Title: Development of RT-RPA-based point-of-care tests for epidemic arthritogenic alphaviruses

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Abstract:

Chikungunya (CHIKV), o'nyong-nyong (ONNV), and Mayaro (MAYV) viruses are transmitted by mosquitoes and known to cause a debilitating arthritogenic syndrome. These alphaviruses have emerged and re-emerged, leading to outbreaks in tropical and subtropical regions of Asia, South America, and Africa. Despite their prevalence, there persists a critical gap in the availability of sensitive and virus-specific point-of-care (POC) diagnostics. Traditional immunoglobulin-based tests such as enzyme-linked immunosorbent assay (ELISAs) often yield cross-reactive results due to the close genetic relationship between these viruses. Molecular diagnostics such as quantitative polymerase chain reaction (qPCR) offer high sensitivity but are limited by the need for specialized laboratory equipment. Recombinase polymerase amplification (RPA), an isothermal amplification method, presents a promising alternative to qPCR, providing rapid results with minimal equipment requirements. Here, we report the development and validation of three virus-specific RPA-based rapid tests for CHIKV, ONNV, and MAYV. These tests demonstrated both speed and sensitivity, capable of detecting 10 viral copies within 20 minutes of amplification, without exhibiting cross-reactivity to related alphaviruses and other arboviruses. Furthermore, we evaluated the clinical potential of these tests using serum and tissue samples from CHIKV, ONNV, and MAYV-infected mice. Ultimately, findings highlight the significance of these rapid and specific POC diagnostics in improving the early detection and management of these arboviral infections.

Introduction

Chikungunya (CHIKV), o'nyong-nyong (ONNV) and Mayaro (MAYV) viruses belong to the family *Togaviridae* and are closely related mosquito-borne alphaviruses[1]. Their genomes consist of approximately 12 kilobases of positive-sense RNA encased within a nucleocapsid, encoding the four nonstructural proteins (nsp1-4) and five structural proteins including three envelope proteins (E1-3), the capsid, and 6K across two open reading frames[2]. While evidence of alphavirus-induced human diseases dates back centuries, CHIKV, ONNV, and MAYV were formally isolated in the 1950s: CHIKV in Tanzania (1952), ONNV in Uganda (1959), and MAYV in Trinidad (1954)[3-5].

CHIKV is primarily transmitted by *Aedes* mosquitoes[1,3]. Following its isolation in 1952 during an outbreak in Tanzania, CHIKV caused several intermittent outbreaks from the 1960s-1980s in the Sub-Saharan Africa[6,7]. Meanwhile, the first record of CHIKV in Asia, was in Thailand in 1958, where it was found to be associated with dengue fever in a significant urban outbreak[6-8]. Several outbreaks in Southeast and South Asia were reported throughout the 1960s [6-8]. Only sporadic cases were reported in the years that followed, until its reemergence in 2004, on Lamu Island, in Kenya[6-9]. This particular outbreak caused a seroprevalence in 75% of the island's population had anti-CHIKV Immunoglobulins (IgM and IgG)l[9]. It subsequently spread to several nearby Islands in the Indian ocean [9]. Of these, the La reunion epidemic of 2005-2006, was particularly severe, with 266,000 cases and over 200 deaths resulting from the more severe disease manifestations, particularly among the older patient population[6,7,9]. Also part of this series of outbreaks was a major CHIKV outbreak in India, from 2005-2006, that resulted in over 1.4 million cases[6-8]. Around this time, outbreaks were also reported

67 in parts of Southeast Asia and also in some pacific islands[6-8]. Then, in 2011, CHIKV
68 reached the western hemisphere, and rapidly diffused through the Caribbean and finally
69 reached the South America in 2014, affecting hundreds of thousands of people along
70 the way [6-8]. Since then, there have been periodic CHIKV outbreaks in all of these
71 regions, as well as some autochthonous cases in parts of Europe, Middle East, and
72 North America[3,6,7]. Overall, according to the European Center for Disease Prevention
73 and Control, CHIKV lays claim to over 100 countries, and in 2024 alone, as of March
74 31, 160,000 new cases and 50 deaths have been reported[10].

75 ONNV, closely related to CHIKV, is limited in its endemicity within Africa,
76 although, reports of the virus reaching countries outside of Africa via international
77 travelers has been previously reported[11-13]. Transmitted by *Anopheles* mosquitoes,
78 ONNV has been implicated in two major outbreaks, that impacted millions of people[1].
79 This first outbreak, spanning 1959-1962, involved countries in both East and West
80 Africa, infecting over 2 million people[12,14]. The second outbreak between 1996-1997,
81 originated in Uganda and then spread to Kenya and Tanzania, with estimated infection
82 rates at 45-68%[1,12]. Following these, isolated cases and smaller outbreaks have been
83 reported across East and West Africa, as well as in travelers returning from those
84 regions[12].

85 Like ONNV, MAYV, is also geographically limited in its spread, causing sporadic
86 outbreaks in Central and South America, since it was first isolated in 1954 in
87 Trinidad[15,16]. Outbreaks have been recorded in Brazil, Bolivia, Venezuela, and Haiti[15,16].
88 Outside of these, wide ranging seroprevalence (21% to 72%) has been reported all
89 across the region, and sporadic cases occur both within the region and abroad, in

90 Europe and the USA through travelers[15,17]. Although primarily transmitted by
91 *Hemagogous* mosquitoes, certain strains of MAYV have the ability to adapt to replicate
92 in and be transmitted by different, more anthropophilic mosquito vectors[15,18,19]. This is
93 noteworthy because this ability had enabled the broader dissemination of related
94 viruses like CHIKV and suggests a possibility of global spread of MAYV[15,18,19].

95 Infection with these viruses typically results in a febrile illness characterized by
96 fever, mild to severe arthralgia, occasionally accompanied by edema, and/or a rash, but
97 can also lead to a debilitating, long-term arthritogenic syndrome[3]. In the case of CHIKV,
98 approximately 40% of infections progress to chronic arthritis, persisting for up to 3
99 months. Long-term manifestations have not been reported for ONNV, whereas, rare
100 chronic arthritis and encephalitic disease has been reported for MAYV[3,20]. Although
101 seldom fatal, the illnesses induced by these viruses substantially impact quality of life.
102 Given the millions of reported cases worldwide, CHIKV, ONNV, and MAYV pose a
103 significant global health risk[1,21].

104 The prevalence of these viruses is anticipated to escalate due to the influences
105 of climate change, urbanization, and international travel[21], necessitating improved
106 monitoring to prevent major outbreaks through effective, point-of-care (POC) diagnostic
107 tools. Diagnosis of CHIKV, ONNV, and MAYV infections primarily rely on laboratory
108 testing owing to clinical overlaps among these viruses with other arboviral diseases like
109 dengue and Zika fever [1,3,21]. Additionally, the geographical co-circulation of CHIKV and
110 ONNV in Africa and CHIKV and MAYV in South America, further complicates accurate
111 diagnosis[1,3,21]. Current diagnostic tests are predominantly either serological or

112 molecular techniques, based on the detection of patient antiviral immunoglobulins (IgM
113 and IgG) or viral RNA, respectively[1].

114 Commercially available serological tests, such as enzyme-linked immunosorbent
115 assays (ELISAs) detect anti-viral IgM and IgG[1]. IgM antibodies can usually be detected
116 in patient serum or plasma within 6 days after infection and persist for 3-4 months after
117 infection[1]. IgG antibodies emerge subsequent to IgM antibodies and can be detected
118 decades after the initial infection[1,22,23]. ELISAs, however, are reported to have low
119 sensitivity and lack specificity since antibodies tend to cross-react between the three
120 viruses due to their genetic similarity[1]. Plaque reduction neutralization tests (PRNTs)
121 are typically considered gold standard assays to distinguish viral infections based on
122 neutralizing antibodies but these tests often require enhanced biosafety laboratories
123 and require expert handling[24]. Moreover, there is evidence suggesting potential cross-
124 reactivity between CHIKV and ONNV in PRNTs as well[1,25-27].

125 Molecular diagnostics, on the other hand, are more sensitive and specific. Of
126 these, reverse transcription (RT)-PCRs or qRT-PCRs are the most popular[1,21]. There
127 are several commercial and/or in-house RT-PCR tests for CHIKV, ONNV, and MAYV.
128 These assays are reported to be sensitive and specific and can reliably detect viruses
129 particularly during the acute phase of the infection (the first 3-4 days), given the high
130 viremia (10^4-10^8 RNA copies/mL in the blood) during that time[1]. However, these assays
131 fall short of being ideal POC diagnostics, especially in more resource-limited settings,
132 due to their reliance on expensive and high precision equipment such as thermal
133 cyclers.

134 Therefore, rapid, inexpensive, sensitive, and specific POC diagnostics are still an
135 unmet need with these three viruses. One possible solution is using isothermal
136 amplification methods to detect viral RNA. These techniques have emerged as faster
137 and less expensive alternatives to PCR, particularly because they do not require precise
138 thermal cycling, and have shorter reaction times[28]. Furthermore, they possess
139 comparable to potentially enhanced sensitivity and specificity, and there are numerous
140 methods to detect their final products, several of which can be accomplished without
141 scanners or fluorescence readers[21,28]. Of these, recombinase polymerase amplification
142 (RPA) is of interest due to its non-complicated assay design, shorter reaction running
143 times, and its potential to be multiplexed[28].

144 RPA was first described in 2006 and it works by using a single pair of single-
145 stranded DNA primers, recombinase proteins (T4 Uvs X and UvsY), single-strand
146 binding proteins (Gp32), and a strand displacing polymerase (B*. subtilis* polymerase
147 large fragment), to isothermally amplify a double-stranded DNA template in the
148 presence of ATP and magnesium acetate[29]. Reactions are typically run at 37°C-42°C
149 for 5-20 minutes[28]. It can be used in the detection of RNA with the addition of a reverse
150 transcription step, either as a separate step or in a one-step reaction[28,30-32].
151 Furthermore, the assay can be configured to comply with several detection modalities,
152 including agarose gel electrophoresis, colorimetric readouts, and rapid test/lateral flow
153 assay (LFA) strips [28-33].

154 There currently are not many RPA tests for the detection of these three
155 alphaviruses[21], with the only example being a CHIKV RPA test designed by Patel et al.,
156 which was sensitive but cross-reactive with ONNV[34]. In this study, we report the

157 development of three virus-specific and sensitive RPA-based diagnostic tests for
158 CHIKV, ONNV, and MAYV. We initially performed multiple sequence alignments
159 (MSAs) to identify genomic targets for primer design, followed by an extensive primer
160 screen, and then finally re-configuring the successful primer pairs to make the assay
161 compatible with LFA-based detection. The assays target E2 for CHIKV, and NSP2 for
162 both MAYV and ONNV and all three tests can detect down to 10 viral copies on LFA
163 strips. The RPA-based rapid tests were further evaluated on serum and tissue samples
164 from clinically relevant mouse infection models for each virus. Ultimately, our results
165 highlight the sensitive and specific nature of RT-RPA-based diagnostics and allude to
166 their value as POC diagnostic tool better characterize these viruses and monitor
167 outbreaks.

168

169 **Methods**

170 **Selecting regions for primer/probe design**

171 MSAs were constructed using NCBI BLAST by first querying a complete genome
172 for each virus (NC_004162.2 for CHIKV, NC_075006.1 for ONNV, NC_003417.1 for
173 MAYV). The search results were filtered down to obtain a set of complete genomes with
174 over 90% similarity to the query sequence. Those sequences were aligned using
175 BLAST's MSA tool and viewed using the NCBI MSA viewer 1.25. The MSA viewer was
176 used to generate a consensus sequence with a 70% threshold, i.e., if >=70% of the
177 aligned sequences have the same nucleotide at the same position in the alignment, the
178 consensus sequence will reflect that, and, if there is less than 70% consensus at a

179 particular position, an ambiguous IUPAC symbol is used instead. The positions with a
180 less than 70% consensus (i.e., with an ambiguous IUPAC symbol in the consensus
181 sequence) were considered “mismatches” while calculating the % non-homology for
182 each gene of interest. Finally, the % mismatch values for the various genes were
183 compared and the top 4 most conserved, i.e., with the lowest % mismatch values were
184 selected for primer design. A list of all the aligned accession numbers is provided in
185 Supplementary Table 1.

186 **Primer and Probe design**

187 Genomes, NC_004162.2, NC_075006.1 and NC_003417.1 for CHIKV, ONNV
188 and MAYV, were respectively used to design primers and probes, within the genomic
189 regions of interest identified through the MSAs, for each virus. Primers were designed
190 manually, ensuring rules: a length of 30-35 nt, a GC content of 40-60% and absence of
191 palindromes or tandem nucleotide repeats[30,33,35]. The target amplicons were all between
192 120-200 bp in size and also had a GC content of 40%-60%. Finally, the designed
193 primers were mapped onto another MSA of the complete genomes of the three viruses
194 to ensure that sufficient mismatch was present to prevent cross reactivity[35]. After the
195 primer screen, probes were designed corresponding to the primer pair identified in the
196 screen for each virus. Nfo probe for the ONNV assay was designed as described by
197 Piepenburg et al., ensuring a complete length of about 45 nt, with at least 30 nt
198 preceding the abasic site, and 15 nt following it[29]. The hybridization probes for the
199 CHIKV and MAYV assays were designed as described by Qian et al., to be around 15-
200 25 nt in size, and binding to a region within the amplicon, avoiding overlap with the
201 primer binding sites[31]. The designed primers were synthesized by Integrated DNA

202 Technologies. A list of the RPA primers and probes designed and used in this study are
203 provided in supplementary Table 2.

204 **RNA extraction**

205 The QiaAmp viral Mini kit (Qiagen) or the PureLink Virl RNA/DNA Mini kit
206 (ThermoFisher) were used for extraction of RNA from serum or culture supernatant for
207 ONNV (NR-51661, BEI Resources Repository), following the manufacturers'
208 instructions. RNA from mouse tissues was extracted using the RNeasy Lipid tissue Mini
209 Kit (Qiagen) following manufacturer instructions. The concentration of the RNA extracts
210 was determined using a NanoDrop One device (ThermoFisher).

211 **cDNA synthesis**

212 Viral gRNA for CHIKV (181/25, BEI Resources Repository) and for MAYV (NR-
213 50081, BEI Resources Repository) and extracted RNA from ONNV (NR-51661, BEI
214 Resources Repository) propagated on Vero cells (CCL-81, ATCC) was used. Viral RNA
215 was converted to cDNA using the SuperScript IV Vilo master mix (ThermoFisher),
216 following the manufacturer's instructions. RNA input of $2x10^7$ viral copies was used in a
217 20 µL cDNA synthesis reaction to obtain cDNA with a concentration of 10^6 copies/µL.
218 For the primer screen, the cDNA stock was diluted to 10^5 copies/µL and 1 µL was used
219 for the RPA reactions. For the sensitivity assays, the cDNA stock was serially diluted to
220 obtain a range of concentrations, to enable inputs ranging from 10^6 copies to 1. For the
221 serum and tissue samples, a fixed volume of RNA (2 µL) was used as input into cDNA
222 synthesis, and 1µL of cDNA was used as input into RPA.

223

224 **RT-RPA reactions using unlabeled primers**

225 RPA reactions were performed using the TwistAmp Basic Lyophilized kit
226 (TwistDx), following the manufacturer's instructions, but scaling measurements down to
227 10 µL reactions. Briefly, lyophilized mix was rehydrated using 29.5 µL of the provided
228 rehydration buffer and 10 µL of nuclease free water, and 7.9 µL of the rehydrated mix
229 was used per 10µL reaction, along with 0.3 µM of each primer, 280 mM magnesium
230 acetate, and the requisite amount of cDNA. The reactions were incubated for 20
231 minutes at either 37°C, 39°C or 41°C, as indicated in the text. Diluted reaction products
232 (1:5) were run on a 2.5% agarose (BioRad) gel in 1x TAE buffer (ThermoScientific) and
233 stained with Gelgreen (Biotium). For the sensitivity assay gels, RPA products were
234 purified using the PureLink PCR purification kit (ThermoFisher), following the
235 manufacturer's instructions.

236 **RT-RPA reactions with labelled primers**

237 For the hybridization method, reactions were set up as described above with one
238 modification—the replacement of the reverse primer with a 5'biotin- or 5'digitonin-
239 labelled version, keeping the concentration the same. The hybridization step was
240 performed as described by Qian et al.[31]. Briefly, after the RPA reaction run, 1 µL of 5
241 µM hybridization probe (final concentration of 0.167 µM) and 19 µL of hybridization
242 buffer (10 mM Tris HCl at pH 8.5) was added to each reaction. This was heated to 95°C
243 for 3 minutes and then allowed to gradually cool to room temperature to enable
244 hybridization. The nfo-probe reactions were performed as described by Piepenburg et
245 al[29]. Briefly, the basic master mix was modified, replacing a portion of the water with nfo
246 or Endonuclease IV (New England Biosciences) at a final concentration of 0.2 U/µL and

247 nfo-probe to a final conc of 0.1 μM. The reverse primer was also replaced with a
248 5'digitonin-labelled version, keeping the concentration the same.

249 **Detection of RPA products on LFA strips**

250 The products from the hybridization and nfo-probe reactions were processed
251 using the HybriDetect 2T LFA strips (Milenia Biosciences), following the manufacturer's
252 instructions. Briefly, unpurified reaction products were directly diluted in the provided
253 assay buffer (1:20 for the nfo-probe reactions and 1:15 for hybridization reactions) and
254 applied to the strip. The strip was then immersed in 70 μL of the provided assay buffer
255 and was allowed to react for 5-30 minutes. The strips were imaged using a smartphone
256 camera.

257 **qRT-PCR**

258 For the CHIKV, ONNV and MAYV qPCRs conducted for this study, the
259 PrimeTime one-step qRT-PCR kit (Integrated DNA Technologies), along with primers
260 and TaqMan probes were used. CT values were recorded using a QuantStudio3 device
261 (ThermoFisher). The primer and probe sequences and the publications they are derived
262 from are as follows:

263 1) CHIKV- 5'AAGCTCCGCGTCCTTTACCAAG 3', 5'CCAAATTGTCCGGGTCCTCCT
264 3', 5'/FAM/CCATGTCTTCAGCCTGGACACCTTT/TAMRA/3'[36].

265 2) ONNV- 5'CGCAGCTTACGGGTTTCATA3', 5'GCAACGCCTTCAGAAACGC3',
266 5'/FAM/TGCTCTACTCTGCATTGCAAGA/BHQ1/3'[37].

267 3) MAYV- 5'AAGCTCTTCCTCTGCATTGC3', 5'TGCTGGAAACGCTCTCTGTA3', 5'/
268 CalFluorOrange560/GCCGAGAGCCCGTTTTTAAAATCAC/ BHQ-1/3'[38].

269 **Preparation of Mouse Samples**

270 Adult AG129 mice were inoculated with 1000 PFU of either CHIKV (181/25
271 strain, BEI Resources), ONNV (NR-50081, BEI Resources), or MAYV (NR-51661, BEI
272 Resources) via subcutaneous injection on the ventral side of the left footpads.
273 Submandibular blood was collected was collected at day 1 and/or day 3 post infection,
274 and into heparin coated tubes and centrifuged at 2000 x g for 10 minutes, to obtain
275 serum. The mice succumbed to the infections at day 3 (CHIKV and ONNV) and at day 5
276 (MAYV) at which point, the liver, spleen, kidneys, and cardiac blood were collected.
277 These samples were subjected to RNA extraction and RT-RPA as described above.

278

279 **Results**

280 In order to obtain primer pairs and probes capable of detecting RNA from all
281 strains of a given virus, we wanted to target them to the most conserved regions of that
282 virus' genome. To identify such targets for primer design, we used BLAST to construct
283 MSAs of a set of complete genomes for each virus. We then analyzed the consensus
284 sequence generated from the MSA to identify the most conserved genomic regions,
285 focusing our analysis on the 4 NSPs, capsid, E1 and E2. The two remaining structural
286 proteins—6K, E3—were overlooked due to their smaller size and therefore less area
287 within which to design a set of primers. The top 4 most conserved genomic regions
288 identified for each virus were as follows: NSP2, NSP4, E2 and E1 for CHIKV; NSP1,

289 NSP2, NSP4, E2 for ONNV; NSP1, NSP2, NSP4 and E1 for MAYV (Fig. 1A-C). Primers
290 were designed within the identified genes for each virus, with one exception: for ONNV,
291 we opted to design primers within E1 rather than NSP4. This was due to its high degree
292 of homology, with less than 40% sequence divergence within the Semliki Forest
293 Serocomplex, the antigenic complex to which the CHIKV, ONNV, and MAYV belong
294 and also due to E1's popularity as a target in existing molecular assays for these
295 viruses[1,39].

296 16 primer pairs were designed for ONNV and 17 for CHIKV and MAYV, spanning
297 the genomic regions identified through the MSAs. It was ensured that the primers
298 followed the stipulations for RPA primer design: a length of 30-35 nt, a GC content of
299 40-60% and absence of palindromes or tandem nucleotide repeats[30,33,35]. Additionally,
300 all designed primers were mapped onto the genomes of the other two viruses, to ensure
301 sufficient mismatches were present to minimize chances of cross-reactivity[35]. To screen
302 the designed primers, viral genomic RNA (gRNA) was first reverse transcribed and then
303 an equivalent of 10^5 viral copies in complementary DNA (cDNA) was used as input into
304 each RPA reaction. The reactions were run for 20 minutes, at three temperatures 37°C,
305 39°C and 41°C, which span the typical RPA temperature range[35]. They were evaluated
306 by running the products out on an agarose gel (Fig. 2A-C). Each primer pair varied in its
307 ability to amplify cDNA, and this activity sometimes influenced by temperature, for
308 example: at 37°C, CHIKV primers #7, 9, 11 fared better than #3, 6, 10, and #7 fared
309 better at 37°C than at 39°C (Fig. 2A). At the end of the screen, several successful
310 primer-pair/reaction-temperature combinations were identified, and the following
311 combinations were selected: #9 at 37°C was identified for CHIKV, #2 at 41°C for ONNV

312 and #1 at 39°C for MAYV. Their respective targets are within the E2 gene for CHIKV
313 and within NSP2 for both ONNV and MAYV. The analytical sensitivity of these three
314 primer-pairs was evaluated by using a serial dilution of cDNA, ranging from 10^6 viral
315 copies to 1 viral copy. Also included were reactions using 10^5 viral copies of the other
316 two viruses to evaluate cross-reactivity of the primers. The limits of detection (LODs) for
317 these three primers, processed via agarose gel electrophoresis, was found to be
318 approximately 10^2 viral copies for CHIKV and ONNV and between 10^3-10^2 viral copies
319 for MAYV, and no cross-reactivity to cDNA from the other two viruses was seen (Fig. 3).
320 Since we sought to design rapid tests for these three viruses, our next step was
321 to re-configure our assays to enable the detection of the reaction products on LFA
322 strips. For this, two approaches were used: hybridization and nfo-probes. At the outset,
323 both approaches involve replacing one of the reaction primers with a 5'labelled version
324 to generate a singly labelled amplification product. The first method involves a separate
325 hybridization step, where the singly-labelled amplification product, is denatured and
326 then hybridized with a labelled probe[32,40]. The second approach involves introducing an
327 nfo-probe, which are labelled probes with an internal abasic site, into the reaction, along
328 with an endonuclease (nfo or Endo IV) that cleaves at that abasic site upon probe
329 hybridization[29]. Ultimately, both approaches result in an amplification product that is
330 doubly labelled—one label with affinity for test line on the strip, and the other, with
331 affinity for the antibody conjugated to the gold nanoparticles (Fig. 4A). The LFA strips
332 we used had two test-bands, one coated with streptavidin and the other with an anti-
333 digitonin antibody. The gold nanoparticles in the strip are conjugated to an anti-FITC
334 antibody. We therefore opted to label the reverse primers using either 5'biotin tag (for

335 CHIKV) or 5'Digitonin tag (for ONNV and MAYV) and labelled all the probes with a
336 5'FAM tag.
337 We chose the hybridization option for the CHIKV and MAYV tests, and the nfo-
338 probe option for ONNV. The reaction temperatures were maintained at the same values
339 that were identified in the agarose gel-based primer screen for the ONNV and MAYV
340 tests. However, for CHIKV, running the reaction at 39°C instead of 37°C resulted in
341 darker coloration of the test-band on the LFA strip, prompting us to modify the running
342 conditions accordingly. Sensitivity of the rapid test was determined in the same manner
343 as described for Figure 3, using a serial dilution of viral cDNA. All three tests were
344 highly sensitive, with the ability to detect down to 10 viral copies, and similar to their un-
345 labeled counterparts (Fig. 3), they did not cross-react with 10^5 cDNA copies from the
346 other two viruses (Fig. 4B-D). Furthermore, we conducted a broader cross-reactivity
347 screen for these three rapid tests using cDNA from other arboviruses and saw no cross
348 reactivity, enabling us to confirm that the designed assays were highly specific to their
349 intended targets (Supplementary Table 3).
350 Having designed sensitive and specific RT-RPA-based rapid tests for CHIKV,
351 ONNV, and MAYV, we then sought to evaluate their performance on clinically relevant
352 samples. For this, adult AG129 mice, which are homozygous interferon
353 alpha/beta/gamma receptor (IFNα/β/γR) knockouts, were inoculated with either CHIKV,
354 ONNV, or MAYV at a dose of 1000 PFU via subcutaneous footpad injection. RNA was
355 then extracted from serum collected 1- and 3-days post-infection (dpi) and from the
356 spleens, livers, and kidneys. The extracted RNA was subjected to the designed RT-
357 RPA tests and also to RT-PCR for comparison (Fig. 5A-C). The CHIKV test was able to

358 detect virus in 2 out of 3 of the 1-dpi serum samples, 3-dpi serum samples, and livers.
359 But all three kidney and spleen samples produced a positive test. The CHIKV RT-PCR
360 test in comparison only detected viral RNA in two samples: one of the 3-dpi serum
361 samples and in one of the livers (Fig. 5A). The ONNV rapid test detected viral RNA in 1
362 out of 2 of the 3-dpi serum samples, spleens, and kidneys and both liver samples (Fig.
363 5B). Several of the RT-RPA-negative ONNV samples did however test positive with RT-
364 PCR. All but one of the MAYV samples tested positive on both RT-RPA and RT-PCR,
365 with the only negative result coming from one of the 1-dpi serum samples (Fig. 5C).

366

367 **Discussion**

368 In this study we describe three RT-RPA based rapid tests for the sensitive and
369 specific detection of CHIKV, ONNV and MAYV. The three viruses are closely related
370 alphaviruses, belonging to the same serocomplex, with ONNV and CHIKV being the
371 closest related viruses in that group[39]. There is currently a lack of sensitive and specific
372 POC diagnostic tools for these three viruses, given the cross reactivity of common
373 serological tests, and the expensive cost of running common molecular diagnostic tests
374 like RT-PCRs[1,21]. We sought to bridge that gap by designing RT-RPA based rapid tests,
375 that utilize the isothermal amplification technique, RPA, and are detected on rapid test
376 strips, making them ideal for use at the POC. We show that our three tests were
377 sensitive, with the ability to detect down to 10 viral copies, and specific, with no cross
378 reactivity to related alphaviruses or other arboviruses.

379 We further demonstrated that our tests are capable of detecting viral RNA from
380 more clinically relevant samples, starting with those obtained from mouse infection
381 models. Mice deficient in interferon signaling, like AG129 mice, are commonly used to
382 model CHIKV, ONNV, and MAYV infections because wildtype mice are not as
383 susceptible and are able to rapidly clear infections from these viruses[41,42]. In our mouse
384 experiments, we were able to detect viral RNA in the day 3 sera more reliably than in
385 the day 1 sera, consistent with the trend of viremia peaking around day 2-3 for arboviral
386 infections in this mouse model[42]. We were also able to detect viral RNA in the spleens
387 and livers, which, along with joints and muscles, are considered common replication
388 sites for alphaviruses in their hosts, allowing for their efficient dissemination
389 thereafter[41,43-45]. We were also able to detect viral RNA in the kidneys of infected mice,
390 and while they not a common target or replication site for alphavirus infections, viral
391 loads have been detected in the kidneys of AG129 mice infected with100 PFU of
392 CHIKV[46]. Additionally, we were only able to produce faint positive test bands on our
393 rapid-test strips for most of these samples. One possible explanation for this could be
394 that these mice succumbed to the infections quite rapidly, and possibly before the
395 viruses had a chance to replicate to produce robust viral loads. It is worth noting that the
396 3 CHIKV infected mice and the 2 ONNV infected mice experienced mortality at day 3,
397 and the 2 MAYV infected mice succumbed to the infection at day 5. This is consistent
398 with what with what is known about the behavior of CHIKV, ONNV, and MAYV
399 infections in AG129, A129, or similar mouse models[36,41,42,45,47-51].

400 One improvement that would further bolster these assays would be optimizing
401 them to function as one-step RT-RPA reactions. Secondly, we had designed the MAYV

402 and ONNV assays to produce products that have a different (digitonin) tag than the
403 CHIKV assay products (which have a biotin tag) because we had initially aimed at
404 multiplexing their detection on our double test-band LFA strips. Further optimization to
405 enable this would allow for the creation of two alphavirus tests, one for the South
406 American viruses-CHIKV and MAYV, and the other for the African viruses-CHIKV and
407 ONNV. Lastly, being able to detect RNA directly from crude biological samples such as
408 plasma/serum, urine, or saliva, without an RNA extraction step, would truly enable field
409 deployment. This is possible and has been attempted before, because compared to
410 PCR, RPA is less susceptible to inhibitors present in crude samples[33,52].

411 Despite their spread and disease severity, approved vaccines and antivirals for
412 these three alphaviruses are limited, with the first approved CHIKV vaccine being as
413 recent as November 2023[53]. As these efforts ramp up with the increasing recognition of
414 the threats posed by these viruses, effective POC diagnostic tools will be critical for the
415 efficient monitoring of outbreaks, and for the dissemination of prophylactics and
416 therapeutics. Given their potential as sensitive, specific, POC diagnostics, we think that
417 the RT-RPA assays described here are a promising step forward towards that goal.

418

419 **Data Availability**

420 The data that support the findings of this study are available within the
421 manuscript or upon request from the corresponding authors.

422

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Conflicts of Interest

BBH is a co-founder of Mir Biosciences, Inc., a biotechnology company focused on T cell-based diagnostics and vaccines for infectious diseases, cancer, and autoimmunity.

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Figures

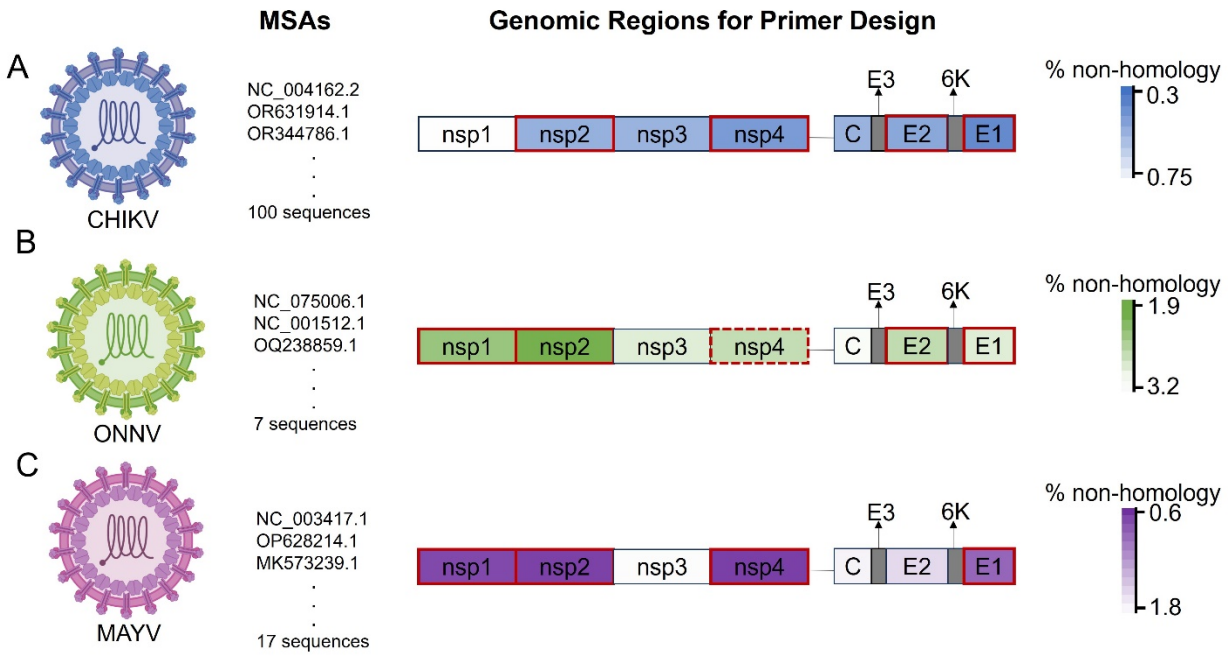

Figure 1: Schematic depicting the regions selected for primer design. Multiple sequence alignments (MSAs) were assembled for (A) CHIKV, (B) ONNV and (C) MAYV using the indicated number of sequences following which the consensus sequence was analyzed focusing on the four NSPs, the Capsid, E2 and E1 to identify the most conserved regions for each virus. The heatmaps show the percent non-homology calculated for each region and the top four most homologous sites (with the darkest shading), highlighted in a red border, were selected for primer design. For ONNV, the analysis identified NSP4, highlighted with a discontinuous border, was identified as the fourth target for primer design, however, we chose to prioritize designing primers in E1 instead.

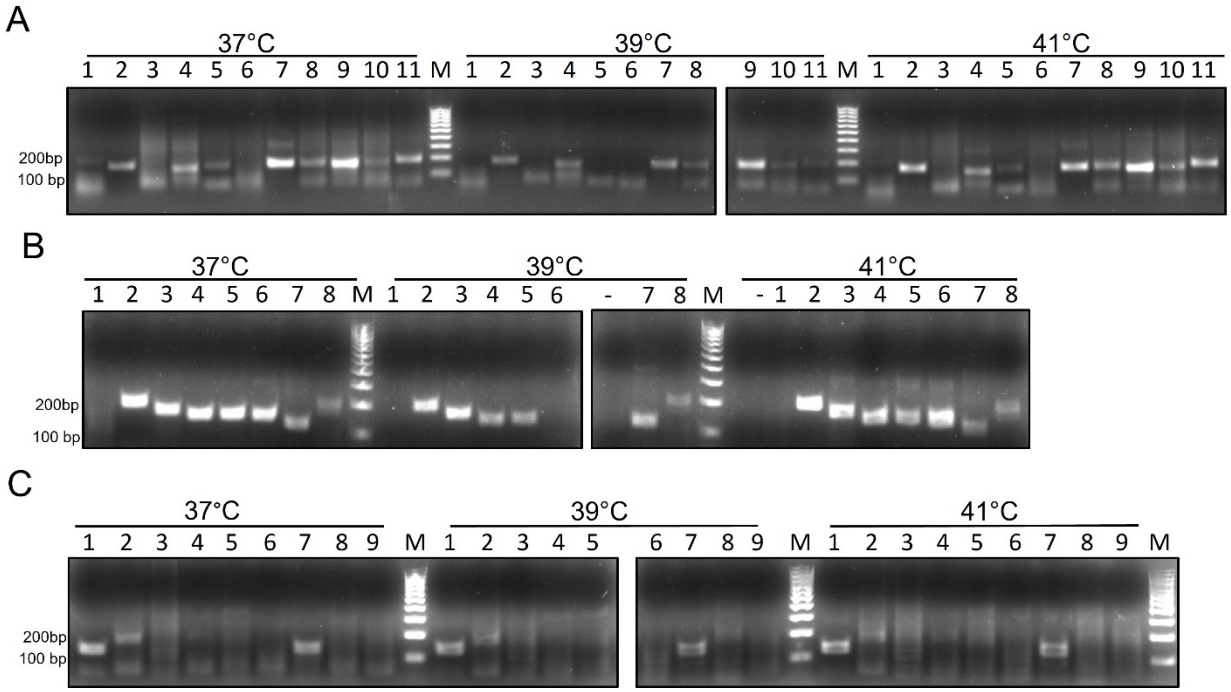

Figure 2: Primer screens conducted by agarose gel electrophoresis of RPA products. All 17 primers for CHIKV, 16 primers for ONNV and 17 primes for MAYV were screened in 20-minute RPA reactions using 10^5 viral copies with cDNA as input, The reactions were run at three temperatures as indicted. Depicted here is a representative subset of those screened primers, A) corresponding to CHIKV, (B) to ONNV, and (C) to MAYV.

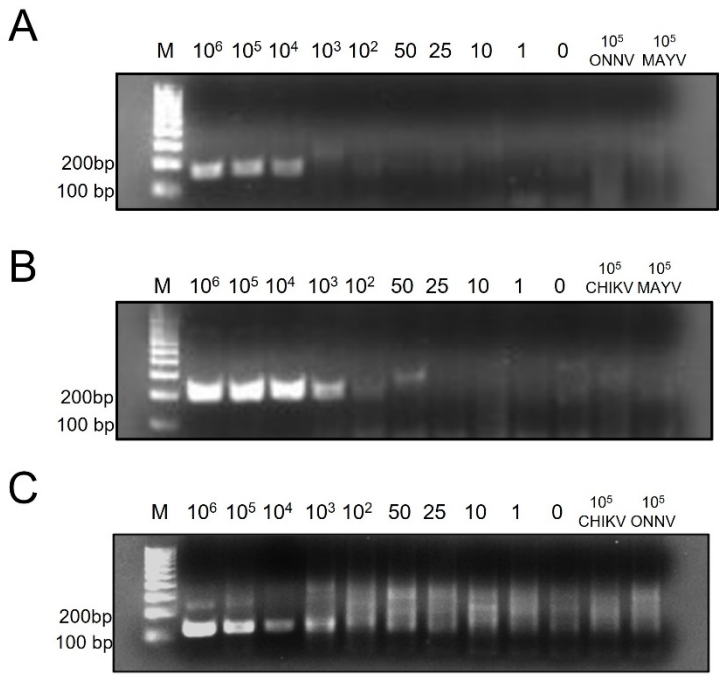

Figure 3: Analytical sensitivities of the designed primers on an agarose gel. Candidate (A) CHIKV, (B) ONNV, and (C) MAYV primer pairs identified in the screen were evaluated on a serial dilution of cDNA inputs ranging from 10^6 viral copes to 1 viral copy. Cross reactivity of these primers was also tested using 10^5 copies of cDNA from the other two viruses. The reaction inputs for each well are denoted in the image. The sensitivity was determined to be 10^2 for CHIKV and ONNV, and between 10^3 and 10^2 for MAYV, with not cross-reactivity.

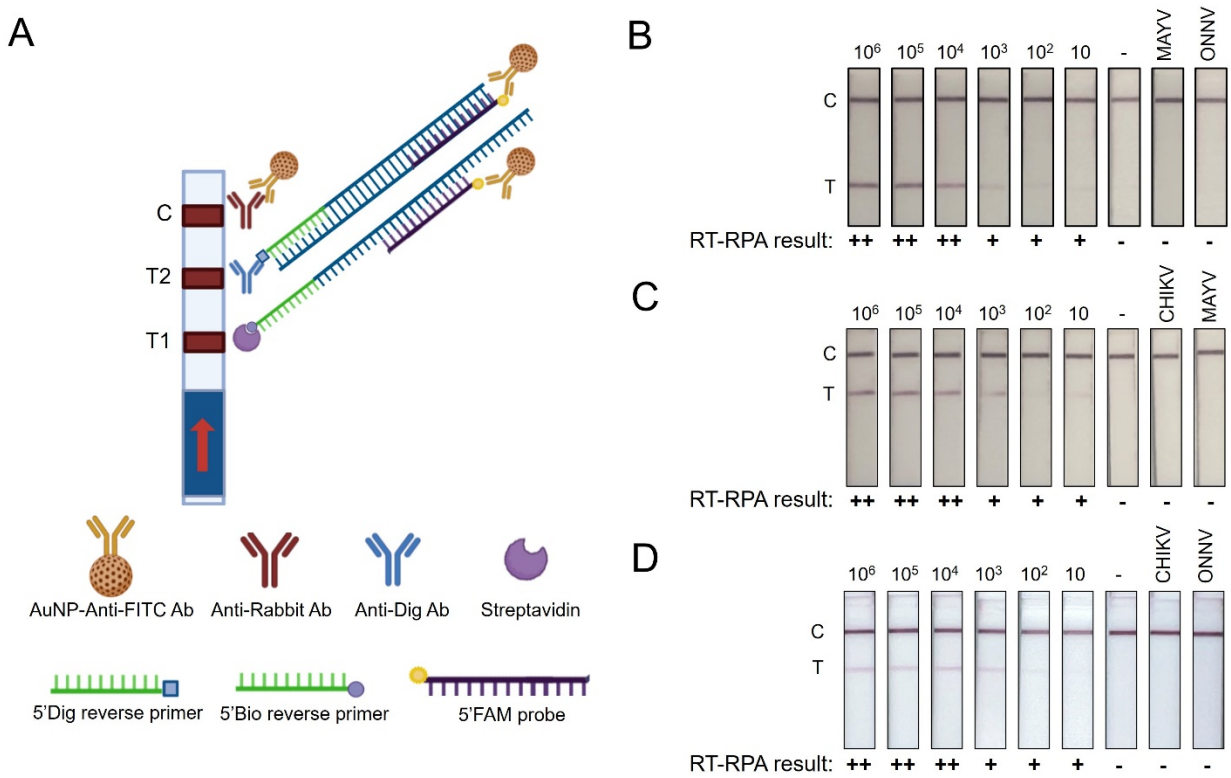

Figure 4: Detecting RPA reactions on LFA strips. A) Schematic representation of the Millenia Biosciences 2T test strips utilized in this study. The strips have two test bands "T1" and "T2," one coated with streptavidin and the other with an anti-Digitonin antibody. The gold nanoparticles (AuNPs) are conjugated to an anti-FITC antibody. Accordingly, all our reverse primers were either tagged with a 5'Biotin tag (CHIKV) or a 5' Digitonin tag (ONNV and MAYV), and their probes- hybridization probe for CHIKV and MAYV or nfo-probe for ONNV- were all 5'FAM tagged. Shown in the figure are depictions of the reaction product from the CHIKV assay, that uses a hybridization probe, binding at T1 and the ONNV assay, that uses a nfo-probe, binding at T2. The MAYV RPA product would appear like the CHIKV product since it also uses hybridization probes, however, it would bind at T2, since its reverse primer has a 5'digitonin tag. (B-D) show the

647 analytical sensitivity of the rapid-test versions of the CHIKV (B), ONNV, (C) and MAYV
648 (D) assays. The reactions used a serial dilution of cDNA as their input. Also included
649 are reactions with 10^5 copies of cDNA from the other two viruses to assess cross-
650 reactivity. RPA reaction products were diluted and applied to the strips and then
651 immersed in the manufacturer-provided assay buffer for up to 30 minutes. The strips
652 were photographed using a smartphone camera. All three assays were able to detect
653 down to 10 copies of viral cDNA and were not cross-reactive.

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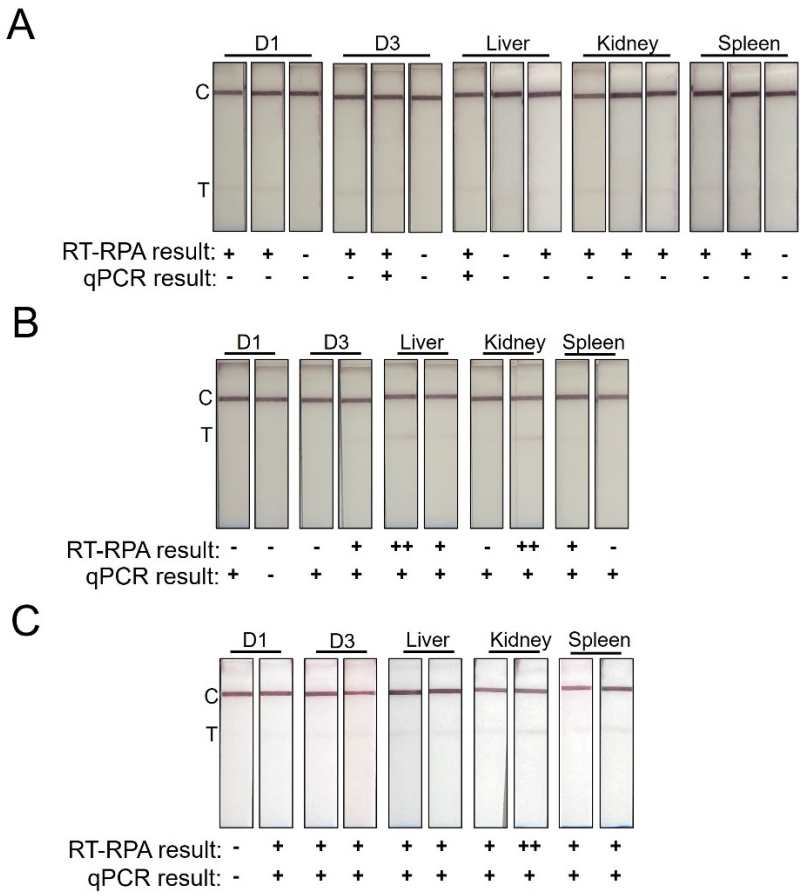

Figure 5: Detecting alphaviruses in serum and tissues from mouse infection models using RT-RPA rapid tests. AG129 mice were infected with 1000 PFU of (A) CHIKV (181/25 strain, n=3), (B) ONNV (NR-50081 strain, n=2) and (C) MAYV (NR-51661 strain, n=2) via subcutaneous footpad injection. Serum was collected 1- and 3-days post infection, and organs were harvested at day 3 for CHIKV and ONNV and at day 5 for MAYV, when the mice experienced mortality. RNA was extracted, reverse-transcribed and subjected to RPA using labelled primers. The reaction products were applied to the test strips and were photographed using a smartphone camera. The extracted RNA from each sample was also subjected to RT-PCR for comparison. The

678 results of both assays for each sample are indicated with a + (or ++) and –, as

679 observed.