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Short Communication

# Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications

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Chikungunya virus (CHIKV) is a mosquito-borne pathogen responsible for epidemics of debilitating arthritic disease. The recent outbreak (2004–2014) resulted in an estimated 1.4–6.5 million cases, with imported cases reported in nearly 40 countries. The development of CHIKV-specific diagnostics and research tools is thus highly desirable. Herein we describe the generation and characterization of the first mAbs specific for the capsid protein (CP) of CHIKV. The antibodies recognized isolates representing the major genotypes of CHIKV, as well as several other alphaviruses, and were reactive in a range of assays including ELISA, Western blot, immunofluorescence and immunohistochemistry (IHC). We have also used the anti-CP mAb 5.5G9 in IHC studies to show that capsid antigen is persistently expressed 30 days post-infection in cells with macrophage morphology in a mouse model of chronic CHIKV disease. These antibodies may thus represent useful tools for further research, including investigations into the structure and function of CHIKV CP, and as valuable reagents for CHIKV detection in a range of settings.

Chikungunya virus (CHIKV) is the aetiological agent of chikungunya fever, first described in 1952 during an epidemic in Tanzania, East Africa (Lumsden, 1955; Robinson, 1955). CHIKV belongs to the *Alphavirus* genus within the *Togaviridae* family and is an enveloped, single-stranded positive-sense RNA virus (Strauss & Strauss, 1994). The 11.5 kb alphavirus genome is capped at its 5′ end and polyadenylated at its 3′ end, and encodes four non-structural proteins (nsP1 to nsP4) and five structural proteins (capsid, E3, E2, 6K and E1) (Strauss & Strauss, 1994).

CHIKV is transmitted to humans by *Aedes aegypti,* and recently also *Aedes albopictus*, mosquitoes. Acute CHIKV disease is characterized by a rapid onset of fever, myalgia and often a rash (usually maculopapular), with chronic disease characterized by episodic, often debilitating, polyarthralgia/polyarthritis (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012). The largest epidemic of CHIKV disease ever reported began in 2004 and has since been responsible for up to 6.5 million human cases, primarily in Africa and Asia, with imported cases reported in over 40 countries (Munasinghe *et al.*, 1966; Lam *et al.*, 2001; Renault *et al.*, 2007; Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Suhrbier *et al.*, 2012; Horwood *et al.*, 2013; Van Bortel *et al.*, 2014). The continued activity of the initial epidemic in conjunction with additional emerging events has led to independent outbreaks in other parts of the globe, such as in Australasia and the Caribbean (Horwood *et al.*, 2013; Viennet *et al.*, 2013; Van Bortel *et al.*, 2014). During the recent epidemic, CHIKV was also clearly associated with occasional severe disease manifestations and mortality, the latter primarily amongst elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010).

The alphavirus capsid protein (CP) is a multifunctional protein that has been shown to act as a serine protease for self-cleavage, bind viral genomic RNA and other CP molecules during nucleocapsid formation, and interact with viral spike proteins during virion formation and egress (Choi *et al.*, 1991). The CP of CHIKV consists of 261 amino acids, which form two major domains. The N-terminal domain has a high degree of positive charge

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A supplementary figure is available with the online Supplementary Material.

implicated in non-specific RNA binding, while the C-terminal domain harbours the globular protease and the binding site for the spike protein (Hong *et al.*, 2006).

The re-emergence of CHIKV, attributable in large part to a mutation allowing efficient transmission by *A. albopictus*, and the current risk it poses to human health, has prompted the demand for new diagnostic and research reagents. Herein we report the generation and characterization of the first monoclonal antibodies (mAbs) to the CP of CHIKV and describe their use in a variety of assays.

CHIKV isolates obtained for these studies included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID EU404186); CHIKV Asian, Thailand strain ($CHIKV_{THAI}$) (GenBank ID FJ457921) and CHIKV Asian, East Timor strain ($CHIKV_{ET}$). To generate mAbs against CHIKV viral proteins, BALB/c mice 6–8 weeks of age were immunized with purified inactivated antigen ($CHIKV_{MAU}$), challenged with live virus ($CHIKV_{THAI}$), followed by a final boost 20 months later with inactivated antigen ($CHIKV_{THAI}$) 4 days prior to fusions for hybridoma production as described previously (Goh *et al.*, 2013). Hybridomas were screened for production of CHIKV-reactive antibodies using fixed-cell ELISA, and positive hybridoma cultures were cloned twice by limit dilution as previously described (Hall *et al.*, 1988; Clark *et al.*, 2007). Eleven hybridomas secreting antibodies reactive to CHIKV proteins were expanded in Hybridoma SFM (Gibco Life Technologies) with 20 % FBS at 37 °C with 5 % $CO_2$, before being weaned off all FBS for the harvesting and clarification of mAbs as culture fluid. Reactivity of these mAbs (1.7B2, 4.1H11, 4.8E2, 4.10A11, 5.1B12, 5.2F8, 5.2H7, 5.4G8, 5.5A11, 5.5D11 and 5.5G9) to various CHIKV strains and related alphaviruses were determined by fixed-cell ELISA (Table 1).

Each mAb recognized the three CHIKV strains used in this study ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) with similar intensity in ELISA, suggesting the epitopes are highly conserved amongst these strains. However, the varying degree of reactivity between the mAbs to the CHIKV antigens, as measured by $OD_{405}$, is likely due to their recognition of different binding sites on the CP and variation in the binding affinity between individual mAbs. To further assess their reactivity towards other closely related alphaviruses, each mAb was also tested against Ross River virus (RRV) T48 strain (GenBank ID GQ433359); Semliki Forest virus (SFV) (GenBank ID NC_003215) and Sindbis virus (SINV) MRE16 strain (GenBank ID AF492770). Three of the mAbs, 5.2H7, 5.5D11 and 5.5G9, reacted with antigens of SFV, RRV and SINV in ELISA, while the remaining eight mAbs recognized SFV and/or RRV but not SINV (Table 1). The isotype of each mAb was also determined using the Mouse typer isotyping kit (Bio-Rad) according to the manufacturer's instructions and found to be either $IgG_1$ or $IgG_{2A}$ (Table 1). When tested for viral neutralizing activity in a micro-neutralization assay (Goh *et al.*, 2013), none of the mAbs neutralized any of the three CHIKV strains *in vitro* (data not shown).

To determine their viral protein specificity, each mAb was assessed for specificity against CHIKV antigens in infected Vero cell lysate by Western blot (Goh *et al.*, 2013). $CHIKV_{MAU}$ antigens were prepared in 4 × NuPAGE LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min. For reduced antigens, 10 mM DTT was added prior to

**Table 1.** Reactivity of CHIKV CP-specific mAbs towards various CHIKV strains and other alphaviruses in ELISA

| Monoclonal antibody* | Reactivity in fixed-plate ELISA | | | | | |
|---|---|---|---|---|---|---|
| | $CHIKV_{MAU}$ | $CHIKV_{THAI}$ | $CHIKV_{ET}$ | $RRV_{T48}$ | SFV | $SINV_{MRE16}$ |
| $5.2H7_{IgG1}$ | ++++ | ++++ | +++++ | +++++ | +++++ | ++++ |
| $5.5D11_{IgG1}$ | ++++ | ++++ | ++++ | +++++ | +++++ | ++++ |
| $5.5G9_{IgG2A}$ | +++++ | +++++ | +++++ | +++++ | +++++ | +++++ |
| $1.7B2_{IgG1}$ | +++++ | +++++ | +++++ | ++ | − | − |
| $4.1H1_{IgG1}$ | +++++ | +++++ | +++++ | ++ | + | − |
| $5.1B12_{IgG2A}$ | +++++ | +++++ | +++++ | +++ | +++ | − |
| $5.5A11_{IgG1}$ | +++++ | ++++ | +++++ | ++ | +++++ | − |
| $4.8E2_{IgG2A}$ | +++ | +++ | +++ | ++ | +++ | − |
| $4.10A11_{IgG1}$ | +++ | +++ | +++ | ++ | +++ | − |
| $5.2F8_{IgG2A}$ | +++ | ++ | +++ | ++ | ++ | − |
| $5.4G8_{IgG1}$ | +++ | ++ | ++ | ++ | ++ | − |
| $G8_{IgG2A}$† | + | + | + | +++++ | +++++ | − |
| $2F2_{IgG1}$‡ | − | − | − | − | − | ++++ |

*The optimal mAb dilution producing the maximum mean $OD_{405}$ reading on $CHIKV_{MAU}$ antigen was determined empirically for each mAb and used for assessment for other virus strains. Scoring: +++++, OD>1.0; ++++, OD=0.75–1.0; +++, OD=0.5–0.75; ++, OD=0.3–0.5; +, OD=0.25–0.3.

†mAb G8 was generated to the E1 protein of RRV and is cross-reactive with CHIKV and SFV (Oliveira *et al.*, 2006).

‡mAb 2F2 was previously raised to the Australian prototype strain MRM39, and has been shown to be SINV-specific.

heating. The proteins were resolved on 4–12 % Bistris precast SDS-PAGE gels (Invitrogen), transferred onto Hybond C nitrocellulose membranes (GE Healthcare), immune-stained and developed as previously described (Clark *et al.*, 2007). To assess the glycosylation status of the target antigen, lysates were treated with PNGase F (Sigma-Aldrich) according to the manufacturer's instructions, prior to analysis by Western blot. All antibodies reacted to a protein band of ~36 kDa, in samples that were reduced or unreduced, as well as PNGase F-treated and untreated material, consistent with recognition of the unglycosylated CHIKV CP (Fig. 1a).

Specificity towards the CHIKV CP was confirmed by testing the mAbs for reactivity with recombinant $CHIKV_{MAU}$ CP (rCap) expressed in COS-7L cells by means of immunofluorescence assay (IFA). $CHIKV_{MAU}$ CP constructs were generated by amplifying the respective CP genes from cDNA synthesized by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, with primer set 'CHIKV Capsid Forward pcDNA' 5′-TATATA GCTAGC ATG GAGTTCATCCCA-ACCCAA-3′, 'CHIKV Capsid Reverse pcDNA' 5′-TATATA GGATCC ACTCCACTCTTCGGCCCC-3′, followed by ligation into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Expression of recombinant E1 (rE1) and E2 (rE2) of $CHIKV_{MAU}$ was also carried out for

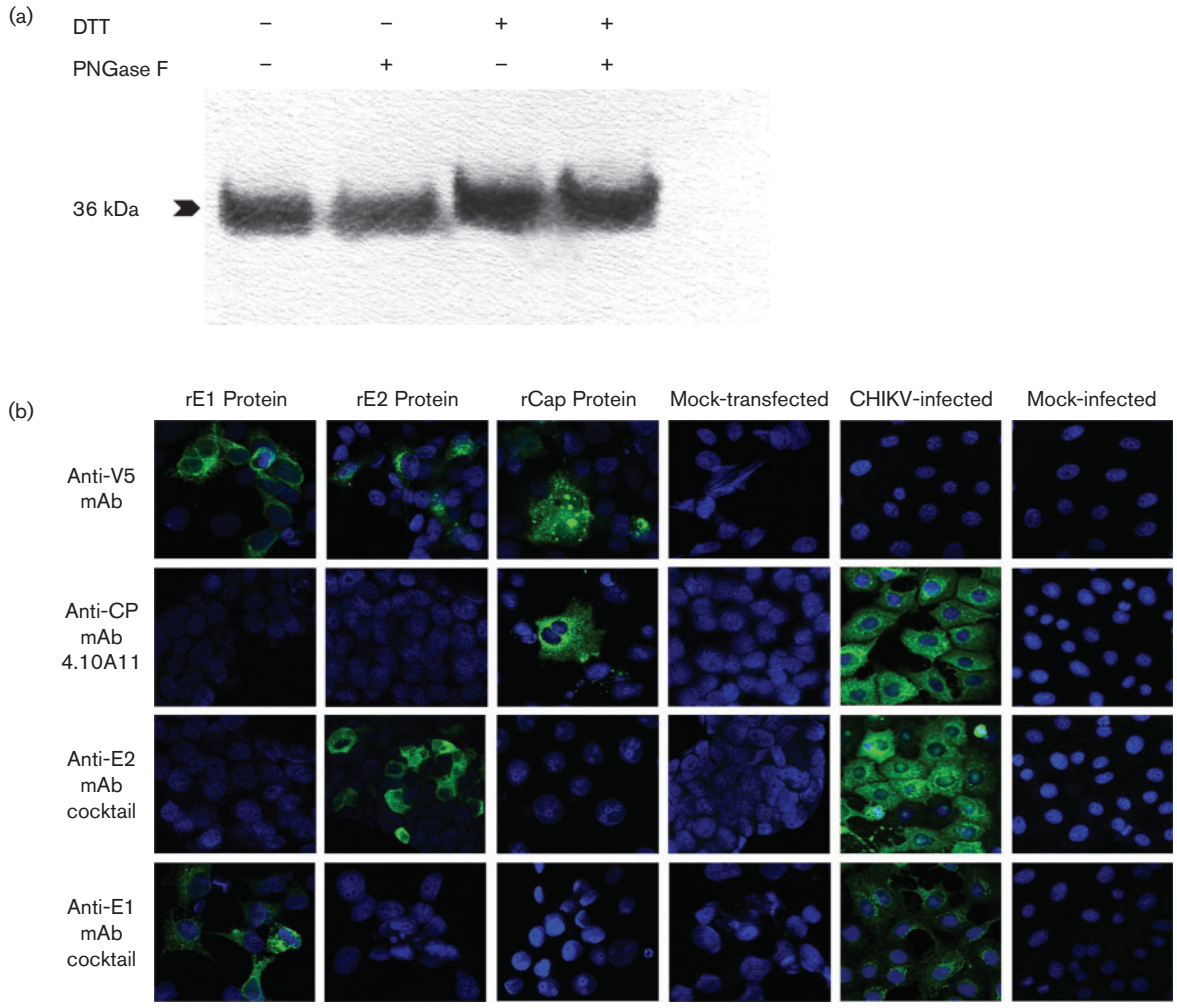


**Fig. 1.** mAb reactivity in Western blot and IFA. (a) Representative mAb 4.10A11 reactions against boiled, reduced (DTT+) or unreduced (DTT−) lysates of $CHIKV_{MAU}$-infected C6/36 cells treated with (+) or without (−) PNGase F. (b) IFA staining of 4.10A11 against transfected/$CHIKV_{MAU}$-infected cells. Cells were probed with mAb 4.10A11 before incubation with an anti-mouse Alexa Fluor 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. A cocktail of five mAbs generated in a previous study was used for the detection of CHIKV E2 (Goh *et al.*, 2013). B10/G8 mAbs (anti-E1 cocktail) were generated to the E1 protein of RRV and are cross-reactive with the E1 protein of CHIKV (Oliveira *et al.*, 2006). Successful expression of recombinant proteins was demonstrated using anti-V5 mAb.

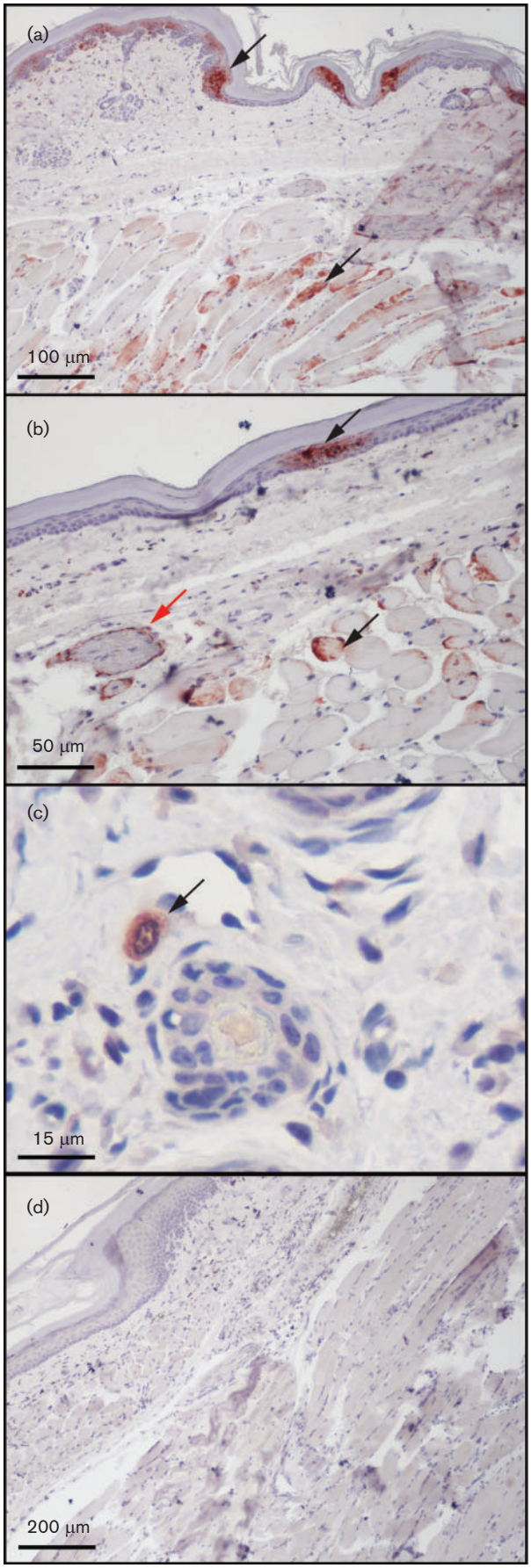


**Fig. 2.** IHC-labelling for CHIKV antigen using capsid-specific mAb 5.5G9. Labelling was observed in epidermal keratinocytes (a, b, top black arrow), skeletal striated muscle cells (a, b, bottom black arrow) and perineural cells (b, red arrow) in tissue sections of acutely infected IRF3/7$^{-/-}$ mice. Macrophage-like cells within connective tissue of CHIKV-infected WT mouse feet 30 days post-infection also stained positive with 5.5G9 (c). No reactivity was observed in the uninfected control (d). Digital micrographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and the NIS-Elements F software and are reproduced without further manipulation.

reference controls as previously described (Goh *et al.*, 2013). Transfected COS-7L cells were fixed onto glass coverslips with 100 % ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid for 1 h at 37 °C. In the case of live virus infection, Vero cells were allowed to grow overnight on glass coverslips before being infected with CHIKV at an m.o.i. of 0.1 for 1 h. Cell monolayers were then washed twice with PBS and incubated at 37 °C in complete growth medium. At 24 h post-infection, Vero cells were fixed and incubated with anti-CHIKV mAbs as described above. Coverslips were then stained, mounted and imaged as described by Goh *et al.* (2013). All 11 mAbs reacted with cells expressing rCap or cells infected with CHIKV, but not the mock-infected/transfected cells or those expressing CHIKV rE1 or rE2 (Figs 1b and S1, available in the online Supplementary Material).

To assess the use of the CHIKV CP-specific mAbs to detect CHIKV in tissues samples, immunohistochemistry (IHC) was performed, as previously described in detail (Goh *et al.*, 2013), on formalin-fixed, paraffin-embedded samples previously prepared from feet of IRF3/7$^{-/-}$ mice infected with CHIKV$_{\text{REUNION}}$ (Rudd *et al.*, 2012) and from wild-type (WT) mice 30 days post-infection. Briefly, deparaffinized sections were subjected to antigen retrieval by heating at 95 °C in a citrate buffer, pH 6 (Target Retrieval Solution, DAKO) for 25 min followed by a 20 min cooling period at room temperature. Following a series of blocking steps, the sections were incubated with undiluted hybridoma culture supernatant of mAb 5.5G9 at 4 °C overnight. Preliminary studies showed that this mAb gave the most intense signal in IHC (data not shown). Antibody binding was visualized using the anti-mouse IgG Envision kit (Dako). Sections were counterstained with Meyer's haematoxylin, mounted and examined under a Nikon Eclipse 51E microscope. Digital micro-photographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and processed with the NIS-Elements F software. Clear staining of keratinocytes and skeletal muscle cells was observed in samples from acutely infected IRF3/7$^{-/-}$ mice with the use of mAb 5.5G9 (Fig. 2), consistent with previous *in situ* hybridization studies in these interferon $\alpha/\beta$-response-deficient mice (Rudd *et al.*, 2012).

An ongoing issue for the field of alphaviral arthritis is understanding the aetiology of chronic inflammatory

disease. Persistence of CHIKV RNA and protein was reported in occasional macrophages (i) in a chronic CHIKV patient 18 months post-onset of disease in the face of a robust host immune response (Hoarau *et al.*, 2010), and (ii) in cynomolgus macaques (*Macaca fascicularis*) 44 days post-CHIKV infection by *in situ* hybridization (Labadie *et al.*, 2010), but has never been described in a mouse model, possibly due to the lack of sensitive reagents. Using the 5.5G9 mAb, we were able to detect CP antigen in scattered macrophage-like cells in connective tissue of the inoculated foot from WT mice 30 days post-infection (Fig. 2). This previously reported mouse model of acute infection and disease (Gardner *et al.*, 2010) thus recapitulates a key feature of chronic disease seen in humans. CP expression on day 30 – well beyond the 4–6 day viraemic period – in this model further supports the view that CHIKV antigen expression persists long-term and is the likely cause of chronic inflammatory disease (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Labadie *et al.*, 2010; Suhrbier *et al.*, 2012). In addition, the ability specifically to detect CHIKV CP-positive cells in paraformaldehyde-fixed, paraffin wax-embedded and decalcified tissue further illustrates the utility of 5.5G9 for CHIKV research. The 5.5G9 mAb may also prove particularly useful for studies of viral persistence, as it allowed the immune-labelling of rare cells with even low levels of CHIKV CP antigen in fixed tissue sections. CHIKV is a biosafety level 3 organism (thus tissue samples must be fixed prior to removal from the biosafety level 3 facility), with joints – necessitating decalcification – and associated tissues often the focus of research for this arthritogenic alphavirus.

In this paper, we report, we believe, the first mAbs generated to the CHIKV CP, and demonstrate their reactivity in ELISA, Western blot and IFA. Our findings suggest these mAbs represent useful research tools and have strong potential in a wide variety of applications. In addition, we have shown that CHIKV antigen can be detected in infected mouse tissue samples by mAb 5.5G9 in IHC. This identifies a further application for these reagents as specific tools for the study of CHIKV pathogenesis. The mAbs generated in this study also recognized different strains of CHIKV ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) representing the two major global lineages of the virus (Asian and East/Central/South African) (Schuffenecker *et al.*, 2006). Furthermore, the three mAbs (5.2H7, 5.5D11 and 5.5G9) that also reacted strongly with the non-CHIKV alphaviruses tested here will also be useful research tools for studying CP in related alphaviruses. The mAbs described in this paper are available from the authors upon request.

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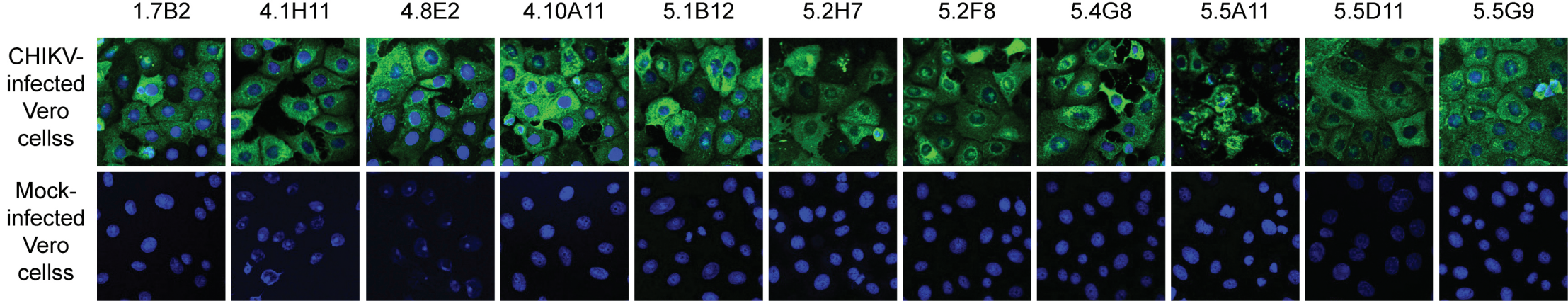
1.7B2
4.1H11
4.8E2
4.10A11
5.1B12
5.2H7
5.2F8
5.4G8
5.5A11
5.5D11
5.5G9
CHIKV-infected Vero cellss
Mock-infected Vero cellss