NTs, as compared to the different OPXV IFAs, could also be due to the generally much lower neutralising antibody titres as compared to binding antibody titres and hence larger variation between individual measurements.

Although serological assays have been described which are able to discriminate between vaccination and infection with MPXV to some extent, those assays rely on pre-absorption with viral antigen [25] or combinations of specific peptides [17] while there is a large overlap of cross-reactive epitopes between the closely related OPXVs. It is known that individuals who received the first-generation poxvirus vaccine against smallpox still have cross-reactive neutralising antibodies against MPXV even 40 years post vaccination [26]. However, in accordance with a recent study, these neutralising antibody titres in individuals with one or two vaccination doses are rather low [12].

The newly optimised ELISA protocol is more suited for the detection of acute infections due to its higher dynamic range which results from quantification over standard curves and its ability to detect IgM antibodies.

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The ELISA enables a higher throughput as compared to IFA and NT, with 20 sera measured per ELISA plate if two serum dilutions are tested. Testing two serum dilutions (1:100 and 1:1000) is advisable to capture the higher dynamic range of acute infections in patients as compared to seroprevalence studies for which the ELISA was initially established at a single 1:100 dilution. One of the two sera with IFA titres of 1:20, which gave higher ELISA signals than the sera with IFA titres of 1:80, was highly positive for PPV, indicating a possible minor cross-reactivity with the VACV-specific lysate used in the ELISA, while the other serum was initially titrated to a titre of 1:320, indicating some ambiguity regarding the exact titre (Figure 3). Some overlap between ELISA results falling into different IFA titres indicates some method-specific discrepancies; yet overall, both assays correlate well, especially for samples with higher IFA titres. Differentiation between lower IgM titres was not possible, which in part could be due to cross-reactivity of the used detection antibodies, as one serum with a high IgM reading by ELISA had an IFA IgM titre of only 1:20 but was highly positive for IgG with a titre of 1:20,480.

It has been shown that during MPXV pathogenesis two viraemic phases occurred that enabled virus dissemination in the infected individual [3]. In contrast, viraemia in vaccinees seems to be rarer [27]. Viraemia in MPXV-infected individuals also seems to be rare [28] and is potentially also clade specific as differences in dissemination are seen between clade I and clade II MPXV. However, viraemia/DNAaemia has been observed during MPXV infection in a prairie dog model, and in two animals viable virus was also found in the blood [29]. Moreover, DNAaemia was also shown to occur in CPXV-infected individuals [30]. This leads to the conclusion that viraemia cannot be excluded in MPXV-infected individuals. Hence, a safe handling procedure was established for BSL-2 laboratories with which at least a five log-level depletion of infectious poxvirus particles could be accomplished in simulated serum samples. The protocol using Triton, Tween and heat can be used to inactivate sera for IFA and ELISA. Due to cytotoxicity, the inactivation protocol is not applicable for lower dilution steps in NT, but NTs based on VACV or other suitable OPXV may be performed in a BSL-2 laboratory under a class II biosafety cabinet.

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Conflict of interest disclosure

The authors declare no conflict of interest.

Supporting tables

Table S1: Titration of OPXV stocks for neutralization tests

Virus stock	**Titre $TCID_{50}$/mL[a]**
MPXV	1.5E+03 ± 7.9E+02
CPXV	1.6E+03 ± 1.4E+02
VACV	9.2E+02 ± 2.6E+02
[a]Average titre ± standard deviation (n=3), target titre 1.0E+03	[a]Average titre ± standard deviation (n=3), target titre

Supporting Figures

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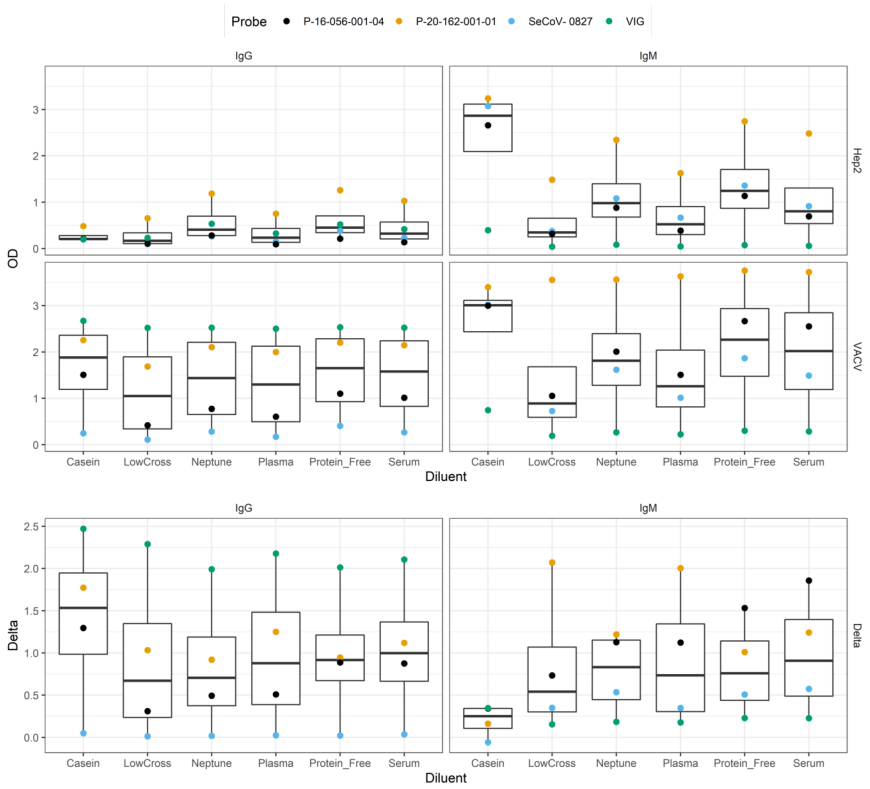

Supporting Figure 1: Impact of different commercial diluent solutions on signal intensities for binding of IgG and IgM to both specific (VACV) and unspecific (HEp2) antigens. Tested samples were tested at 1:100 dilution or 1:500 dilutions (VIG only) in the specified diluents. Sample P-16-056-001-04 had IFA titres of 1:5120 (IgG) and 1:1280 (IgM), sample P-20-162-001-01 had IFA titres of 1:1280 for both IgG and IgM antibodies, and VIG is highly positive for IgG but negative for IgM while sample SeCoV-0827 is negative for both IgG and IgM. Casein blocking buffer was prepared in-house as described, LowCross-Buffer was obtained from Candor, Neptune, Plasma Sample, Protein-Free, and General Serum diluents were all obtained from ImmunoChemistry. For IgG detection, dilution in the established in-house casein blocking buffer enabled the lowest background signals with the highest specific signals, leading to the overall highest difference between specific and unspecific signals. In contrast, due to very high unspecific binding signals in casein blocking buffer, almost no signal difference remained in casein blocking buffer for IgM detection. All commercial diluents tested significantly lowered unspecific binding for IgM detection. Due the fact that unspecific binding was most reduced when using LowCross-Buffer, this buffer was used for IgM detection in the ELISA, although also specific signals on VACV lysate were slightly reduced as compared to the other diluents tested.

Supporting Figure 2: Comparison of ELISA signals (VACV-specific signals minus HEp2 signals) for native and sera inactivated with triton/tween + heat. Almost perfect agreement between measurement ($R^2$0.9921, slope 0.9661, y-intercept -0.004592 of linear regression) indicates that congruent results can be obtained from native and inactivated sera.

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