

1 **Development of real-time reverse transcriptase PCR assays for the detection of Punta Toro**
2 **virus and Pichinde virus.**

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28

29 **Abstract**

30

31 **Background:** Research with high biocontainment pathogens such as Rift Valley fever virus
32 (RVFV) and Lassa virus (LASV) is expensive, potentially hazardous, and limited to select
33 institutions. Surrogate pathogens such as Punta Toro virus (PTV) for RVFV infection and
34 Pichinde virus (PICV) for LASV infection allow research to be performed under more
35 permissive BSL-2 conditions. Although used as infection models, PTV and PICV have no
36 standard real-time RT-PCR assays to detect and quantify pathogenesis. PTV is also a human
37 pathogen, making a standardized detection assay essential for biosurveillance. Here, we
38 developed and characterized two real-time RT-PCR assays for PICV and PTV by optimizing
39 assay conditions and measuring the limit of detection (LOD) and performance in multiple
40 clinical matrices.

41 **Methods:** Total nucleic acid from virus-infected Vero E6 cells was used to optimize TaqMan-
42 minor groove binder (MGB) real-time RT-PCR assays. A 10-fold dilution series of nucleic acid
43 was used to perform analytical experiments with 60 replicates used to confirm assay limits of
44 detection (LODs). Serum and whole blood spiked with 10-fold dilutions of PTV and PICV virus
45 were assessed as matrices in a mock clinical context. The second derivative method with the
46 Roche LightCycler 480 software version 1.5.1 was used to determine Cq.

47 **Results:** Optimized PTV and PICV assays had LODs of 1000 pfu/ml and 100 pfu/ml,
48 respectively, and this LOD was confirmed in 60/60 (PTV) and 58/60 (PICV) positive replicates.
49 Preliminary mock clinical LODs remained consistent in serum and whole blood for PTV and
50 PICV at 1000 pfu/ml and 100 pfu/ml. An exclusivity panel showed no cross reaction with near
51 neighbors.

52 **Conclusions:** PTV and PICV Taq-man MGB based real-time RT-PCR assays developed here
53 showed relevant sensitivity and reproducibility in samples extracted from a variety of clinical
54 matrices. These assays will be useful as a standard by researchers for future experiments utilizing
55 PTV and PICV as infection models, offering the ability to track infection and viral replication
56 kinetics during research studies.

57

58 **Background**

59

60 Both Rift Valley fever virus (RVFV) and Lassa fever virus (LASV) are highly pathogenic
61 viruses endemic to Africa. RVFV, within the *Bunyaviridae* family, is a mosquito-borne,
62 biosafety level (BSL)-3 select agent of major public health and economic concern, affecting
63 humans and livestock throughout Africa (Adam et al., 2009; Daubney et al., 1931; Digoutte and
64 Peters, 1989; Durand et al., 2003; Madani et al., 2003; Meegan et al., 1979) and the Arabian
65 Peninsula (Shoemaker et al., 2002). LASV, within the *Arenaviridae* family, is a BSL-4 select
66 agent responsible for approximately 500,000 infections yearly in West Africa (Birmingham and
67 Kenyon, 2001; Buckley et al., 1970). Both of these viruses can result in a hemorrhagic fever
68 syndrome and can cause large outbreaks in endemic regions.

69

70 Research with either of these pathogens is hazardous, expensive, and limited to studies at select
71 institutions by approved individuals. As such, BSL-2 infection models for both viruses have been
72 developed: Punta Toro virus (PTV) for RVFV infection and Pichinde virus (PICV) for LASV
73 infection. While these models have been used for multiple pathogenesis and therapeutics studies
74 (Anderson et al., 1990; Buchmeier and Rawls, 1977; Fisher et al., 2003; Gowen et al., 2005;
75 Gowen et al., 2006a; Gowen et al., 2006c; Jahrling et al., 1981; Lucia et al., 1989; Perrone et al.,
76 2007; Smee et al., 1993), there are no real-time PCR assays described in the literature for these
77 viruses. The availability of well characterized assays to monitor viral replication kinetics would
78 aid these research efforts.

79

80 PTV infection in mice (Gowen et al., 2006a) and hamsters (Anderson et al., 1990; Fisher et al.,
81 2003; Perrone et al., 2007) results in disease similar to RVFV infection in humans and is an
82 established BSL-2 surrogate infection model for RVFV. PTV, a mosquito-transmitted
83 bunyavirus, typically causes a mild and self-limiting infection in humans but may progress to an
84 acute, febrile illness (Bartelloni and Tesh, 1976). Both RVFV and PTV consist of three RNA
85 segments: the L, S, and M segments (Lihoradova et al., 2013; Xu et al., 2007). The L segment
86 encodes the viral polymerase, the S segment contains the nucleoprotein and the nonstructural
87 protein NSs, and the M segment encodes the two glycoproteins Gn and Gc as well as the
88 nonstructural protein NSm.

89
90 PICV causes a similar disease in hamsters (Buchmeier and Rawls, 1977; Gowen et al., 2005;
91 Gowen et al., 2006c; Smee et al., 1993) and guinea pigs (Jahrling et al., 1981; Lucia et al., 1989)
92 as LASV infection in humans. Both PICV and LASV are arenaviruses with a genome comprised
93 of the L and S RNA segments (Liang et al., 2010; Lukashevich 2013). The L segment encodes
94 the viral polymerase and the Z protein, and the S segment encodes the nucleoprotein and the
95 glycoprotein precursor GPC which is cleaved to yield the glycoproteins GP1 and GP2.

96
97 In this study, we designed two TaqMan-based real-time RT-PCR assays for detection of PTV
98 and PICV. These assays were characterized and evaluated using cell culture supernatant from
99 PTV or PICV infected cells and mock clinical samples. Overall, these assays could benefit the
100 scientific community using animal models as surrogates for RVFV and LASV infection as well
101 as biosurveillance for PTV infections in humans.

102

103 **Methods**

104

105 *Viruses and cells.* The Adames strain of PTV and the CO AN 4763 strain of PICV were provided
106 by Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, Galveston,
107 TX). Each virus was initially passaged in Vero E6 cells to generate stock virus from the cell
108 culture supernatant. Virus stock titers were determined by standard plaque assay using 0.6%
109 (w/v) SeaKem ME agarose (Lonza, Basel, Switzerland) and a secondary overlay containing 5%
110 neutral red (Life Technologies, Grand Isle, NY). Vero E6 cells were maintained in complete
111 Eagle's Minimum Essential Medium (cEMEM, Lonza, Basel, Switzerland) supplemented with
112 10% (v/v) fetal bovine serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin G (Life
113 Technologies), and 100 mg/ml streptomycin (Life Technologies). Cells were incubated at 37°C
114 with 5% CO₂. RNA from the cell culture supernatant was purified using Trizol LS (Life
115 Technologies) and the Qiagen EZ1 robot with the EZ1 Virus Mini Kit (Qiagen, Valencia, Ca)
116 according the manufacturer's directions.

117

118 *Real-time RT-PCR assay design and downselection.* Primers and TaqMan-minor groove binder
119 (MGB) probe pairs were designed for PICV and PTV using Primer Express version 2.0 (Applied

120 Biosystems, Foster City, CA) and AlleleID 7.73 (PREMIER Biosoft, Palo Alto, CA).
121 Primer/probe pairs (see Supplementary Table 1) were designed for PICV (L segment, GenBank#
122 JN378748; S segment, GenBank# JN378747) and PTV (S segment, GenBank# EF201835; M
123 segment, GenBank# DQ363407.1; L segment, GenBank# DQ363408.1). Primers and probe
124 were ordered from Life Technologies.

125
126 Initial primer down selection was accomplished by testing for amplicon formation using purified
127 nucleic acid from virus-infected cell culture supernatant and SYBR Green (Life Technologies),
128 diluted according to manufacturer's protocol. Resultant amplicons were run on an ethidium
129 bromide gel; primer pairs were selected based on a single, clean PCR product of the correct size.
130 Downselected primer pairs were then evaluated using with the appropriate TaqMan-MGB probe
131 and the Invitrogen SuperScript One-Step RT-PCR Kit (Life Technologies) with added bovine
132 serum albumin (BSA) 20 mg/ml (Sigma, St. Louis, MO). Assays were run on the Roche
133 LightCycler 2.0 (Roche Applied Science, Indianapolis, IN) or the LightCycler 480 (Roche) with
134 a final concentration of 4 mM MgSO₄ and 0.25 mg/ml BSA with the following cycling
135 conditions: 50°C for 15 min (1 cycle); 95°C for 5 min (1 cycle); 95°C for 1 sec and 60°C for 20
136 sec (45 cycles); and 40°C for 30 sec (1 cycle). A single fluorescence read was taken at the end of
137 each 60°C step, and a sample was considered positive if the C_q value was less than 40 cycles.

138
139 Sensitivity testing was conducted for each assay using total nucleic acid isolated from cell
140 culture supernatants from PICV and PTV infected Vero E6 cells. These supernatants were
141 previously titered by plaque assay, so the limit of detection (LOD) was determined based on the
142 number of pfu/ml. Purified RNA was serially diluted 10 fold into water, and 5 µl of the diluted
143 RNA was run with each assay in triplicate. The preliminary LOD was determined based on 3/3
144 replicates being positive (<40 C_q), and 60 replicates at this preliminary LOD was conducted for
145 LOD confirmation.

146
147 *Mock Clinical LOD determination.* Preliminary LODs for PTV or PICV in water, serum, and
148 whole blood (BioreclamationIVT, Baltimore, MD) were determined by serially diluting known
149 concentrations of virus into matrix in triplicate. RNA at each dilution was extracted using TRIzol
150 LS and the EZ1 (Qiagen) according to the manufacturer's instructions, and real-time RT-PCR
151 was performed on the extracted RNA with SuperScript One-Step RT-PCR Kit as described
152 previously.

153 *Exclusivity/Inclusivity panel.* PTV and PICV probes and primers were tested against a panel of
154 extracted viral nucleic acid samples. West Nile virus (UCC# Flavi022) and dengue virus
155 serotypes 1-4 (UCC# Flavi029, UCC# Flavi030, UCC# Flavi031, and UCC# Flavi032) were
156 provided by the Unified Culture Collection (UCC) maintained at USAMRIID. Rift Valley fever
157 virus, Lassa fever virus (strains Josiah, Weller, and Pinneo), Mozambique virus, Junín virus,
158 Machupo virus (Carvalo), Mobala virus, and Heartland virus are all maintained at USAMRIID.

159 Real-time PCR was performed with SuperScript One-Step RT-PCR Kit as described before. PTV
160 and PICV were used as positive controls.

161
162 *Statistical analysis.* Cq values were calculated using the second derivative method with the
163 Roche LightCycler 480 software version 1.5.1. GraphPad Prism v. 6.04 graphing software
164 (GraphPad, La Jolla, CA) was used to plot sample data.

165

166 **Results and Discussion**

167

168 *Assay Design/Optimization*

169 Initial evaluations identified optimal primer and probe concentrations for each assay
170 combination, and preliminary downselection testing identified a final assay for each virus (Table
171 1). The assays target the highly conserved polymerase genes, and empirical testing determined
172 an optimal annealing temperature of 60°C with optimal primer and probe concentrations of 0.5
173 and 0.2 µM, respectively. Sanger sequencing confirmed the amplicon as virus-specific (Table 1).

174

175 *Analytical LOD determination.*

176 To have confidence in assay sensitivity, we conducted analytical evaluations including a
177 preliminary LOD and a confirmation of LOD using nucleic acid extracted from virus-infected
178 Vero E6 cell culture supernatant. Testing of eight, 10-fold serial dilutions from each virus
179 ranging from 10⁶ to 10⁻¹ pfu/ml with each assay identified the preliminary LOD in which all
180 three replicates were positive (Figure 1a). This testing showed the preliminary LODs were 1,000
181 pfu/ml and 100 pfu/ml for the PTV and PICV assay, respectively. In each case, we confirmed the
182 preliminary LOD in a statistically robust manner (Figure 1b) using 60 replicates at the
183 preliminary LOD. For PTV, all 60 replicates fell below the cutoff with an average Cq of 37.73,
184 and 58/60 replicates were positive for the PICV assay with an average Cq of 36.16. The
185 coefficient of variation for PTV and PICV was 1.15% and 2.13%, respectively. Exclusivity
186 testing of primers and probes against a panel of extracted viral RNA were all negative.

187

188 *Mock Clinical LOD determination.*

189 Inhibitors of PCR are found in complex matrices such as blood (Akane et al., 1994) or stool
190 (Monteiro et al., 1997; Widjoatmodjo et al., 1992), and these inhibitors can carry through RNA
191 preparation methods, affecting real-time RT-PCR results and impacting assay sensitivity
192 (Kramvis et al., 1996). To further characterize these assays, we spiked each virus into water,
193 human sera, and whole blood and performed six, 10-fold serial dilutions with matrix followed by
194 nucleic acid extraction to re-establish preliminary LODs. Final concentrations ranged from 10⁶ to
195 10¹ pfu/ml. For each matrix tested, water, serum, or whole blood, the LOD for PTV remained
196 consistent at 1,000 pfu/ml as determined by 3/3 replicates falling below the 40 Cq cutoff value
197 (Figure 2a). Similarly for PICV, the LOD remained unchanged when compared to the analytical
198 characterizations at 100 pfu/ml for all three matrices tested (Figure 2b). In both instances,

199 evaluation criteria dictated Cq values greater than 40 be considered negative and given a final
 200 value of 40. Overall, these evaluations showed little to no impact or inhibition due to clinical
 201 matrix carryover for these assays.

202

203 **Conclusion**

204

205 Both RVFV and LASV are highly pathogenic viruses of significant public health and economic
 206 concern, and both are considered biothreat agents. Use of RVFV is limited to BSL-3 conditions,
 207 and LASV is restricted to the highest level of containment, BSL-4. Due to these restrictions and
 208 the limited access to these viruses, BSL-2 models for both viruses have been developed for
 209 pathogenesis and therapeutic studies. To date, the authors are unaware of published real-time
 210 RT-PCR assays for either PTV or PICV, so we developed and characterized real-time PCR
 211 assays for both PICV and PTV.

212

213 A series of primers and probes for each virus targeting the highly conserved polymerase gene
 214 were optimized and tested for assay LOD and performance in multiple clinical matrices. These
 215 assays will help track viral kinetics when utilizing PTV and PICV as infection models in animal
 216 models. Since PTV can also clinically infect humans, this assay can be used for biosurveillance
 217 studies or for diagnostics.

218

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 222 are not necessarily endorsed by the U.S. Army. The authors would also like to thank Cindy Rossi
 223 and the UCC for supplying some of the viruses used in the study.

224

225 **Figure Legends and Tables**

226

227 **Table 1. Primers for PTV and PICV**

228

Virus	Primers/probe	Sequence (5'-3')	Conc. (μ M)	Amplicon
PICV	F3512	CATGTGTGGCCCCCATTT	0.5	63 bp
	R3574	TCAGTTGTTAGGCAAAGTGGTCTT	0.5	
	P3532S-MGB	6FAM-AATGGTCCATTGACACGG-MGBNFQ	0.2	
PTV	F430	CAGATAGCTGCTGCCATTTTACA	0.5	66 bp
	R495	GCTTTTAAGTTTCCCAGCCAAA	0.5	
	P454S-MGB	6FAM-CTCATTATTGTGGGCTCAT-MGBNFQ	0.2	

229

230 **Figure 1. Analytical LOD for the PTV and PICV assays.**

231 Preliminary and confirmatory LODs were performed with primer probe combinations listed in
 232 Table 1. A) Preliminary LODs were determined as having 3/3 replicate Cq values below 40
 233 using serial 10-fold dilutions of PTV and PICV extracted RNA from water. Error bars represent

234 the standard deviation of three replicates. B) Confirmatory LODs were demonstrated with 60
 235 replicates of PTV and PICV RNA extracted from water. Numbers in parenthesis represent the
 236 number of replicates with Cq values below the cutoff line. Each replicate is shown as an
 237 individual point with the bars representing the mean and standard deviation. In each assay,
 238 replicates that had no amplification curves or Cq values falling above 40 were given a base value
 239 of 40.

240

241 **Figure 2. Mock clinical LODs for the PTV and PICV assays.**

242 Total RNA extracted from 10-fold serial dilutions of (A) PTV and (B) PICV spiked into water,
 243 sera, and whole blood were extracted in triplicate and tested with the final PTV and PICV assay
 244 listed in Table 1. In each assay, replicates that had no amplification curves or Cq values falling
 245 above 40 were given a base value of 40. Error bars represent the standard deviation of three
 246 replicates.

247

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