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Cécile Khou, Marco Aurelio Díaz-Salinas, Anaëlle da Costa, Christophe Préhaud, Patricia Jeannin, Philippe V. Afonso, Marco Vignuzzi, Monique Lafon, Nathalie Pardigon

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1 **Comparative analysis of neuroinvasion by Japanese encephalitis virulent**  
2 **and vaccine strains in an *in cellulo* model of human blood-brain barrier**

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26

27 **ABSTRACT**

28 Japanese encephalitis virus (JEV) is the major cause of viral encephalitis  
29 in South East Asia. It has been suggested that JEV gets access to the central  
30 nervous system (CNS) as a consequence of a preceding inflammatory process  
31 which leads to the blood-brain barrier (BBB) disruption and viral neuroinvasion.  
32 However, what happens at early times of JEV contact with the BBB is poorly  
33 understood. In the present work, we evaluated the ability of both a virulent and  
34 a vaccine strain of JEV (JEV RP9 and SA14-14-2, respectively) to cross an *in*  
35 *cellulo* human BBB model consisting of hCMEC/D3 human endothelial cells  
36 cultivated on permeable inserts above SK-N-SH human neuroblastoma cells.  
37 Using this system, we demonstrated that both JEV RP9 and SA14-14-2 are  
38 able to cross the BBB without disrupting it at early times post-addition.  
39 Furthermore, this BBB model was able to discriminate between the virulent RP9  
40 and the vaccine SA14-14-2 strains, as demonstrated by the presence of almost  
41 10 times more RP9 infectious particles that crossed the BBB than SA14-14-2  
42 particles at a high MOI. Besides contributing to the understanding of early  
43 events in JEV neuroinvasion, this *in cellulo* BBB model represents a suitable  
44 and useful system to study the viral determinants of JEV neuroinvasiveness and  
45 the molecular mechanisms by which this flavivirus crosses the BBB at early  
46 times of neuroinvasion.

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## 52 INTRODUCTION

53 *Flaviviruses* such as Japanese encephalitis virus (JEV) are arthropod-  
54 borne viruses (arbovirus) that are transmitted through the bite of an infected  
55 mosquito and may cause serious human diseases [1]. JEV is the main  
56 causative agent of viral encephalitis in South East Asia, with an annual  
57 incidence of around 68 000 cases [2]. About 30% of the cases are fatal, and  
58 half of the survivors present neurological sequelae [3]. To date, no specific  
59 treatment against JEV exists [3]. However, Japanese encephalitis is a  
60 preventable disease as vaccines have been developed: the live-attenuated JEV  
61 SA14-14-2 strain, as well as a recombinant vaccine and an inactivated one,  
62 also based on the JEV SA14-14-2 strain [4, 5]. The live-attenuated vaccine was  
63 obtained empirically after several passages of the JEV SA14 virulent strain in  
64 primary hamster kidney cells [6]. Although highly efficient, cases of post-vaccine  
65 encephalitis were also reported [7], suggesting that the vaccine strain JEV  
66 SA14-14-2 is still neurovirulent in humans.

67 JEV has a positive-sense RNA genome encoding a single polyprotein  
68 flanked by two untranslated regions (UTR) at its 5' and 3' ends. This polyprotein  
69 is co- and post-translationally cleaved into three structural proteins (capsid C,  
70 membrane prM and envelope E) involved in viral particle assembly and  
71 antigenicity and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A,  
72 NS4B and NS5) involved in genome replication, viral particle assembly and  
73 evasion of innate immunity [1]. Due to an error-prone NS5 polymerase that  
74 frequently introduces mutations in the viral genome during replication, a

75 *Flavivirus* population is not clonal, but rather a mix of multiple viral genomic  
76 species (aka quasispecies) [8, 9].

77 JEV is a neuroinvasive and neurovirulent virus. It is associated with  
78 neuroinflammation of the central nervous system (CNS) [10], and disruption of  
79 the blood-brain barrier (BBB), as shown *in vivo* in murine and simian models  
80 [10, 11]. Expression levels of tight junction proteins involved in maintaining BBB  
81 functions such as occludin, claudin-5 and zonula occludens 1 (ZO-1) are  
82 significantly decreased in symptomatic JEV-infected mice, suggesting physical  
83 disruption of the BBB [11]. However, it seems that BBB disruption occurs after  
84 infection of the CNS cells in a mouse model of JEV-induced encephalitis [11]  
85 and that inflammatory response of infected astrocytes and pericytes plays a key  
86 role in BBB leakage [11-13], suggesting that JEV can cross the BBB before  
87 disrupting it. Indeed early studies of JEV infection of mouse brain demonstrated  
88 that the virus was transported across the cerebral endothelium by endocytosis  
89 [14]. Vesicular transport of cellular cargoes through endothelial cells is known  
90 as transcytosis [15], but it is unclear whether this mechanism also applies to the  
91 transport of JEV.

92 In contrast to virulent JEV strains as RP9, the vaccine strain SA14-14-2  
93 was shown to be essentially non-neuroinvasive and non-neurovirulent in  
94 weanling ICR mice, but is still highly neurovirulent in neonates [16]. JEV SA14-  
95 14-2 genome displays 57 nucleotide differences positioned along the genome  
96 when compared to the parental strain SA14, leading to 25 amino-acid  
97 substitutions [16]. Mutations in the E protein seem to attenuate JEV  
98 neurovirulence [17, 18], while mutations in the 5' UTR, capsid C and NS1-NS2A  
99 protein coding regions have been found to attenuate JEV neuroinvasiveness in

100 a mouse model [18-20]. Despite the identification of these attenuating  
101 mutations, the specific amino-acids contributing to the attenuation of JEV SA14-  
102 14-2 are unknown.

103         Encephalitis incidents have occurred after vaccination with the SA14-14-  
104 2 JEV strain, but no virus could be recovered from them [7]. Whether these  
105 neurological adverse events originated from virus reversion to a virulent  
106 phenotype, a specific viral neuroinvasive and neurovirulent sub-population or  
107 from host determinants is still unknown [7]. In any case, the JEV vaccine strain,  
108 although much less neurovirulent and neuroinvasive than its parental  
109 counterpart, must have crossed the BBB in order to reach the CNS and initiate  
110 encephalitis.

111         The BBB is the physical and physiological barrier between the brain and  
112 the blood compartments in vertebrates, and it is comprised of a network of  
113 different cell types including the brain microvascular endothelium along with  
114 pericytes, astrocytes, microglia and the basement membrane [21]. Many BBB  
115 models have been developed in order to facilitate studies on the biology and  
116 pathophysiology of its diverse components, as well as to evaluate drug  
117 transport to the brain [22]. The brain microvascular endothelial cell line  
118 hCMEC/D3 exhibits a stable growth and endothelial marker characteristics that  
119 makes it suitable to form a reproducible and easy-to-grow BBB *in cellulo*.  
120 hCMEC/D3 monolayer displays good restricted permeability to paracellular  
121 tracers and retains most of the transporters and receptors present on *in vivo*  
122 BBB [23]. Accordingly, hCMEC/D3 cells have been used to investigate host-  
123 pathogen interactions with human pathogens that affect the CNS [24, 25].

124 In the present study, we evaluated the ability of both a virulent and a  
125 vaccine strain of JEV (JEV RP9 and SA14-14-2, respectively) to cross an *in*  
126 *cellulo* human BBB model consisting of hCMEC/D3 human endothelial cells  
127 cultivated on permeable supports above SK-N-SH human neuroblastoma cells.  
128 Using this system, we demonstrated that both JEV RP9 and SA14-14-2 strains  
129 are able to cross the BBB without disrupting it at early times post-addition. More  
130 importantly this BBB model is discriminant as about 10 times more RP9 than  
131 SA14-14 infectious particles may cross the barrier at a high MOI. Besides  
132 contributing to the understanding of early events in JEV neuroinvasion, this *in*  
133 *cellulo* BBB model represents a useful tool to examine the viral determinants of  
134 JEV neuroinvasiveness and the molecular mechanisms by which this flavivirus  
135 cross the BBB.

136

## 137 **MATERIAL AND METHODS**

### 138 **Cell lines and JEV strains**

139 Human endothelial cells hCMEC/D3 [23], were maintained at 37°C on rat  
140 collagen diluted at 100µg/mL in water (Cultrex; catalog no. 3443-100-01) in  
141 EndoGro medium (Merck Millipore; catalog no. SCME004) supplemented with  
142 5% fetal bovine serum (FBS) and 10mM HEPES buffer (Sigma-Aldrich; catalog  
143 no. 83264). hCMEC/D3 cells can form tight junctions when cultured for 6 days  
144 at 37°C. Human neuroblastoma cells SK-N-SH (ATCC HTB-11) were  
145 maintained at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented  
146 with 10% heat-inactivated FBS. *Cercopithecus aethiops* monkey kidney Vero  
147 cells were maintained at 37°C in DMEM supplemented with 5% heat-inactivated

148 FBS. *Aedes albopictus* mosquito cells C6/36 were maintained at 28°C in  
149 Leibovitz medium (L15) supplemented with 10% heat-inactivated FBS.  
150 A molecular cDNA clone of JEV genotype 3 strain RP9 was kindly provided by  
151 Dr. Yi-Ling Lin [26]. This plasmid was modified in our laboratory as previously  
152 described [27]. To produce infectious virus, the molecular clone  
153 (pBR322(CMV)-JEV-RP9) was transfected into C6/36 cells using Lipofectamine  
154 2000 (Life Technologies; catalog no. 11668-019). Once a cytopathic effect was  
155 visible, viral supernatant was collected and used to infect C6/36 cells. As  
156 hCMEC/D3 monolayer is very sensitive to any change of medium, we used  
157 viruses produced from cells grown in the same medium as the one used to grow  
158 endothelial cells (EndoGro medium). A CD.JEVAX® (JEV SA14-14-2) vaccine  
159 vial was kindly provided by Dr. Philippe Dussart (Institut Pasteur of Phnom  
160 Penh, Cambodia). The vaccine was reconstituted with 500µL of DMEM. 250µL  
161 of reconstituted vaccine were used to infect Vero cells for 7 days. Viral  
162 supernatants were collected and used to infect C6/36 cells cultivated in  
163 EndoGro medium supplemented with 2% FBS. Both JEV RP9 and SA14-14-2  
164 viral supernatant stocks were collected 3 days after infection and the infectious  
165 titer was determined in Vero cells through a focus-forming assay (see below).

## 166 **Antibodies**

167 Mouse hybridomas producing the monoclonal antibody 4G2 anti-  
168 *Flavivirus* E protein were purchased from the ATCC (catalog no. HB-112), and a  
169 highly-purified antibody preparation was produced by RD Biotech (Besançon,  
170 France). Mouse monoclonal antibody anti-JEV NS5 was kindly provided by Dr.  
171 Yoshiharu Matsura [28]. Horseradish peroxidase (HRP)-conjugated goat anti-  
172 mouse IgG antibody was obtained from Bio-Rad Laboratories (catalog no. 170-

173 6516). Alexa Fluor 488-conjugated goat anti-mouse IgG antibody was obtained  
174 from Jackson ImmunoResearch (catalog no. 115-545-003).

#### 175 **Evaluation of JEV neuroinvasive capacity**

176  $5 \cdot 10^4$  hCMEC/D3 cells were seeded on 12-well Transwell<sup>®</sup> permeable  
177 inserts (Corning; catalog no. 3460) in EndoGro medium supplemented with 5%  
178 FBS for 5 days.  $2 \cdot 10^5$  SK-N-SH cells were seeded in 12-well tissue culture  
179 plates in EndoGro supplemented with 2% FBS. Permeable inserts containing  
180 hCMEC/D3 cells were then transferred in these culture plates and medium was  
181 replaced by EndoGro medium supplemented with 2% FBS. Aliquots of virus  
182 were diluted the next day in 50 $\mu$ L of EndoGro medium supplemented with 2%  
183 FBS, heated at 37°C and then added to the cells. Cells were incubated at 37°C  
184 until collection.

#### 185 **Focus-forming assay (FFA)**

186 Vero cells were seeded in 24-well plates. Ten-fold dilutions of virus  
187 samples were prepared in DMEM and 200 $\mu$ L of each dilution was added to the  
188 cells. The plates were incubated for 1h at 37°C. Unabsorbed virus was removed  
189 and 800 $\mu$ L of DMEM supplemented with 0.8% carboxymethyl cellulose (CMC),  
190 5 mM HEPES buffer, 36 mM sodium bicarbonate, and 2% FBS were added to  
191 each well, followed by incubation at 37°C for 48h for JEV RP9 or for 72h for  
192 JEV SA14-14-2. The CMC overlay was aspirated, and the cells were washed  
193 with PBS and fixed with 4% paraformaldehyde for 20 min, followed by  
194 permeabilization with 0.1% Triton X-100 for 5 min. After permeabilization, the  
195 cells were washed with PBS and incubated for 1h at room temperature with  
196 anti-E antibody (4G2), followed by incubation with HRP-conjugated anti-mouse  
197 IgG antibody. The assays were developed with the Vector VIP peroxidase

198 substrate kit (Vector Laboratories; catalog no. SK-4600) according to the  
199 manufacturer's instructions. The viral titers were expressed in focus-forming  
200 units (FFU) per milliliter.

### 201 **Lucifer Yellow (LY) permeability assays**

202 LY dye migration through the BBB monolayers was performed as  
203 previously described [24]. Briefly, Transwell® inserts containing hCMEC/D3  
204 monolayers were transferred in culture wells containing 1.5 mL of Hanks'  
205 Buffered Salt Solution (HBSS) supplemented with 10 mM of HEPES buffer, 1  
206 mM of sodium pyruvate and 50µM of LY (Sigma-Aldrich; catalog no. L0144).  
207 The culture medium inside the Transwell® inserts was replaced with 500µL of  
208 HBSS buffer. Cells were incubated at 37°C for 10 min. Permeable inserts were  
209 then transferred in culture well containing 1.5 mL of HBSS buffer and incubated  
210 at 37°C for 15 min. They were then transferred in culture well containing 1.5 mL  
211 of HBSS buffer and incubated at 37°C for 20 min. Concentrations of LY in the  
212 wells were determined using a fluorescent spectrophotometer (Berthold,  
213 TriStar<sup>2</sup> LB 942). The emission at 535 nm was measured with an excitation light  
214 at 485 nm. The endothelial permeability coefficient of LY was calculated in  
215 centimeters/min (cm/min), as previously described [29].

### 216 **Virus infections**

217  $10^5$  hCMEC/D3 cells were seeded on coverslips in 24-well tissue culture  
218 plates in EndoGro medium supplemented with 5% FBS. After 5 days, cell  
219 medium was replaced with 1 mL of EndoGro medium supplemented with 2%  
220 FBS.  $10^5$  SK-N-SH cells were seeded on coverslips in 24-well tissue culture  
221 plates in DMEM supplemented with 2% FBS. Aliquots of virus were diluted in  
222 200µL of medium and added to the cells. Plates were incubated for 1h at 37°C.

223 Unabsorbed virus was removed and 1mL of EndoGro or DMEM supplemented  
224 with 2% FBS was added to the cells, followed by incubation at 37°C until  
225 collection.

### 226 **Immunofluorescence analysis (IFA)**

227 All the following steps were performed at room temperature. Cells were  
228 fixed with 4% paraformaldehyde for 20 min followed by permeabilization with  
229 0.1% Triton X-100 for 5 min. After permeabilization, the cells were washed with  
230 PBS and incubated for 5 min with PBS containing 1% BSA. The cells were then  
231 washed with PBS and incubated for 1h with anti-JEV NS5 antibody diluted at  
232 1:200 in PBS, followed by incubation with Alexa Fluor 488-conjugated anti-  
233 mouse IgG antibody diluted at 1:500 in PBS. The coverslips were mounted with  
234 ProLong gold antifade reagent with DAPI (Life Technologies; catalog no.  
235 P36931). The slides were examined using a fluorescence microscope (EVOS  
236 FL Cell Imaging System).

### 237 **Gene expression studies**

238  $5 \cdot 10^4$  hCMEC/D3 cells were seeded on 12-well Transwell® insert filters  
239 in EndoGro medium supplemented with 5% FBS for 5 days.  $2 \cdot 10^5$  SK-N-SH  
240 cells were seeded in 12-well tissue culture plates in EndoGro supplemented  
241 with 2% FBS. Transwell® containing hCMEC/D3 cells were then transferred in  
242 these culture plates and medium was replaced by EndoGro medium  
243 supplemented with 2% FBS. Cells were incubated at 37°C. At 24h post-contact,  
244 total RNA of hCMEC/D3 cells were extracted using NucleoSpin RNA kit  
245 (Macherey-Nagel; catalog no. 740955.50) following the manufacturer's  
246 instructions. 200 ng of total RNA were used to produce cDNA using the  
247 SuperScript II Reverse Transcriptase (Thermo Fisher; catalog no. 18064014)

248 according to the manufacturer's instructions. Quantitative PCR were performed  
249 on 2µL of cDNA using SYBR Green PCR Master Mix (Thermo Fisher; catalog  
250 no. 4309155) according to the manufacturer's instructions. The CFX96 real-time  
251 PCR system (Bio-Rad) was used to measure SYBR green fluorescence with the  
252 following program: an initial PCR activation at 95°C (10 min), 40 cycles of  
253 denaturation at 95°C (15s) and annealing-extension at 60°C (1 min). Results  
254 were analyzed using the CFX Manager Software (Bio-Rad) gene expression  
255 analysis tool. GAPDH was used as the reference gene. Primers used in gene  
256 expression studies are listed in Table 1.

### 257 **Quantification of JEV RNA copies number**

258 Total RNA from JEV BBB-crossing samples was extracted using  
259 NucleoSpin®RNA kit (Macherey-Nagel; 740955.50) according to the  
260 manufacturer's instructions. The number of JEV RNA copies present in BBB-  
261 crossing samples was determined by RT-qPCR using TaqMan® Fast Virus 1-  
262 Step Master Mix kit (Applied Biosystems®, 4444432) according to the  
263 manufacturer's instructions. The forward and reverse primers (Sigma-Aldrich®)  
264 were 5'GAAGATGTCAACCTAGGGAGC3' and  
265 5'TGGCGAATTCTTCTTTAAGC3' respectively, while  
266 [6FAM]AAGAGCCGTGGGAAAGGGAGA[BHQ1] was the probe for the assay.  
267 JEV RNA copies were calculated from a standard curve generated by  
268 amplifying known amounts of *in vitro*-transcribed RP9 NS5 gene region cloned  
269 and under SP6 promotor control. The *in vitro* transcription was performed using  
270 mMESAGE mMACHINE™ SP6 kit (Invitrogen, Thermo Fisher Scientific,  
271 AM1340) following the manufacturer's instructions.

### 272 **Statistical analysis**

273 Unpaired two-tailed *t* test, Mann-Whitney test and ANOVA test corrected  
274 with Tukey method for multiple comparisons were used to compare  
275 experimental data. GraphPad Prism 7 was used for these statistical analyses.

276

## 277 **RESULTS**

278 **hCMEC/D3 cell monolayers grown on permeable inserts form a BBB**

279 **whose properties are not affected by SK-N-SH cells presence.** A basic *in*

280 *cellulo* model to study JEV neuroinvasion should consist of two main

281 components: 1) a cell monolayer mimicking the BBB, and 2) a brain tissue-

282 derived cell line permissive to JEV. Based on our previous work [24], we chose

283 to use hCMEC/D3 human endothelial cells monolayers cultivated on permeable

284 inserts and place these inserts in wells in which human neuroblastoma SK-N-

285 SH cells were grown, in order to partly mimic the brain parenchyma. Relevant

286 parameters of a functional BBB model, such as permeability and presence of

287 cell transporters and receptors specific of hCMEC/D3 cells were evaluated

288 when the endothelial cells were or not grown above SK-N-SH monolayers (Fig.

289 1). Permeability measurement of hCMEC/D3 monolayers through evaluation of

290 Lucifer Yellow (LY) passage showed no significant difference whether SK-N-SH

291 cells were present or not (Fig. 1A, + or - respectively). Moreover, the relative

292 RNA level of genes coding for proteins involved both in cell receptors (Fig. 1B)

293 and transporters (Fig. 1C) characteristic of endothelial barriers were similar in

294 the two conditions, suggesting that the culture of neuroblastoma cells under the

295 inserts on which hCMEC/D3 were grown did not disturb the endothelial cell

296 intrinsic BBB properties and actually makes of this *in cellulo* BBB model a useful

297 tool to study the neuroinvasion ability of JEV.

298

299 **JEV SA14-14-2 is less replicative than JEV RP9 in SK-N-SH cells.**

300 Neuroblastoma SK-N-SH cells are susceptible to both the virulent JEV RP9  
301 strain and the SA14-14-2 attenuated strain [27, 30]. However, a direct  
302 comparison between replication of these two JEV strains in that cell line has not  
303 been described. We thus evaluated replication of each JEV strain in SK-N-SH  
304 cells at 24 and 48 hpi (Fig. 2). As expected, both JEV strains infected the  
305 neuroblastoma-derived cell line as demonstrated by the detection of a viral  
306 antigen (NS5 protein) through immunofluorescence assays (Fig. 2A). However,  
307 the viral progeny of JEV SA14-14-2 vaccine strain produced in SK-N-SH cells at  
308 24 and 48 hpi was significantly lower than that of JEV RP9 (1.7 and 1.2 log<sub>10</sub>  
309 less at 24 and 48 hpi respectively, Fig. 2B), suggesting that JEV SA14-14-2 is  
310 less neurovirulent than JEV RP9 in human cell cultures.

311

312 **Neither JEV RP9 nor JEV SA14-14-2 infects hCMEC/D3 cells after they**

313 **form a BBB.** In order to examine the susceptibility of our hCMEC/D3 BBB  
314 model to JEV infection, the cells were grown 6 days on coverslips to allow the  
315 BBB to form, then inoculated with either RP9 or SA14-14-2 JEV strain (Fig. 3).  
316 The presence of the NS5 viral protein as infection evidence was assessed by  
317 immunofluorescence microscopy as described in the Material and Methods  
318 section. No fluorescence signal was observed in hCMEC/D3 BBB-forming  
319 monolayer either at 24 or 48 hpi (Fig. 3A). Surprisingly, hCMEC/D3 cells could  
320 be infected by either JEV strains when they were inoculated after only one day  
321 of culture (ie not forming of a BBB), as detected through the same  
322 immunofluorescence approach (Fig. 3B). Moreover, in this condition, both JEV

323 strains produced infectious viral progeny in hCMEC/D3, although the RP9 viral  
324 titer was significant higher by around 2 log than that observed for SA14-14-2  
325 (Fig. 3C). These results suggest that hCMEC/D3 cells are not susceptible to  
326 JEV infection when they already have formed a barrier, but they are JEV  
327 permissive before tight junctions formation.

328

329 **Neither JEV RP9 nor JEV SA14-14-2 disrupts the BBB when added for 6h.**

330 It has been suggested that JEV infects brain tissue cells as a consequence of a  
331 preceding inflammatory process which leads to the BBB disruption and viral  
332 neuroinvasion [31, 32]. However, the very early events of JEV BBB crossing are  
333 still poorly understood. In order to evaluate the neuroinvasive ability of JEV in  
334 our BBB model at early times post-addition, hCMEC/D3 cells cultivated on  
335 permeable inserts to form a BBB above SK-N-SH cells monolayer were  
336 exposed to either JEV RP9 or SA14-14-2 virus addition (MOI=1 or 10; Fig. 4).  
337 The permeability of the BBB at 6 hpi in the presence of the 2 different JEV  
338 strains did not show a significant difference when compared to that of the mock-  
339 infected condition (Fig. 4A), suggesting that the BBB model was not disturbed  
340 either by the JEV strains or the MOIs used.

341

342 **More JEV RP9 infectious particles may cross the *in cellulo* BBB model**  
343 **than JEV SA14-14-2.**

344 Since the BBB permeability was not affected by the addition of either virus, we  
345 examined the viral crossing of each strain by evaluating the quantity of viral  
346 RNA and infectious particles in the supernatants under the inserts (Fig. 4B and  
347 C). The number of viral RNA copies detected for both viruses was 1.7 log<sub>10</sub>

348 higher when a MOI of 10 was used in comparison to a MOI of 1 (Fig. 4B),  
349 suggesting that the higher the JEV viral load, the greater the number of viral  
350 particles crossing the BBB. Of note, there was no significant difference in the  
351 viral RNA copy number between the JEV strains for each MOI (MOI=1 or =10,  
352 Fig. 4B). However, the infectious titers of the JEV particles that crossed the  
353 BBB was surprisingly different between the RP9 and SA14-14-2 strains, as  
354 about 3 times more RP9 infectious particles were found in the supernatants  
355 under the inserts than SA14-14-2 when an MOI of 1 was used, and close to 10  
356 times for a MOI of 10 (Fig. 4C). Calculation of the specific infectivity for JEV  
357 RP9 and SA14-14-2 strains as the ratio between the detected JEV RNA copy  
358 number per infectious focus-forming unit did not show a significant difference  
359 between the 2 viral stocks (Fig. 5A). Interestingly, the specific infectivity for the  
360 RP9 BBB-crossing samples was significantly lower than that observed for the  
361 vaccine strain SA14-14-2 with a 3 to 10 fold decrease for MOI=1 and =10  
362 respectively (Fig. 5B). These results indicate that more JEV RP9 infectious  
363 particles may cross our BBB model than SA14-14, and demonstrate that this *in*  
364 *cellulo* barrier is capable of discriminating between 2 viruses with different  
365 neuroinvasive capabilities.

366

## 367 **DISCUSSION**

368 Several lines of research in either *in vivo* and *in vitro* systems have  
369 suggested that JEV infects brain tissue cells as a consequence of a preceding  
370 inflammatory process which would lead to the BBB disruption and viral  
371 neuroinvasion [31, 32]. While *in vivo* approaches are useful to understand the  
372 systemic viral disease, *in vitro* models are also useful because they allow

373 studying the molecular mechanisms that govern viral pathogenesis. In this  
374 regard, previous approaches have been used to characterize JEV  
375 neuroinvasion properties at late times of infection, mainly 24 hpi or later [30, 33-  
376 35]. However, knowledge relative to the early times of JEV contact with the BBB  
377 is poor, if not null.

378 In this study, we have used an *in cellulo* model of a human BBB to  
379 compare JEV RP9 virulent and JEV SA14-14-2 vaccine strain ability to cross  
380 the BBB at early times post-addition. We have shown that both JEV RP9 and  
381 SA14-14-2 are able to cross the BBB without disrupting it at 6 hpi (Fig. 4). This  
382 finding is very relevant because it suggests that JEV could be able to get  
383 access to the CNS and establish a primary infection there without the preceding  
384 need of inflammatory cytokines that could lead to BBB disruption prior CNS  
385 cells viral infection as it is currently thought [31, 32].

386 Moreover, the fact that both JEV RP9 and SA14-14-2 strains crossed the  
387 BBB without infecting its endothelial cells, nor disrupting the barrier, also  
388 suggests that JEV is able to cross the BBB in a transcellular way through the  
389 endothelial cells or in a paracellular way between the endothelial cells. These  
390 observations are consistent with other studies conducted *in vivo* in mice and  
391 monkeys [11, 16, 36]. Observations of JEV-infected suckling mice brain by  
392 electron-microscopy suggested that JEV crosses the BBB endothelial cells by  
393 transcytosis [14]. Regardless of these observations, currently there is no  
394 published data to support this hypothesis from biochemical, genetics or  
395 functional approaches. The combination of these approaches, together with the  
396 use of our *in cellulo* BBB model and JEV strains with different neuroinvasive

397 capabilities such as the ones used in this work would be useful to identify which  
398 cell mechanisms are "highjacked" by these pathogens to cross the BBB.

399         Interestingly, our specific infectivity data suggest that JEV RP9 infectious  
400 particles crossed the BBB more efficiently than JEV SA14-14-2 (Fig. 5).  
401 Comparison of the transcriptome from hCMEC/D3 cells forming a BBB to which  
402 either JEV RP9, SA14-14-2 or no virus was added for 6h showed no significant  
403 difference in the levels of gene expression (fold-change threshold of 2, data not  
404 shown). This suggests that an early cell response is not responsible for the  
405 differential BBB crossing of JEV RP9 versus JEV SA-14-14-2 particles we  
406 observed (Fig. 4C), and that it most likely stems from viral factors. It is also  
407 possible that the difference in the JEV RP9 and SA14-14-2 infectious particles  
408 ability to cross the *in cellulo* BBB relies on specific protein interactions, for  
409 example, interaction of the viral particle with a strain-specific cell surface  
410 receptor for viral entry. A full characterization of the viral particles that are able  
411 to cross the BBB including by deep-sequencing of their RNA content and  
412 examining the endothelial cells forming the BBB after contact with either virus  
413 by electron microscopy, together with uncovering specific cell receptor(s) for  
414 JEV strains could help solving these issues.

415         Surprisingly, we found that hCMEC/D3 were permissive to both RP9 and  
416 SA14-14-2 strains only when the BBB formation was not completed (Fig. 3B),  
417 suggesting that formation of tight junctions between these cells could make the  
418 JEV cell entry receptor(s) inaccessible to the virus. Based on our data and  
419 considering the current model of JEV neuroinvasion that suggests disruption of  
420 the BBB following CNS viral infection [11], endothelial cells from a disrupted  
421 barrier might become permissive to JEV because of better accessibility to cell

422 entry receptor(s), and these cells, upon infection, could in turn become a new  
423 source of viral production contributing to JEV infection of the CNS.

424 In conclusion, our study demonstrates that both JEV RP9 and SA14-14-2  
425 are able to cross a BBB model without disrupting it at early times post-addition  
426 and that the BBB formed by human endothelial cells represents a useful  
427 discriminant *in cellulo* model to characterize viral determinants of JEV  
428 neuroinvasiveness as well as a tool to study the molecular mechanisms by  
429 which these pathogens cross the BBB.

430

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443

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- 563
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- 565

566 **Table 1. Primers used for quantification of tight junctions, receptors and**  
 567 **transporters encoding genes.**

Gene	Forward primer	Reverse primer	Reference
TFRC	5'-ATG CTG ACA ATA ACA CAA-3'	5'-CCA AGT AGC CAA TCA TAA-3'	[37]
AGER	5'-CTC GAA TGG AAA CTG AAC AC-3'	5'-CTG GTA GTT AGA CTT GGT CTC-3'	[37]
LRP1	5'-GCA TCC TGA TCG AGC ACC TG-3'	5'-GCC AAT GAG GTA GCT GGT GG-3'	[37]
INSR	5'-TGT TCA TCC TCT GAT TCT CTG-3'	5'-GCT TAG ATG TTC CCA AAG TC-3'	[38]
LEPR	5'-GGA AAT CAC ACG AAA TTC AC-3'	5'-GCA CGA TAT TTA CTT TGC TC-3'	[38]
BCAM	5'-GCT TTC CTT ACC TCT AAA CAG-3'	5'-GAA GGT GAT AGA ACT GAG CG-3'	[38]
SLC6A8	5'-TGA GAG AAT GAG ATT TCT GCT TGT-3'	5'-TAG GGC TCA CAG GGA TGG-3'	[37]
SLC3A2	5'-TTG GCT CCA AGG AAG ATT-3'	5'-GAG TAA GGT CCA GAA TGA CA-3'	[37]
SLC2A1	5'-GAG ACA CTT GCC TTC TTC-3'	5'-GCT TTG TAG TTC ATA GTT CG-3'	[37]
SLC7A5	5'-TTG ACA CCA CTA AGA TGA T-3'	5'-GTA GCA ATG AGG TTC CAA-3'	[37]
SLC7A1	5'-CCT CCT GAG ACA TCT TTG-3'	5'-CTG GAA TAT GAC GGG AAG-3'	[37]
SLC16A1	5'-ACA CAA AGC CAA TAA GAC-3'	5'-ACA GAA TCC AAC ATA GGT A- 3'	[37]
ABCB1	5'-GCC TGG CAG CTG GAA GAC AAA TAC ACA AAA TT-3'	5'-CAG ACA GCA GCT GAC AGT CCA AGA ACA GGA CT-3'	[37]
ABCG2	5'-TGG CTG TCA TGG CTT CAG TA-3'	5'-GCC ACG TGA TTC TTC CAC AA-3'	[37]
ABCC1	5'-ACC AAG ACG TAT CAG GTG GCC- 3'	5'-CTG TCT GGG CAT CCA GGA T- 3'	[37]
ABCC2	5'-CCA ATC TAC TCT CAC TTC AGC GAG A-3'	5'-AGA TTC CAG CTC AGG TCG GTA CC-3'	[37]
ABCC4	5'-AAG TGA ACA ACC TCC AGT TCC A- 3'	5'-CCG GAG CTT TCA GAA TTG AC-3'	[37]

Gene	Forward primer	Reverse primer	Reference
ABCC5	5'-AGT GGC ACT GTC AGA TCA AAT T- 3'	5'-TTG TTC TCT GCA GCA GCA AAC-3'	[37]
STRA6	5'-TTT GGA ATC GTG CTC TCC G-3'	5'-AAG GTG AGT AAG CAG GAC AAG-3'	[38]
SLC38A5	5'-TGT CAG TGT TCA ACC TCA G-3'	5'-GTG GAT GGA GTA GGA CGA-3'	[38]
SLC1A1	5'-GTT ATT CTA GGT ATT GTG CTG G- 3'	5'-CTG ATG AGA TCT AAC ATG GC-3'	[38]
PLVAP	5'-CAA TGC AGA GAT CAA TTC AAG G-3'	5'-ACG CTT TCC TTA TCC TTA GTG-3'	[38]
CXCL8	5'-TCT TGG CAG CCT TCC TGA TT-3'	5'-TTA GCA CTC CTT GGC AAA ACT G-3'	[39]
CXCL10	5'-TGG CAT TCA AGG AGT ACC TCT C-3'	5'-CTT GAT GGC CTT CGA TTC TG-3'	[40]
GAPDH	5'-AGC CAC ATC GCT CAG ACA CC-3'	5'-GTA CTC AGC GCC AGC ATC G- 3'	[37]

568

569

## 570 FIGURE LEGENDS

571 **Fig. 1. The presence of SK-N-SH cells under hCMEC/D3 BBB-forming cells**

572 **does not affect the BBB properties.** hCMEC/D3 were cultivated on

573 Transwell® inserts. Five days after seeding, SK-N-SH (SK) cells were cultivated

574 or not in wells under the Transwell® inserts (white and black bars respectively).

575 **A)** Twenty-four hours after adding the SK-N-SH cells (+) or not (-), BBB

576 permeability to LY was measured. **B)** and **C)** hCMEC/D3 BBB-forming cells total

577 RNA was extracted and receptors (B) and transporters (C) typical of the BBB-

578 encoding genes were quantified by RT followed by qPCR as described in

579 Material and Methods. Graphs show the results from two independent

580 experiments performed by duplicates.

581 **Fig. 2. JEV RP9 is more replicative than JEV SA14-14-2 in SK-N-SH**  
582 **neuroblastoma cells.** SK-N-SH cells were infected at MOI 0.1 for 24 or 48h by  
583 the indicated JEV strain. **A)** The infected cells were analyzed at the indicated  
584 times post-infection by immunofluorescence staining for the presence of the  
585 NS5 protein (in green). The images were taken at a x200 magnification, the cell  
586 nuclei were stained by DAPI (in blue). **B)** Supernatants of SK-N-SH cells  
587 infected by JEV RP9 (black bar) or JEV SA14-14-2 (white bar) were titrated in  
588 Vero cells. The arithmetic means  $\pm$  standard deviation of three independent  
589 experiments performed in triplicate is shown. Asterisks indicate a significant  
590 difference between RP9 and SA14-14-2 in each one of the times post-infection  
591 evaluated (\*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ).

592 **Fig. 3. Infection of hCMEC/D3 cells by JEV strains.** hCMEC/D3 were  
593 cultured on coverslips for either 6 days (**A**) or 1 day (**B**), so that they form or not  
594 a BBB respectively. Cells were then inoculated with the indicated JEV strain at  
595 MOI=0.1 and analyzed at 24 and 48 hpi by immunofluorescence staining for the  
596 presence of the NS5 protein (in green). The images were taken at a x200  
597 magnification, the cells nuclei are stained by DAPI (in blue). **C)** Supernatants  
598 from hCMEC/D3 cells that do not form a BBB and infected by JEV RP9 (black  
599 bar) or JEV SA14-14-2 (white bar) were collected at 24 and 48 hours post-  
600 infection and their viral titer was determined as described in Material and  
601 Methods. The arithmetic means  $\pm$  standard deviation of three independent  
602 experiments performed by triplicate is shown. Asterisks indicate a significant  
603 difference between RP9 and SA14-14-2 in each one of the times post-infection  
604 evaluated (\*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ).

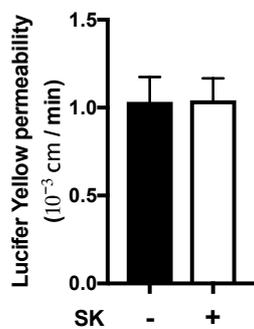
605 **Fig. 4. JEV RP9 and JEV SA14-14-2 may cross the *in cellulo* BBB model**  
606 **without disrupting it. A)** hCMEC/D3 cells were cultivated on Transwell®  
607 inserts. Five days after seeding, SK-N-SH cells were added to the wells under  
608 the Transwell® insert. 24h later JEV RP9 or SA-14-14-2 was added either at  
609 MOI=1 or =10 to the BBB as indicated. Permeability to Lucifer Yellow was  
610 assayed in the hCMEC/D3 cells 6 h post-addition as described in Material and  
611 Methods. **B)** *In cellulo* BBBs were generated as indicated above and either JEV  
612 strain was added at MOI=1 or =10. After 6 h, total RNA was extracted from JEV  
613 BBB-crossing samples under the inserts and the number of JEV RNA copies  
614 was determined by RT-qPCR as described in Material and Methods. **C)** Either  
615 JEV RP9 (black bars) or SA14-14-2 (white bars) BBB-crossing samples were  
616 collected after 6 h post-addition and their viral titer was determined as described  
617 in Material and Methods. The arithmetic means  $\pm$  standard deviation of at least  
618 two independent experiments performed by triplicate is shown. Asterisks  
619 indicate a significant difference between the RP9 and SA14-14-2 titers in each  
620 one of the MOIs evaluated in the BBB-crossing experiments (\*\*\*\*,  $P < 0.0001$ ).

621 **Fig. 5. The specific infectivity of JEV RP9 is decreased after BBB-**  
622 **crossing. A)** Both the number of viral RNA copies and the infectious titers for  
623 either JEV RP9 or JEV SA14-14-2 stocks used for the JEV BBB-crossing  
624 experiments were determined as described in Material and Methods. The  
625 specific infectivity of both stocks was calculated by dividing the viral RNA copies  
626 number/ml by the FFU/ml of each viral stocks. B) The specific infectivity of the  
627 JEV RP9 and SA14-14-2 BBB-crossing samples was calculated as indicated  
628 above using the data from Fig. 4B and 4C. The arithmetic means  $\pm$  standard  
629 deviation of at least two independent experiments performed by triplicate is

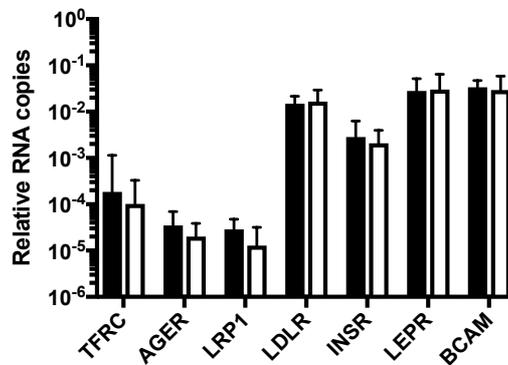
630 shown. Asterisks indicate a significant difference between the specific infectivity  
631 of RP9 and SA14-14-2 in each one of the MOIs evaluated in the BBB-crossing  
632 experiments (\*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.0001$ ).

Fig. 1

A)



B)



C)

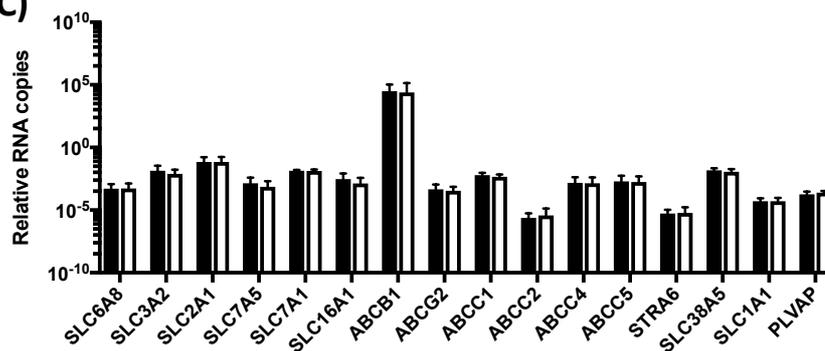
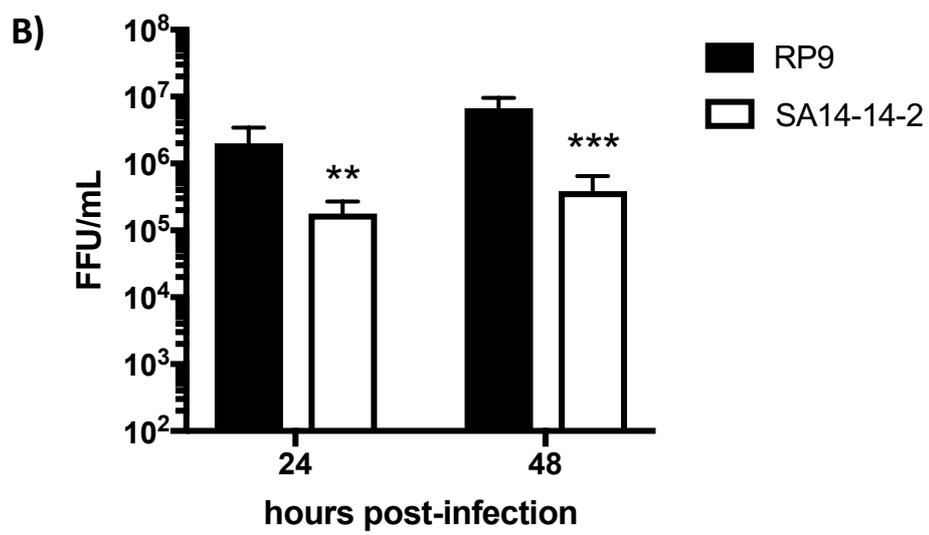
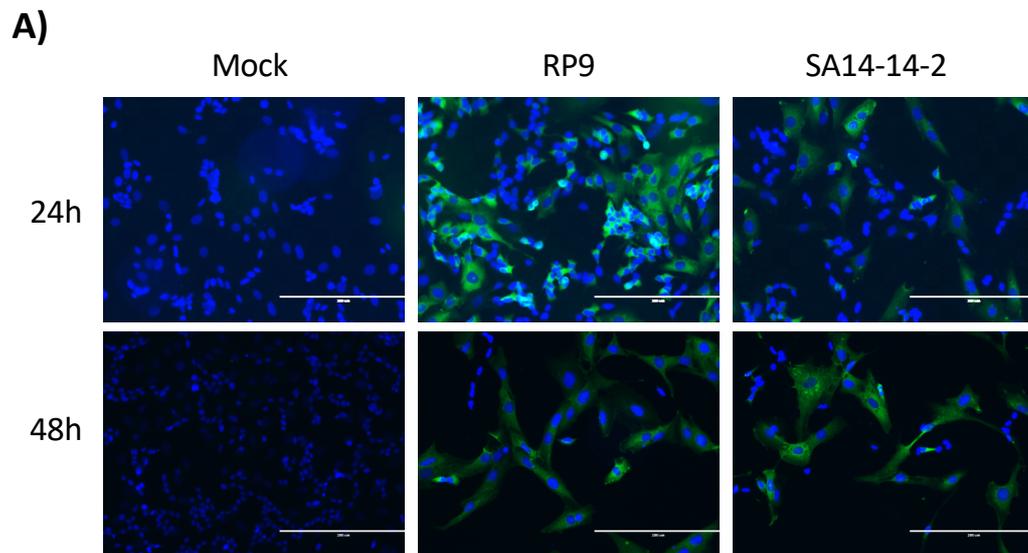


Fig. 2



**Fig. 3**

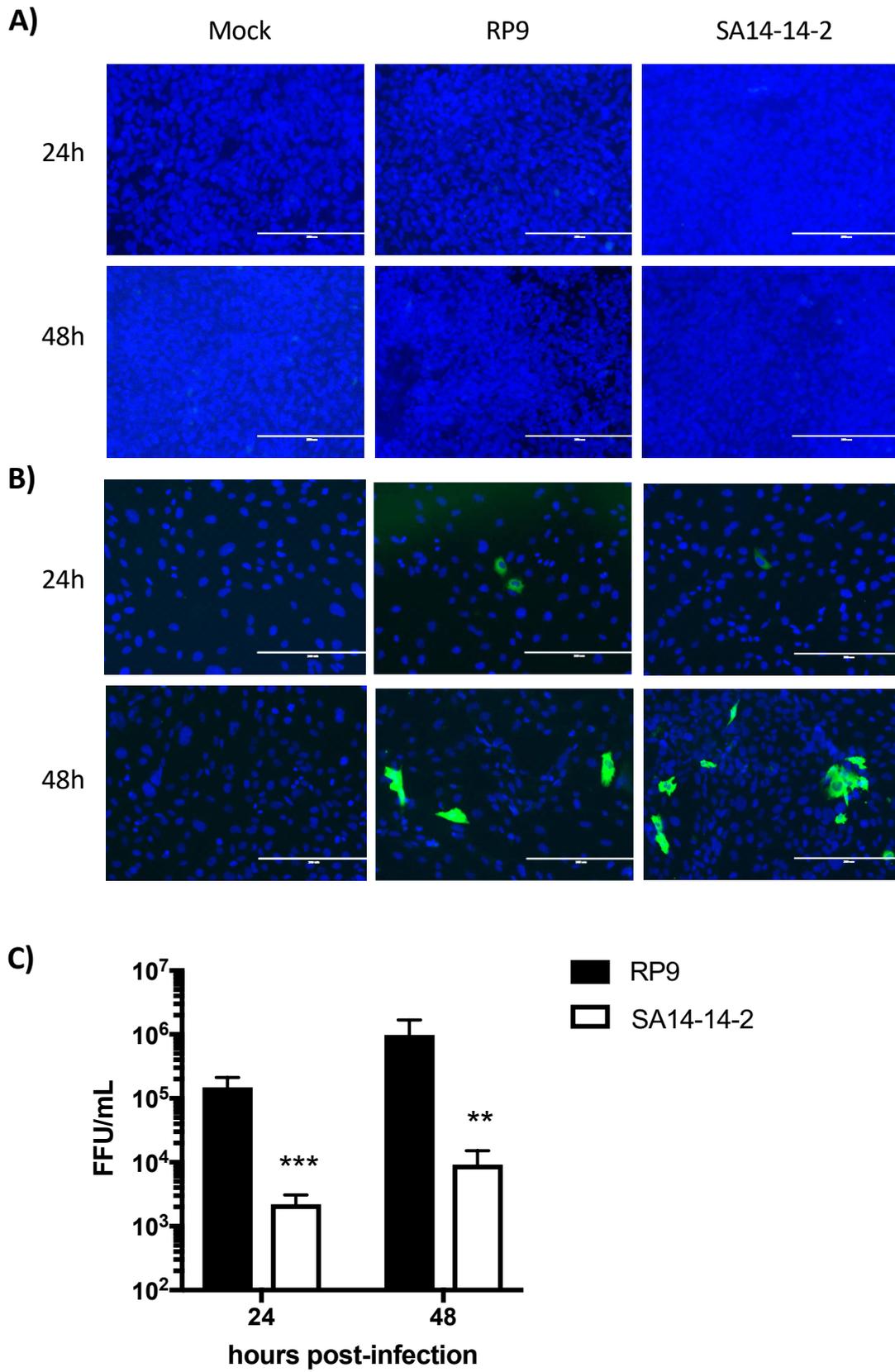


Fig. 4

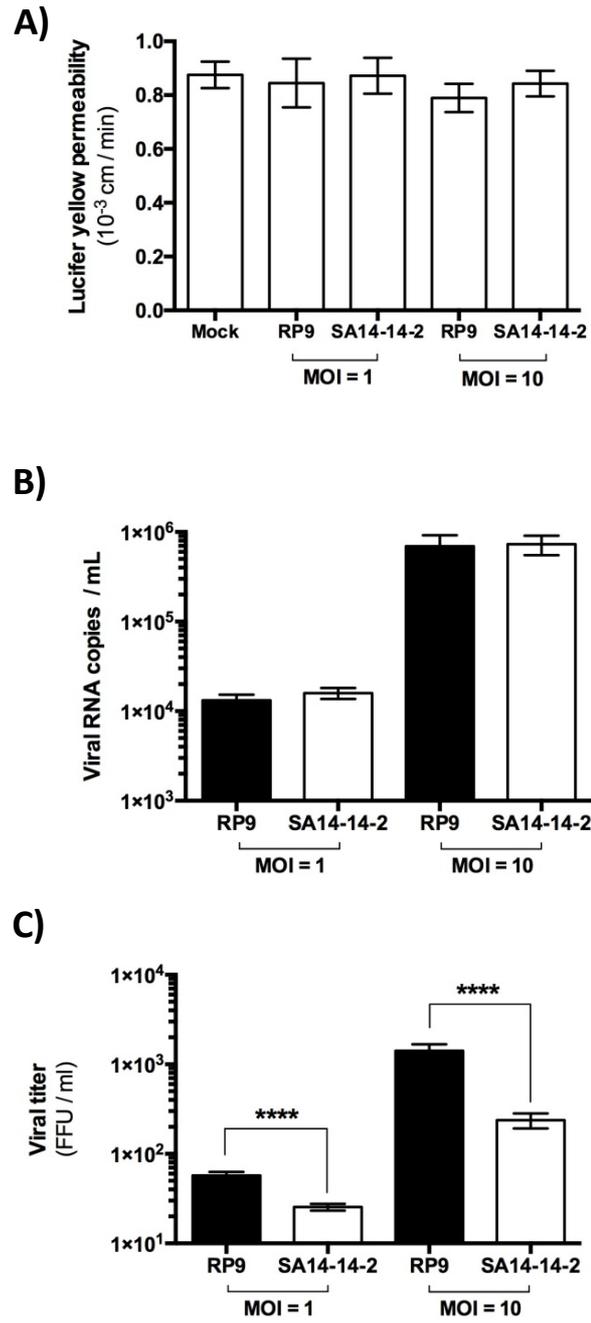
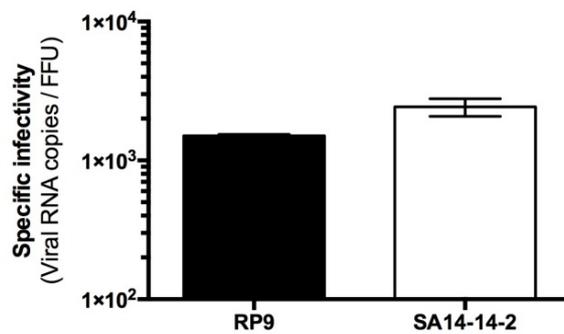


Fig. 5

A)



B)

