Nunes, S. F., Hamers, C., Ratinier, M., Shaw, A., Brunet, S., Hudelet, P., and Palmarini, M. (2014) *A synthetic biology approach for a vaccine platform against known and newly emerging serotypes of bluetongue virus.* Journal of General Virology . ISSN 0022-1317

Copyright © 2014 The Authors

http://eprints.gla.ac.uk/96853/

Deposited on: 02 October 2014

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk

JVI Accepts, published online ahead of print on 20 August 2014
J. Virol. doi:10.1128/JVI.02183-14
Copyright © 2014, American Society for Microbiology. All Rights Reserved.

JVI Accepts published online ahead of print

1 **A Synthetic Biology Approach For a Vaccine Platform Against Known and Newly Emerging**
2 **Serotypes of Bluetongue Virus**
3
4 Sandro Filipe Nunes[1], Claude Hamers[2], Maxime Ratinier[1], Andrew Shaw[1], Sylvie Brunet[2],
5 Pascal Hudelet[2] and Massimo Palmarini[1]*
6
7
8 [1]MRC - University of Glasgow Centre for Virus Research, Glasgow, United Kingdom.
9 [2]Merial Animal Health, Lyon, France.
10
11 **Running title**: Bluetongue vaccines using synthetic biology
12 **Keywords:** Synthetic biology, Bluetongue virus, vaccines
13
14 ***Corresponding Author**
15 Massimo Palmarini
16 MRC - University of Glasgow Centre for Virus Research
17 464 Bearsden Road
18 Glasgow G61 1QH
19 Scotland (UK)
20 Tel: +44 (0)141-330 2541
21 E-mail: massimo.palmarini@glasgow.ac.uk
22
23

JVI Accepts published online ahead of print

24 **ABSTRACT**

25

26 Bluetongue is one of the major infectious diseases of ruminants and is caused by
27 Bluetongue virus (BTV), an arbovirus existing in nature in at least 26 distinct serotypes. Here,
28 we describe the development of a vaccine platform for BTV. The advent of synthetic biology
29 approaches and the development of reverse genetics systems, has allowed the rapid and
30 reliable design and production of pathogen genomes which can be subsequently
31 manipulated for vaccine production. We describe BTV vaccines based on "synthetic" viruses
32 in which the outer core proteins of different BTV serotypes are incorporated into a common
33 tissue-culture adapted backbone. As a means of validation for this approach, we selected
34 two BTV-8 synthetic reassortants and demonstrated their ability to protect sheep against
35 virulent BTV-8 challenge. In addition, to further highlight the possibilities of genome
36 manipulation for vaccine production, we also designed and rescued a synthetic BTV chimera
37 containing a VP2 protein including regions derived from both BTV-1 and BTV-8.
38 Interestingly, while the parental viruses were neutralized only by homologous antisera, the
39 chimeric proteins could be neutralized by both BTV-1 and BTV-8 antisera. These data
40 suggest that neutralizing epitopes are present in different areas of the BTV VP2 and likely
41 "bivalent" strains eliciting neutralizing antibodies for multiple strains can be obtained.

42 **Importance**

43 Overall, this vaccine platform can significantly reduce the time taken from the identification
44 of new BTV strains to the development and production of new vaccines, as the viral
45 genomes of these viruses can be entirely synthesised *in vitro*. In addition, these vaccines can
46 be brought quickly in the market as they alter the approach, but not the final product, of

47 existing commercial products.

48

49

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

50 **INTRODUCTION**

51 Bluetongue is a major infectious disease of ruminants and is caused by Bluetongue virus
52 (BTV), an arbovirus in the *Reoviridae* family transmitted from infected to uninfected
53 mammalian hosts by *Culicoides* biting midges (1, 2).

54 BTV has a genome composed of ten linear double-stranded RNA (dsRNA) segments
55 encoding for seven structural and four non-structural proteins (3-5). The BTV virion is an
56 icosahedral particle assembled as a triple layered capsid (6, 7). There are at least 26 BTV
57 serotypes (BTV-1 to BTV-26) circulating worldwide. Serotypes are determined primarily by
58 differences in the outer capsid protein VP2, which mediates viral entry into the cell and is
59 the target for neutralizing antibodies in infected animals (8-13). VP2, and to a lesser extent
60 VP5, interact with the VP7 protein, the major component of the underlying core (14).

61 BTV infection in mammalian hosts results in inapparent to severe clinical symptoms
62 generally associated with damage to small blood vessels (2, 15, 16). Serotype-specific
63 neutralizing antibodies are generated upon infection in both naturally or experimentally
64 infected ruminants and provide little or no protection against heterologous serotypes (17,
65 18).

66 Traditionally, bluetongue endemic regions have been limited to tropical and sub-tropical
67 areas of the world (19, 20). However, in the last 15 years, similarly to some other arbovirus
68 infections, bluetongue has expanded its geographical limits. Since 1998, outbreaks caused
69 by various BTV serotypes have been increasingly observed in Northern Africa and Europe
70 (21-23).

71 Vaccination of susceptible livestock remains the most effective strategy for the control of
72 BTV epidemics. Currently, only two different types of vaccines are available commercially:
73 live attenuated vaccines, traditionally obtained from the successive passage of BTV in

JVI Accepts published online ahead of print

74 embryonated eggs or tissue culture, and inactivated whole-virus vaccines, in which BTV
75 viruses are grown in tissue culture and later chemically inactivated.
76 Live attenuated vaccines have been used for decades in South Africa where Bluetongue is
77 endemic (24). These vaccines elicit strong neutralizing antibody and likely cell-mediated
78 immune responses and confer long term protection against homologous BTV infection (18).
79 However, their use in Southern Europe, although effective in most cases, has been a cause
80 of concern as some strains have been proven to be (i) poorly attenuated, (ii) teratogenic and
81 affecting pregnancy, (iii) transmitted to non-vaccinated animals and (iv) reassorted with
82 wild type viruses (25-29). For these reasons, the use of live attenuated viruses for BTV
83 control in Europe was discontinued and several vaccine manufacturers developed whole-
84 virus inactivated vaccines (18, 30). These vaccines were proven to protect vaccinated
85 animals against homologous BTV challenge. Although the duration of immunity induced by
86 inactivated vaccines is shorter compared to that induced by live vaccines, their use helped
87 to control and eventually eliminate BTV-1 and BTV-8 from Central and Northern Europe (18,
88 31-33).
89 The advent of synthetic biology approaches and the development of reverse genetics
90 systems, has allowed the rapid and reliable design and production of pathogen genomes
91 which can be subsequently manipulated for vaccine production. In the present study, we
92 describe the development of a strategy for the design and production of inactivated BTV
93 vaccines that can significantly reduce the time taken from the identification of a new BTV
94 emerging strain, to the development and production of a new vaccine.
95
96

JVI Accepts published online ahead of print

97 **MATERIALS AND METHODS**

98 **Cells.** BSR, VERO and BHK_{21} cells were grown in Dulbecco's modified Eagle's medium
99 (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were incubated at 35°C or
100 37°C, depending on the experimental setting, in a humidified incubator with 5% $CO_{2.}$ BSR
101 cells were used for the recovery of "synthetic" BTV (sBTV) reassortants (5, 36). BHK21 cells
102 were used for the production of BTV reassortants according to industry practice (20, 32),
103 while VERO cells are cells recommended by the OIE (World Animal Health Organization) for
104 the diagnosis of BTV infection in ruminants by seroneutralization assays (34).

105 **Plasmids.** Plasmids used for the rescue of BTV-1 of BTV-8 by reverse genetics were
106 described previously (4). Similar plasmids containing segment 2 (encoding for VP2) or
107 segment 6 (encoding VP5) of the reference strains of BTV-2 to -24 and the recently
108 described BTV-25 and -26 were synthesized commercially (Genscript) (Table 1). In addition,
109 a chimeric BTV-1/BTV-8 segment 2 was also synthesised in which the nucleotide sequence
110 encoding amino acid residues 220 to 429 of the BTV-8 VP2 were introduced into the
111 homologous region of the BTV-1 segment 2.

112 **Recovery of "synthetic" BTV (sBTV) reassortants**. Synthetic BTV reassortants were rescued
113 *in vitro* by reverse genetics as previously described (4, 35). Briefly, DNA plasmids containing
114 the eight genomic segments of the BTV-1 backbone and each of the segments 2 and 6 of the
115 26 different BTV serotypes were linearized with restriction enzymes and purified by phenol-
116 chloroform extraction. The linearized plasmids were then used as templates for the in vitro
117 synthesis of capped BTV-like RNA using mMESSAGE mMACHINE T7 Ultra Kit (Ambion),
118 following the manufacturer's instructions. RNA was then extracted by phenol-chloroform
119 and further purified using the RNeasy Mini Kit (Qiagen). For the rescue of synthetic BTV
120 virus, monolayers of BSR cells in 12-well plates were transfected twice with BTV RNAs using

JVI Accepts published online ahead of print

121 Lipofectamine 2000. Firstly, 1x1011 RNA molecules of each segment encoding VP1, VP3,
122 VP4, NS1, VP6 and NS2 were diluted in Opti-MEM I Reduced Serum Medium containing
123 0.5U/µl of RNAsin Plus (Promega) for 5 min and mixed with Lipofectamine 2000 diluted in
124 Opti-MEM I Reduced Serum Medium. After 25 min of incubation at room temperature, the
125 mixture was added drop-wise to the cells and transferred to a humidified incubator at 35 C.
126 After 16-18h a second transfection was carried out using 1x1011 molecules of all the BTV
127 segments. sBTVs were plaque purified from supernatants of transfected cultures displaying
128 cytopathic effect. Viruses obtained by reverse genetics based on the reference strain of BTV-
129 1 from South Africa and the European BTV-8 strain (NET2006/04) were described previously
130 (4). Each sBTV reassortant is formed by a "backbone" (i.e. all the non-structural and
131 enzymatic proteins in addition to VP7 and VP3) of BTV-1 and an outer capsid layer
132 comprising VP2 and VP5 (or only the VP2) of a heterologous serotype. Each synthetic virus is
133 referred in this study with "B1" (indicating the backbone of BTV-1), followed by the serotype
134 of the VP2 (encoded by Seg-2) and VP5 (encoded by Seg-6) combination used. For example,
135 $_{B1}$BTV-8$^{VP2/VP5}$ is a synthetic virus containing the backbone of BTV-1 with the VP2 and VP5 of
136 BTV-8. Synthetic reassortants with a heterologous VP2 - VP5 combination have an additional
137 number referring to the serotype from which the VP5 was obtained. For example, $_{B1}$BTV-
138 24$^{VP2(4-VP5)}$ has the backbone of BTV-1, the VP2 from BTV-24 and the VP5 from BTV-4. An
139 additional synthetic BTV was rescued incorporating the chimeric BTV-1/BTV-8 VP2 segment
140 and designated as $_{B1}$BTV-1$^{(8-VP2-220-429).}$
141 **sBTV plaque phenotype and replication kinetics**. Viral plaque phenotypes of each sBTV
142 reassortant were assessed in VERO cells. Cell monolayers were infected with 10-fold viral
143 dilutions in DMEM for 2 h at 37°C, after which media containing the virus was removed, the
144 cells washed with PBS, followed by incubation with 3 ml of media containing 1.2% Avicel®

JVI Accepts published online ahead of print

145 (FMC Biopolymer) at 37°C for 72 h. Plates were fixed with 4% formaldehyde for 1 h. The
146 monolayer was incubated with a primary rabbit antibody against the BTV VP7 protein (4),
147 followed by an anti-rabbit HRP-conjugated secondary antibody and finally stained with
148 TrueBlue™ peroxidase staining kit (KPL). The replication kinetics of each synthetic virus was
149 assessed in BHK_{21} cells as already described (4) using an MOI of 0.001.

150 **Inactivated vaccine production**. $_{B1}$BTV-8$^{VP2/VP5}$ and $_{B1}$BTV-8^{VP2} were grown for 48 h in BHK_{21}
151 cells in 50 l bioreactors at 37°C in 5% CO_2 humidified atmosphere at the Merial S.A.S.
152 laboratory using standard industrial procedures. Prior to inactivation, virus cultures were
153 treated with chloroform, mechanically homogenised with Ultra-Turrax T50 (IKA), clarified by
154 filtration and centrifugation. Formaldehyde (0.5mg/ml) was then added to the filtrated
155 supernatant, which was subsequently treated twice with binary ethyleneimine (1.5 mM).
156 This inactivation step was carried out at 37°C for 24h. Complete inactivation of the virus was
157 confirmed by serially passaging the treated supernatants three times in VERO cells. The
158 inactivated supernatant was filtrated in high flow capacity filters, concentrated twice and
159 purified by immobilized metal ion affinity chromatography. Antigen dosage was then
160 measured through dot-blot analysis of the VP2 protein in parallel with an ELISA for the VP7
161 protein in order to use amounts of antigen identical to the commercial BTVPUR AlSap®
162 vaccines (30). Vaccines were blended with aluminium hydroxide and saponin.

163 **Vaccination and BTV challenge.** All animal experiments were carried out in accordance with
164 EU legislation and conducted in a high containment animal facility. 4 to 5 months old
165 Lacaune crossbred sheep, confirmed to be seronegative for BTV-8 by serum neutralisation
166 at the beginning of the study, were divided into four groups: G1 (n=6) was the unvaccinated
167 control, G2 (n=5) was vaccinated with $_{B1}$BTV-8$^{VP2/VP5}$, G3 (n=5) was vaccinated with $_{B1}$BTV-

JVI Accepts published online ahead of print

168 8^{VP2} and G4 (n=5) was vaccinated with a low antigen dose (1/10th compared to the dose
169 used in G3) of $_{B1}$BTV-8^{VP2}. Sheep were vaccinated subcutaneously with a single dose of 1 ml
170 of each sBTV vaccine while control sheep were not vaccinated.
171 21 days after vaccination, individual rectal temperatures were recorded and serum samples
172 were collected before sheep were challenged intradermally with 3ml of a virulent North
173 European BTV-8 strain ($1x10^7$ genome copy number/ml) distributed among approximately
174 30 separate injection points. Sheep were monitored daily up to 14 days post-challenge for
175 rectal temperature and clinical signs (general and body condition, congestion and/or
176 oedema, hypersalivation, nasal discharge/crusts, plaintive bleating, swollen lymph nodes,
177 locomotion difficulty, respiratory and digestive problems). A clinical score was assigned for
178 the following signs: between 0 and 3 for general condition, 0 and 1 for body condition and 0
179 to 4 for hyperthermia defined as body temperature above 40°C. Any other clinical signs
180 scored 1 if present. The individual scores for each sheep were added together, resulting in a
181 daily clinical score. Blood samples were collected on days 5, 7, 9, 12 and 14 post-challenge in
182 order to detect BTV RNA as an indication of viremia by qRT-PCR (see below). Neutralising
183 antibody titres in the serum of each animal were also measured at day 14 post-challenge
184 (see below).
185 **Neutralization assays.** The presence of neutralizing antibodies in vaccinated and control
186 sheep was assessed by microneutralization assays as already described (36). Sera were
187 collected at the beginning of the study before vaccination (-21 days), at the time of BTV-8
188 challenge (Day 0) and 2 weeks post-challenge (Day 14). The 50% protective dose (PD_{50}) for
189 each serum sample, defined as the serum dilution that inhibits BTV infection in 50% of VERO
190 cell cultures, was then determined using a linear regression after angular transformation.
191 Samples below the detection limit of 0.48 Log_{10} PD_{50} were considered negative.

JVI Accepts published online ahead of print

192 **Quantitative RT-PCR.** RNA was extracted from blood samples using the QIAamp Viral RNA
193 Mini Kit (Qiagen) according to the manufacturer's instructions. One-step quantitative RT-
194 PCR for the amplification of a conserved region of segment 10 was performed using TaqMan
195 EZ RT-PCR core reagent (Applied Biosystems). Serial ten-fold dilutions of RNA standard *in*
196 *vitro* transcribed from the BTV segment 10 were used to obtain a standard curve.
197

JVI Accepts published online ahead of print

198 **RESULTS**

199 **Rescue of synthetic BTV viruses (sBTV).** We obtained DNA plasmids containing
200 commercially synthesised genomic segments encoding VP2 and VP5 (outer core proteins) of
201 the reference strains of BTV-1 to -26. Plasmids were designed in order to be used in BTV
202 rescue experiments as already described (4, 35). Initially, cells were transfected using
203 homologous Seg-2 and Seg-6 from each serotype, in addition to plasmids containing
204 genomic segments forming the "backbone" of the synthetic viruses (Seg-1, -3, -4, -5, -7, -8, -
205 9 and -10) encoding the remaining structural, enzymatic and non-structural proteins from
206 BTV-1. The above strategy was successful for the rescue of 16 different BTV serotypes
207 resulting in the generation of the following synthetic BTV viruses: $_{B1}$BTV-1$^{VP2/VP5}$, $_{B1}$BTV-
208 2$^{VP2/VP5}$, $_{B1}$BTV-3$^{VP2/VP5}$, $_{B1}$BTV-4$^{VP2/VP5}$, $_{B1}$BTV-6$^{VP2/VP5}$, $_{B1}$BTV-8$^{VP2/VP5}$, $_{B1}$BTV-9$^{VP2/VP5}$, $_{B1}$BTV-
209 11$^{VP2/VP5}$, $_{B1}$BTV-13$^{VP2/VP5}$, $_{B1}$BTV-17$^{VP2/VP5}$, $_{B1}$BTV-20$^{VP2/VP5}$, $_{B1}$BTV-21$^{VP2/VP5}$, $_{B1}$BTV-22$^{VP2/VP5}$,
210 $_{B1}$BTV-23$^{VP2/VP5}$, $_{B1}$BTV-25$^{VP2/VP5}$ and $_{B1}$BTV-26$^{VP2/VP5}$. Seg-2 and Seg-6 of BTV-25 were
211 synthesised using the untranslated regions of the homologous segments of BTV-1.
212 For those synthetic viruses that we were not able to rescue using homologous VP2 and VP5,
213 we attempted rescue experiments using each Seg-2 (encoding VP2) in combination with the
214 remaining 25 heterologous Seg-6 (encoding VP5). Following this approach, two new sBTV
215 were generated: $_{B1}$BTV-14$^{VP2(6\text{-}VP5)}$, rescued using the VP2 of BTV-14 and the VP5 of BTV-6,
216 and $_{B1}$BTV-24$^{VP2(4\text{-}VP5)}$, rescued using the VP2 of BTV-24 and the VP5 of BTV-4. Other
217 strategies used to rescue the synthetic viruses with the outer core proteins of BTV-5, BTV-7,
218 BTV-10, BTV-12, BTV-15, BTV-16, BTV-18 and BTV-19 included the use of Seg-2 with the
219 untranslated region of BTV-1 and the use of homologous VP2, VP5 and VP7. In none of these
220 cases we were able to rescue stable viruses that could be propagated beyond one passage
221 after transfection, suggesting that infectious viruses were generated but they were unstable

JVI Accepts published online ahead of print

222 (data not shown). We sequenced a portion of segments 2 and 6 for all of the reassortants
223 successfully rescued to confirm the identity of each virus produced. In addition, we
224 repeated each rescue at least three times independently (using two different plasmid
225 preparations) in order to rule out that the failure in rescuing certain reassortants was due to
226 deleterious mutations arising by chance during the rescue experiments.

227 **Replication kinetics of synthetic viruses.** Replication kinetics of the rescued synthetic
228 viruses were assessed in BHK_{21} cells using a low MOI (0.001) in order to simulate the initial
229 production of a viral master stock for vaccine production (Fig. 1A). 14 of the 16 synthetic
230 viruses released virus into the supernatant of infected cells reaching titers higher than $1x10^4$
231 $TCID_{50}$ per ml at 72h post-infection. Differences in the replication kinetics between viruses
232 reaching titers > 10^6 $TCID_{50}$/ml at 72h p.i. (e.g. $_{B1}BTV\text{-}1^{VP2\text{-}P5}$, $_{B1}BTV\text{-}9^{VP2/VP5}$) and those with
233 lower titers (< 10^6 $TCID_{50}$/ml) were especially evident in the first 24h. Indeed, the titers of
234 the three reassortants with lower yields at 72h p.i. ($_{B1}BTV\text{-}2^{VP2/VP5}$, $_{B1}BTV\text{-}25^{VP2/}$VP5, and
235 $_{B1}BTV\text{-}26^{VP2/VP5}$) increased 10 to 1000 fold by 120 hpi, reaching levels compatible with the
236 other reassortants produced in this study (data not shown).

237 The size of the plaques induced in VERO cells appeared to be largely, but not always,
238 correlated to the titers reached in replication kinetic assays in BHK_{21} cells (Fig. 1B).

239 **Vaccination with inactivated synthetic BTV-based vaccines incorporating the BTV-8 VP2**
240 **protein confers protection against homologous challenge.** In order to validate the use of
241 synthetic viruses as inactivated vaccines against BTV, we carried out vaccination trials using
242 $_{B1}BTV\text{-}8^{VP2/VP5}$ and $_{B1}BTV\text{-}8^{VP2}$ (Fig. 2). These strains were inactivated with binary
243 ethyleneimine and prepared in an industrial setting at the Merial S.A.S. laboratory similarly
244 to the existing inactivated commercial vaccines (30).

245 For the vaccine trial, sheep were assigned into 4 groups: an unvaccinated control group

246 (G1) and three other groups in which animals were inoculated with vaccine preparations
247 containing identical antigen payload dose to commercial vaccines of $_{B1}$BTV-8$^{VP2/VP5}$ (G2) and
248 $_{B1}$BTV-8^{VP2} (G3) and a lower antigen dose (1/10th) of $_{B1}$BTV-8^{VP2} (G4). Animals were
249 subsequently challenged intra-dermally with a virulent BTV-8 strain (Fig. 2 and Table 2).
250 As anticipated, all six unvaccinated control sheep (G1) showed fever and developed clinical
251 signs commonly associated with BTV infection. The peak of fever was reached at day 7 post-
252 challenge (41.3 ±0.2^{o}C) in which all animals exhibited congestion of ears, eyes, nostrils and
253 lips, edema in the lips and erythema (redness of skin). Due to the severity of the clinical
254 signs, one sheep had to be euthanized at day 10 post-challenge. Viremia was detected
255 throughout the 14 days in all animals of the group. In sheep vaccinated with $_{B1}$BTV-8$^{VP2/VP5}$
256 (G2) no increase in rectal temperature was observed in any of the vaccinated animals and
257 only mild respiratory signs were observed in one animal on days 7 and 8 post-challenge.
258 Viremia was not detected in any of the animals of this group.
259 $_{B1}$BTV-8^{VP2} was used in the vaccination of groups G3 and G4. In both vaccination groups, the
260 presence of BTV-8 VP2 protein prevented fever and viremia. However, one animal in G3 did
261 not seem to respond to vaccination and exhibited mild clinical signs of infection. Viremia
262 was observed in this sheep to similar levels observed in the unvaccinated controls.
263 Interestingly, 4 of the 5 animals in G4 did not develop neutralizing antibodies after
264 vaccination but were protected against virulent BTV challenge. These data reinforce the idea
265 that the presence of detectable neutralizing antibodies in vaccinated animal is not
266 absolutely required for protection against BTV infection.
267 **Synthetic BTVs with a chimeric BTV-1 and BTV-8 VP2 are neutralized by both BTV-1 and**
268 **BTV-8 antisera**. In addition to using the entire VP2 coding sequence of individual serotypes,
269 we also tested whether it was possible to generate chimeric VP2 proteins cross-reacting

JVI Accepts published online ahead of print

JVI Accepts published online ahead of print

270 with different serotypes. To achieve this, we designed a DNA plasmid encoding a chimeric
271 VP2 protein in which amino acid residues from position 220 to 429 of BTV-1 were
272 substituted by the homologous region of BTV-8 VP2 (Fig. 3A). The design of this chimera was
273 based on the presence of conserved residues in the BTV-1 and BTV-8 VP2 junction sites,
274 while including divergent areas where neutralizing epitopes had been identified in previous
275 studies (37-40). This novel VP2 segment was processed for reverse genetics and used in the
276 successful rescue of a synthetic virus termed $_{B1}$BTV-1$^{(8\text{-}VP2\text{-}220\text{-}429)}$.
277 To determine if the introduction of the BTV-8 VP2 region into the BTV-1 VP2 changed the
278 antigenicity of the resulting chimeric proteins, we carried out virus neutralization assays of
279 wild type BTV-1, BTV-8 and $_{B1}$BTV-1$^{(8VP2\text{-}220\text{-}429)}$ with sheep antisera raised against BTV-1 or
280 BTV-8. Unlike parental viruses, which were neutralised only by the homologous antisera,
281 $_{B1}$BTV-1$^{(8VP2\ 220\text{-}429)}$ was cross-neutralized by both BTV-1 and BTV-8 antisera, suggesting that
282 the design of “bivalent” synthetic viruses is theoretically feasible (Fig. 3B).
283
284

JVI Accepts published online ahead of print

285 **DISCUSSION**

286 In the present study we devised a novel vaccine platform for the production of inactivated
287 synthetic viruses which facilitates vaccine production against current and newly emerging
288 BTV serotypes. Importantly, the ability for these vaccines to be scaled to an industrial scale
289 has been proven.

290 Our vaccine platform is based on the design and rescue of viruses containing the VP2 (or
291 VP2 and VP5) of distinct BTV serotypes with a viral "backbone" already adapted to tissue
292 culture conditions and vaccine production. We have validated this approach by selecting
293 two reassortants, $_{B1}$BTV-8$^{VP2/VP5}$ and $_{B1}$BTV-8^{VP2}, which have been prepared in an industrial
294 setting and proved to protect sheep as effectively as current commercial inactivated
295 vaccines based upon wild type strains of BTV-8 (30).

296 If a new outbreak is caused by a currently known BTV serotype, pre-prepared "off-the-shelf"
297 sBTV viruses, which have been validated for the initial steps of master-stock vaccine
298 preparation, could be used in an accelerated vaccine production pipeline. In case of an
299 emerging BTV strain of a previously unknown serotype (or a particularly divergent strain
300 within a known serotype), the nucleotide sequence of segment 2 (encoding the VP2 protein)
301 is synthesised and cloned into a DNA plasmid vector which, in parallel with the tissue culture
302 adapted BTV backbone, is used for the rescue of a replication competent sBTV virus by
303 reverse genetics (Fig. 4). These vaccines are safe given that the inactivation step eliminates
304 the risk of releasing replication competent pathogenic viruses into the environment. In
305 addition, no secondary effects post-vaccination were observed in any of the vaccinated
306 animals in our trials.

307 The development of veterinary vaccines from discovery to regulatory approval and their use
308 in the market is an extremely costly and lengthy process. Hence, vaccines for emerging

JVI Accepts published online ahead of print

309 diseases, especially if caused by pathogens with multiple distinct serotypes (and therefore
310 requiring multiple products) are an especially big challenge for industry. In most cases, for
311 obvious economic reasons, vaccine production initiates only after an outbreak occurs in an
312 area where there is a potential market. In order to respond to BTV outbreaks, vaccine
313 manufacturers have been able to significantly reduce the time needed to introduce new
314 products into the market. However, it can take approximately 1 to 6 months for a vaccine
315 manufacturer to acquire a new BTV strain from the field and a further 14-20 months to
316 develop, test, produce a new vaccine and take it through provisional market authorization.
317 These are conservative figures, and assume that every step runs smoothly, but it does
318 happen that a seed vaccine strain may not pass all of the many steps required for vaccine
319 development. Because all of the elements of the vaccines described in this study are derived
320 synthetically, the acquisition of a viral isolate by industry and many of the quality control
321 steps required by manufacturers prior to the introduction of field samples into the vaccine
322 pipeline (e.g. presence of adventitious agents) can be bypassed and/or carried out more
323 easily. In addition, a deeper understanding of possible barriers regulating reassortment of
324 different genomic segments between specific BTV serotypes can also result in a further
325 reduction in the time taken to obtain a new master seed for a new reassortant. Based upon
326 current industry experience, the synthetic biology approach to vaccine development could
327 save 6 months across the entire vaccine pipeline.
328 The timeline of vaccine production can be absolutely critical to halting the spread of a newly
329 introduced BTV serotype. Most bluetongue outbreaks in temperate regions will have a
330 limited diffusion in the first vector season after introduction, but will spread considerably
331 and cause extensive damage in the following year (41).
332 We have used and tested a single "backbone" in the current study based on BTV-1. It is

JVI Accepts published online ahead of print

333 likely that other backbones might be useful in order to increase the ability to rescue any BTV
334 serotype/strain. We failed in the current study to obtain stable synthetic viruses containing
335 the VP2 of BTV-5, -7, -10, -12, -15, -16, -18, -19. These serotypes don't seem to have any
336 particular phylogenetic feature in common that could explain their unsuccessful rescue with
337 a BTV-1 backbone (Fig. 5). Hence, more studies with additional backbones (or additional
338 segment 2 sequences) will certainly shed light on the "compatibility" of different BTV
339 proteins or genomic segments.
340 To further highlight the possibilities of genome manipulation for vaccine production, we also
341 designed and rescued a synthetic BTV chimera containing a VP2 protein including regions
342 derived from both BTV-1 and BTV-8. Interestingly, while the parental viruses were
343 neutralized only by homologous antisera, the chimeric proteins could be neutralized by both
344 BTV-1 and BTV-8 antisera. These data suggest that neutralizing epitopes are present in
345 different areas of the VP2 and likely "bivalent" strains eliciting neutralizing antibodies for
346 multiple strains can be obtained. Thus, the potential exists to develop products made by 2
347 or 3 synthetic strains with chimeric VP2 that may be effective against multiple serotypes.
348 Several potential strategies for vaccination against BTV have been developed in the last
349 decade (42). In mouse models, immunization with VP2 and VP5 expressed in bacteria has
350 recently been shown to protect against lethal BTV challenge (43). Mice were also protected
351 against BTV when VP2 was expressed in vectors such as Bovine herpesvirus 4 (BoHV-4) (44),
352 Equine herpesvirus 1 (EHV-1) (45) or modified Vaccinia Ankara (MVA) (46).
353 In sheep, virus-like particles (VLPs) derived using recombinant baculovirus expression
354 systems have been shown to protect against virulent BTV challenge (47, 48). Vesicular
355 stomatitis viral replicons expressing VP2 or similar vaccines based on canarypoxviruses have
356 also been shown to be potentially effective vaccines for bluetongue (49, 50).

JVI Accepts published online ahead of print

357 Vaccines based on reverse genetics include the generation of live attenuated vaccines in
358 which the VP2 and VP5 proteins of BTV-1 and BTV-8 were introduced into an attenuated
359 BTV-6 backbone (51). Disabled-infectious-single-cycle (DISC) BTV vaccines have also proven
360 to be effective in experimental trials in sheep. These vaccines are particularly interesting as
361 they are generated by rescuing BTV in cell lines expressing one of the viral proteins *in trans*
362 and therefore might possess most of the benefits of the live attenuated vaccines without
363 their inherent risks (52).
364 However, the passage from a potential vaccine developed in an experimental setting to an
365 industrial product ready to be used in the market is affected by several economic, scientific
366 and regulatory issues that are particularly complex for emerging diseases. The vaccine
367 platform that we have developed has the advantage of utilising synthetic biology in order to
368 curtail the developmental period, whilst simultaneously equating to an existing commercial
369 product and its known qualities. In turn, this allows the product to be rapidly introduced
370 into the market when necessary.
371

372 **ACKNOWLEDGEMENTS**

373 This work was funded by a BBSRC Industrial partnership Award. Sandro Filipe Nunes, Pascal
374 Hudelet and Massimo Palmarini are co-inventors in a patent application covering the
375 synthetic vaccine platform described in this study (US Patent and Trademark Office - US
376 20130337010 A1). We thank Mariana Varela for useful suggestions. This document is
377 provided for scientific purposes only. Any reference to a brand or trademark herein is for
378 informational purposes only and is not intended for a commercial purpose or to dilute the
379 rights of the respective owner(s) of the brand(s) or trademark(s). ®All Marks are the
380 property of their respective owners.

JVI Accepts published online ahead of print

381 **REFERENCES**

382 1. **Mellor, P. S., M. Baylis, and P. P. Mertens.** 2009. Bluetongue. Academic Press,
383 London.

384 2. **Caporale, M., L. Di Gialleonorado, A. Janowicz, G. Wilkie, A. Shaw, G. Savini, P. A.**
385 **Van Rijn, P. Mertens, M. Di Ventura, and M. Palmarini.** 2014. Virus and Host Factors
386 Affecting the Clinical Outcome of Bluetongue Virus Infection. J Virol.

387 3. **Belhouchet, M., F. Mohd Jaafar, A. E. Firth, J. M. Grimes, P. P. Mertens, and H.**
388 **Attoui.** 2011. Detection of a fourth orbivirus non-structural protein. PLoS One
389 **6:**e25697.

390 4. **Ratinier, M., M. Caporale, M. Golder, G. Franzoni, K. Allan, S. F. Nunes, A.**
391 **Armezzani, A. Bayoumy, F. Rixon, A. Shaw, and M. Palmarini.** 2011. Identification
392 and characterization of a novel non-structural protein of bluetongue virus. PLoS
393 Pathog **7:**e1002477.

394 5. **Roy, P.** 2008. Functional mapping of bluetongue virus proteins and their interactions
395 with host proteins during virus replication. Cell biochemistry and biophysics **50:**143-
396 157.

397 6. **Gouet, P., J. M. Diprose, J. M. Grimes, R. Malby, J. N. Burroughs, S. Zientara, D. I.**
398 **Stuart, and P. P. Mertens.** 1999. The highly ordered double-stranded RNA genome
399 of bluetongue virus revealed by crystallography. Cell **97:**481-490.

400 7. **Patel, A., and P. Roy.** 2014. The molecular biology of Bluetongue virus replication.
401 Virus Res **182:**5-20.

JVI Accepts published online ahead of print

402 8. **Kahlon, J., K. Sugiyama, and P. Roy.** 1983. Molecular basis of bluetongue virus
403 neutralization. J Virol **48:**627-632.

404 9. **Huismans, H., and B. J. Erasmus.** 1981. Identification of the serotype-specific and
405 group-specific antigens of bluetongue virus. Onderstepoort J Vet Res **48:**51-58.

406 10. **Hofmann, M. A., S. Renzullo, M. Mader, V. Chaignat, G. Worwa, and B. Thuer.**
407 2008. Genetic characterization of toggenburg orbivirus, a new bluetongue virus,
408 from goats, Switzerland. Emerg Infect Dis **14:**1855-1861.

409 11. **Maan, S., N. S. Maan, K. Nomikou, E. Veronesi, K. Bachanek-Bankowska, M. N.**
410 **Belaganahalli, H. Attoui, and P. P. Mertens.** 2011. Complete genome
411 characterisation of a novel 26th bluetongue virus serotype from Kuwait. PLoS One
412 **6:**e26147.

413 12. **Maan, S., N. S. Maan, A. R. Samuel, S. Rao, H. Attoui, and P. P. Mertens.** 2007.
414 Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue
415 virus serotypes. J Gen Virol **88:**621-630.

416 13. **Shaw, A. E., M. Ratinier, S. F. Nunes, K. Nomikou, M. Caporale, M. Golder, K. Allan,**
417 **C. Hamers, P. Hudelet, S. Zientara, E. Breard, P. Mertens, and M. Palmarini.** 2013.
418 Reassortment between two serologically unrelated bluetongue virus strains is
419 flexible and can involve any genome segment. J Virol **87:**543-557.

420 14. **Zhang, X., M. Boyce, B. Bhattacharya, S. Schein, P. Roy, and Z. H. Zhou.** 2010.
421 Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5
422 resembles enveloped virus fusion proteins. Proc Natl Acad Sci U S A **107:**6292-6297.

423 15. **Erasmus, B. J.** 1975. Bluetongue in sheep and goats. Aust Vet J **51:**165-170.

JVI Accepts published online ahead of print

424 16. **MacLachlan, N. J., J. E. Crafford, W. Vernau, I. A. Gardner, A. Goddard, A. J. Guthrie,**
425 **and E. H. Venter.** 2008. Experimental reproduction of severe bluetongue in sheep.
426 Vet Pathol **45:**310-315.

427 17. **Zientara, S., and J. M. Sanchez-Vizcaino.** 2013. Control of bluetongue in Europe. Vet
428 Microbiol **165:**33-37.

429 18. **Savini, G., N. J. MacLachlan, J. M. Sanchez-Vizcaino, and S. Zientara.** 2008. Vaccines
430 against bluetongue in Europe. Comp Immunol Microbiol Infect Dis **31:**101-120.

431 19. **Maclachlan, N. J.** 2011. Bluetongue: history, global epidemiology, and pathogenesis.
432 Prev Vet Med **102:**107-111.

433 20. **Wilson, A. J., and P. S. Mellor.** 2009. Bluetongue in Europe: past, present and future.
434 Philos Trans R Soc Lond B Biol Sci **364:**2669-2681.

435 21. **Purse, B. V., H. E. Brown, L. Harrup, P. P. Mertens, and D. J. Rogers.** 2008. Invasion
436 of bluetongue and other orbivirus infections into Europe: the role of biological and
437 climatic processes. Rev Sci Tech **27:**427-442.

438 22. **Purse, B. V., P. S. Mellor, D. J. Rogers, A. R. Samuel, P. P. Mertens, and M. Baylis.**
439 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev
440 Microbiol **3:**171-181.

441 23. **Saegerman, C., D. Berkvens, and P. S. Mellor.** 2008. Bluetongue epidemiology in the
442 European Union. Emerg Infect Dis **14:**539-544.

443 24. **Verwoerd, D. W.** 2012. History of Orbivirus research in South Africa. Journal of the
444 South African Veterinary Association **83:**Art. #532.

JVI Accepts published online ahead of print

445 25. **Batten, C. A., S. Maan, A. E. Shaw, N. S. Maan, and P. P. Mertens.** 2008. A European
446 field strain of bluetongue virus derived from two parental vaccine strains by genome
447 segment reassortment. Virus Res **137:**56-63.

448 26. **Veronesi, E., K. E. Darpel, C. Hamblin, S. Carpenter, H. H. Takamatsu, S. J. Anthony,**
449 **H. Elliott, P. P. Mertens, and P. S. Mellor.** 2010. Viraemia and clinical disease in
450 Dorset Poll sheep following vaccination with live attenuated bluetongue virus
451 vaccines serotypes 16 and 4. Vaccine **28:**1397-1403.

452 27. **Savini, G., A. Cannas, C. Casaccia, L. Di Gialleonardo, A. Leone, C. Patta, and P.**
453 **Nicolussi.** 2010. Risk factors associated with the occurrence of undesired effects in
454 sheep and goats after field vaccination with modified-live vaccine against bluetongue
455 virus serotypes 2, 4 and 16. Vet Microbiol **146:**44-50.

456 28. **Veronesi, E., C. Hamblin, and P. S. Mellor.** 2005. Live attenuated bluetongue vaccine
457 viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera:
458 Ceratopogonidae). Vaccine **23:**5509-5516.

459 29. **Darpel, K. E., C. A. Batten, E. Veronesi, A. E. Shaw, S. Anthony, K. Bachanek-**
460 **Bankowska, L. Kgosana, A. bin-Tarif, S. Carpenter, U. U. Muller-Doblies, H. H.**
461 **Takamatsu, P. S. Mellor, P. P. Mertens, and C. A. Oura.** 2007. Clinical signs and
462 pathology shown by British sheep and cattle infected with bluetongue virus serotype
463 8 derived from the 2006 outbreak in northern Europe. Vet Rec **161:**253-261.

464 30. **Hamers, C., S. Galleau, R. Chery, M. Blanchet, L. Besancon, C. Cariou, B. Werle-**
465 **Lapostolle, P. Hudelet, and S. Goutebroze.** 2009. Use of inactivated bluetongue virus
466 serotype 8 vaccine against virulent challenge in sheep and cattle. Vet Rec **165:**369-
467 373.

JVI Accepts published online ahead of print

468 31. **Di Emidio, B., P. Nicolussi, C. Patta, G. F. Ronchi, F. Monaco, G. Savini, A. Ciarelli,**
469 **and V. Caporale.** 2004. Efficacy and safety studies on an inactivated vaccine against
470 bluetongue virus serotype 2. Veterinaria italiana **40:**640-644.

471 32. **Szmaragd, C., A. J. Wilson, S. Carpenter, J. L. Wood, P. S. Mellor, and S. Gubbins.**
472 2010. The spread of bluetongue virus serotype 8 in Great Britain and its control by
473 vaccination. PLoS One **5:**e9353.

474 33. **Baetza, H. J.** 2014. Eradication of bluetongue disease in Germany by vaccination. Vet
475 Immunol Immunopathol **158:**116-119.

476 34. **OIE.** 2014. Manual of diagnostic tests and vaccines for terrestrial animals, Paris.

477 35. **Boyce, M., C. C. Celma, and P. Roy.** 2008. Development of reverse genetics systems
478 for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. J
479 Virol **82:**8339-8348.

480 36. **Hamers, C., S. Galleau, R. Chery, M. Blanchet, L. Besancon, C. Cariou, B. Werle-**
481 **Lapostolle, P. Hudelet, and S. Goutebroze.** 2009. Use of inactivated bluetongue virus
482 serotype 8 vaccine against virulent challenge in sheep and cattle. The Veterinary
483 record **165:**369-373.

484 37. **Wilson, W. C., K. A. Bernard, B. A. Israel, and J. O. Mecham.** 2008. Bluetongue virus
485 serotype 17 sequence variation associated with neutralization. DNA Seq **19:**237-240.

486 38. **DeMaula, C. D., H. W. Heidner, P. V. Rossitto, C. M. Pierce, and N. J. MacLachlan.**
487 1993. Neutralization determinants of United States bluetongue virus serotype ten.
488 Virology **195:**292-296.

JVI Accepts published online ahead of print

489 39. **Gould, A. R., and B. T. Eaton.** 1990. The amino acid sequence of the outer coat
490 protein VP2 of neutralizing monoclonal antibody-resistant, virulent and attenuated
491 bluetongue viruses. Virus Res **17:**161-172.

492 40. **Pierce, C. M., P. V. Rossitto, and N. J. MacLachlan.** 1995. Homotypic and heterotypic
493 neutralization determinants of bluetongue virus serotype 17. Virology **209:**263-267.

494 41. **Meroc, E., C. Herr, B. Verheyden, J. Hooyberghs, P. Houdart, M. Raemaekers, F.**
495 **Vandenbussche, K. De Clercq, and K. Mintiens.** 2009. Bluetongue in Belgium:
496 episode II. Transbound Emerg Dis **56:**39-48.

497 42. **Calvo-Pinilla, E., J. Castillo-Olivares, T. Jabbar, J. Ortego, F. de la Poza, and A.**
498 **Marin-Lopez.** 2014. Recombinant vaccines against bluetongue virus. Virus Res
499 **182:**78-86.

500 43. **Mohd Jaafar, F., M. Belhouchet, D. Vitour, M. Adam, E. Breard, S. Zientara, P. P.**
501 **Mertens, and H. Attoui.** 2014. Immunisation with bacterial expressed VP2 and VP5
502 of bluetongue virus (BTV) protect alpha/beta interferon-receptor knock-out (IFNAR)
503 mice from homologous lethal challenge. Vaccine.

504 44. **Franceschi, V., A. Capocefalo, E. Calvo-Pinilla, M. Redaelli, C. Mucignat-Caretta, P.**
505 **Mertens, J. Ortego, and G. Donofrio.** 2011. Immunization of knock-out alpha/beta
506 interferon receptor mice against lethal bluetongue infection with a BoHV-4-based
507 vector expressing BTV-8 VP2 antigen. Vaccine **29:**3074-3082.

508 45. **Ma, G., M. Eschbaumer, A. Said, B. Hoffmann, M. Beer, and N. Osterrieder.** 2012.
509 An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8
510 bluetongue virus (BTV-8) induces protection in a murine infection model. PLoS One
511 **7:**e34425.

JVI Accepts published online ahead of print

512 46. **Calvo-Pinilla, E., F. de la Poza, S. Gubbins, P. P. Mertens, J. Ortego, and J. Castillo-**
513 **Olivares.** 2014. Vaccination of mice with a modified Vaccinia Ankara (MVA) virus
514 expressing the African horse sickness virus (AHSV) capsid protein VP2 induces virus
515 neutralising antibodies that confer protection against AHSV upon passive
516 immunisation. Virus Res **180:**23-30.

517 47. **Roy, P., D. H. Bishop, H. LeBlois, and B. J. Erasmus.** 1994. Long-lasting protection of
518 sheep against bluetongue challenge after vaccination with virus-like particles:
519 evidence for homologous and partial heterologous protection. Vaccine **12:**805-811.

520 48. **Stewart, M., C. I. Dovas, E. Chatzinasiou, T. N. Athmaram, M. Papanastassopoulou,**
521 **O. Papadopoulos, and P. Roy.** 2012. Protective efficacy of Bluetongue virus-like and
522 subvirus-like particles in sheep: presence of the serotype-specific VP2, independent
523 of its geographic lineage, is essential for protection. Vaccine **30:**2131-2139.

524 49. **Kochinger, S., N. Renevey, M. A. Hofmann, and G. Zimmer.** 2014. Vesicular
525 stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus
526 serotype 8 induces complete protection of sheep against challenge infection. Vet Res
527 **45:**64.

528 50. **Boone, J. D., U. B. Balasuriya, K. Karaca, J. C. Audonnet, J. Yao, L. He, R. Nordgren,**
529 **F. Monaco, G. Savini, I. A. Gardner, and N. J. Maclachlan.** 2007. Recombinant
530 canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid
531 proteins of bluetongue virus induces high level protection in sheep. Vaccine **25:**672-
532 678.

533 51. **van Gennip, R. G., S. G. van de Water, M. Maris-Veldhuis, and P. A. van Rijn.** 2012.
534 Bluetongue viruses based on modified-live vaccine serotype 6 with exchanged outer

JVI Accepts published online ahead of print

535 shell proteins confer full protection in sheep against virulent BTV8. PLoS One
536 **7:**e44619.

537 52. **Celma, C. C., M. Boyce, P. A. van Rijn, M. Eschbaumer, K. Wernike, B. Hoffmann, M.**
538 **Beer, A. Haegeman, K. De Clercq, and P. Roy.** 2013. Rapid generation of replication-
539 deficient monovalent and multivalent vaccines for bluetongue virus: protection
540 against virulent virus challenge in cattle and sheep. J Virol **87:**9856-9864.

541 53. **Guindon, S., F. Delsuc, J. F. Dufayard, and O. Gascuel.** 2009. Estimating maximum
542 likelihood phylogenies with PhyML. Methods Mol Biol **537:**113-137.

543 54. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA
544 substitution. Bioinformatics **14:**817-818.

545

546

547

548

549

550

551

552

553

554

555

JVI Accepts published online ahead of print

556 **Table 1. Genbank accession numbers of the BTV viral segments used in this study.**

	Genbank Accession Number		
Serotype	**Segment 2**	**Segment 6**	**BTV-1 Backbone**
BTV-1	FJ969720	FJ969723	
BTV-2	AJ585123	JN255867	**Segment 1**
BTV-3	AJ585124	AJ586697	FJ969719
BTV-4	AJ585125	AJ586699	
BTV-5	AJ585126	AJ586700	**Segment 3**
BTV-6	AJ585127	AJ586703	FJ969721
BTV-7	AJ585128	AJ586704	
BTV-8	AM498052	AM498056	**Segment 4**
BTV-9	AJ585130	AJ586708	FJ969722
BTV-10	AJ585131	AJ586709	
BTV-11	AJ585132	AJ586710	**Segment 5**
BTV-12	AJ585133	AJ586711	FJ969724
BTV-13	AJ585134	AJ586713	
BTV-14	AJ585135	AJ586714	**Segment 7**
BTV-15	AJ585136	AJ586716	FJ969725
BTV-16	AJ585137	AJ586719	
BTV-17	AJ585138	AJ586720	**Segment 8**
BTV-18	AJ585139	AJ586721	FJ969726
BTV-18	AJ585140	AJ586722	
BTV-20	AJ585141	AJ586723	**Segment 9**
BTV-21	AJ585142	AJ586724	JN848767
BTV-22	AJ585143	AJ586725	
BTV-23	AJ585144	AJ586727	**Segment 10**
BTV-24	AJ585145	AJ586730	FJ969728
BTV-25	EU839840	EU839842	
BTV-26	HM590642	JN255159	

557

558

559

JVI Accepts published online ahead of print

560 **Table 2. BTV-8 neutralizing antibody response in vaccinated and control sheep.**

561

562

Groups	Animal	BTV-8 Neutralizing Antibody Titres*		
		Day -21	Day 0	Day 14
G1 Unvaccinated control	10349	<0.48	<0.48	0.89
	10372	<0.48	<0.48	1.12
	10375	<0.48	<0.48	2.08
	10412	<0.48	<0.48	1.28
	10434	<0.48	<0.48	n.a.
	20133	<0.48	<0.48	1.20
	Mean ±SD	**<0.48**	**<0.48**	**1.31 ±0.45**
G2 $_{B1}$BTV-8$^{VP2/VP5}$	10358	<0.48	0.53	2.08
	10376	<0.48	0.80	2.08
	10379	<0.48	0.72	1.76
	10414	<0.48	1.20	1.20
	20152	<0.48	0.72	1.76
	Mean ±SD	**<0.48**	**0.79 ±0.25**	**1.78 ±0.36**
G3 $_{B1}$BTV-8^{VP2}	10348	<0.48	0.72	1.68
	10360	<0.48	0.80	1.85
	10371	<0.48	<0.48	1.76
	10374	<0.48	<0.48	2.16
	10424	<0.48	0.53	1.76
	Mean ±SD	**<0.48**	**<0.60 ±0.15**	**1.84 ±0.19**
G4 $_{B1}$BTV-8^{8VP2} (Low Dose)	10356	<0.48	<0.48	1.77
	10363	<0.48	<0.48	2.64
	10385	<0.48	0.72	1.20
	10420	<0.48	<0.48	1.37
	20103	<0.48	<0.48	1.76
	Mean ±SD	**<0.48**	**<0.53 ±0.11**	**1.75 ±0.56**

563 *The neutralizing titre in each sheep was expressed as log_{10} of 50 per cent protective dose
564 (PD_{50}).

565

566

567

568

JVI Accepts published online ahead of print

569 **FIGURE LEGENDS**

570

571 **Figure 1. Replication kinetics and plaque phenotype of synthetic viruses. A.** Viral
572 replication kinetics of synthetic viruses in BHK_{21} cells. Confluent monolayers were infected
573 with each sBTV reassortant at a MOI of 0.001 and the virus supernatants titrated as
574 described in Materials and Methods. **B**. Morphology of plaques induced by synthetic viruses
575 in VERO cells. Immunostaining of viral plaques was performed using a BTV-1 VP7 antibody,
576 followed by a HRP-conjugated secondary antibody and staining with TrueBlue.

577 **Figure 2. Sheep vaccinated with $_{B1}$BTV-8$^{VP2/VP5}$ and $_{B1}$BTV-8^{VP2} are protected against wild**
578 **type BTV-8 challenge.** Vaccine trials were carried out using four groups of Lacaune
579 crossbred sheep. Group 1 were not vaccinated while Groups 2 and 3 were vaccinated with
580 either $_{B1}$BTV-8$^{VP2/VP5}$ or $_{B1}$BTV-8^{VP2}, respectively. An additional group of sheep was also
581 vaccinated with $_{B1}$BTV-8^{VP2} (Group 4) but with a lower vaccine dose that used in current
582 vaccine formulations. 21 days post vaccination each group was challenged with a virulent
583 BTV-8 strain and each animal was assessed for the onset of fever, clinical signs and viremia.
584 14 of 15 vaccinated animals in groups 2-4 were protected against BTV-8 challenge. One
585 animal in Group 3 developed fever and displayed viremia.

586 **Figure 3. A synthetic virus with a chimeric BTV-1/BTV-8 VP2 is replication competent and**
587 **cross-reacts with BTV-1 and BTV-8 antisera. A**. Protein alignment of BTV-1 and BTV-8 VP2.
588 **B**. Plaque morphology of parental and chimeric synthetic reassortants ($_{B1}$BTV-1$^{(BTV\text{-}8\text{-}220\text{-}429)}$)
589 (left). Neutralization assays (right) of BTV-1, BTV-8 and $_{B1}$BTV-1$^{(8VP2\text{-}220\text{-}429)}$ using BTV-1 and
590 BTV-8 antisera as described in Materials and Methods. BTV-1 and BTV-8 are neutralized only
591 by homologous antisera. On the other hand, $_{B1}$BTV-1$^{(8VP2\text{-}220\text{-}429)}$ is neutralized by both BTV-1

JVI Accepts published online ahead of print

592 and BTV-8 antisera.

593 **Figure 4. A vaccine platform based on inactivated synthetic viruses against current and**
594 **emerging BTV serotypes.** The strategy used for the development of a vaccine platform
595 based on synthetic reassortants formed by a "backbone" based on cell-culture adapted BTV-
596 1, and the outer core VP2 protein (or VP2 and VP5) of the serotype of interest. "Off-the-
597 shelf" or newly-obtained vaccine strains will be used for vaccine development depending on
598 the BTV strain involved in the outbreak to be contained.

599 **Figure 5. Phylogenetic relationships of the genomic segments encoding VP2, VP5 and VP7**
600 **of the BTV reference strains.** Unrooted maximum likelihood phylogenetic trees of the
601 genomic segments encoding for VP2, VP5 and VP7 of the BTV reference strains. Trees were
602 estimated using PhyML (53) with 1000 bootstraps under the best-fit model of nucleotide
603 substitution determined using Modeltest (54). Branch lengths are drawn to scale of
604 nucleotide substitutions per site with the bars representing 0.5 substitution/site. In red are
605 indicated the serotypes for which we have not been able to rescue stable synthetic viruses
606 in a BTV-1 backbone.

607

JVI Accepts published online ahead of print

A

Infectious viral titre ($TCID_{50}$/ml)

$_{B1}$BTV-1$^{VP2/VP5}$ | $_{B1}$BTV-2$^{VP2/VP5}$ | $_{B1}$BTV-3$^{VP2/VP5}$ | $_{B1}$BTV-4$^{VP2/VP5}$ | $_{B1}$BTV-6$^{VP2/VP5}$ | $_{B1}$BTV-8$^{VP2/VP5}$

$_{B1}$BTV-9$^{VP2/VP5}$ | $_{B1}$BTV-11$^{VP2/VP5}$ | $_{B1}$BTV-13$^{VP2/VP5}$ | $_{B1}$BTV-14$^{VP2(6-VP5)}$ | $_{B1}$BTV-17$^{VP2/VP5}$ | $_{B1}$BTV-20$^{VP2/VP5}$

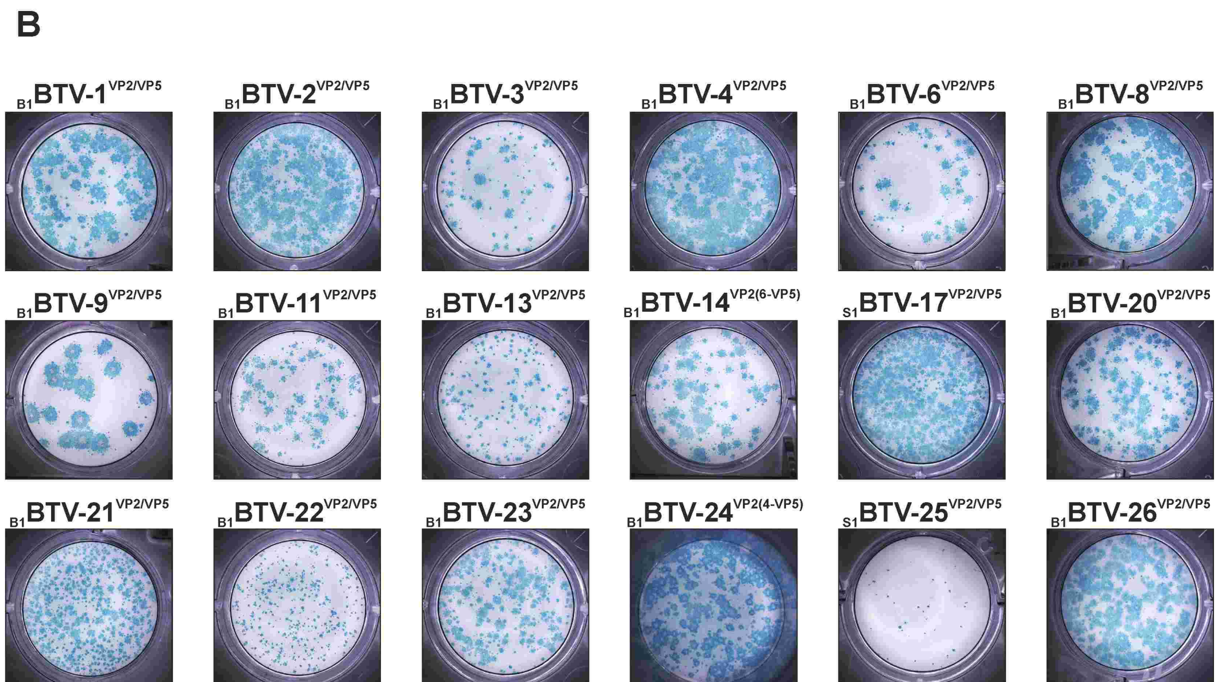
$_{B1}$BTV-21$^{VP2/VP5}$ | $_{B1}$BTV-22$^{VP2/VP5}$ | $_{B1}$BTV-23$^{VP2/VP5}$ | $_{B1}$BTV-24$^{VP2(4-VP5)}$ | $_{S1}$BTV-25$^{VP2/VP5}$ | $_{B1}$BTV-26$^{VP2/VP5}$

10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9

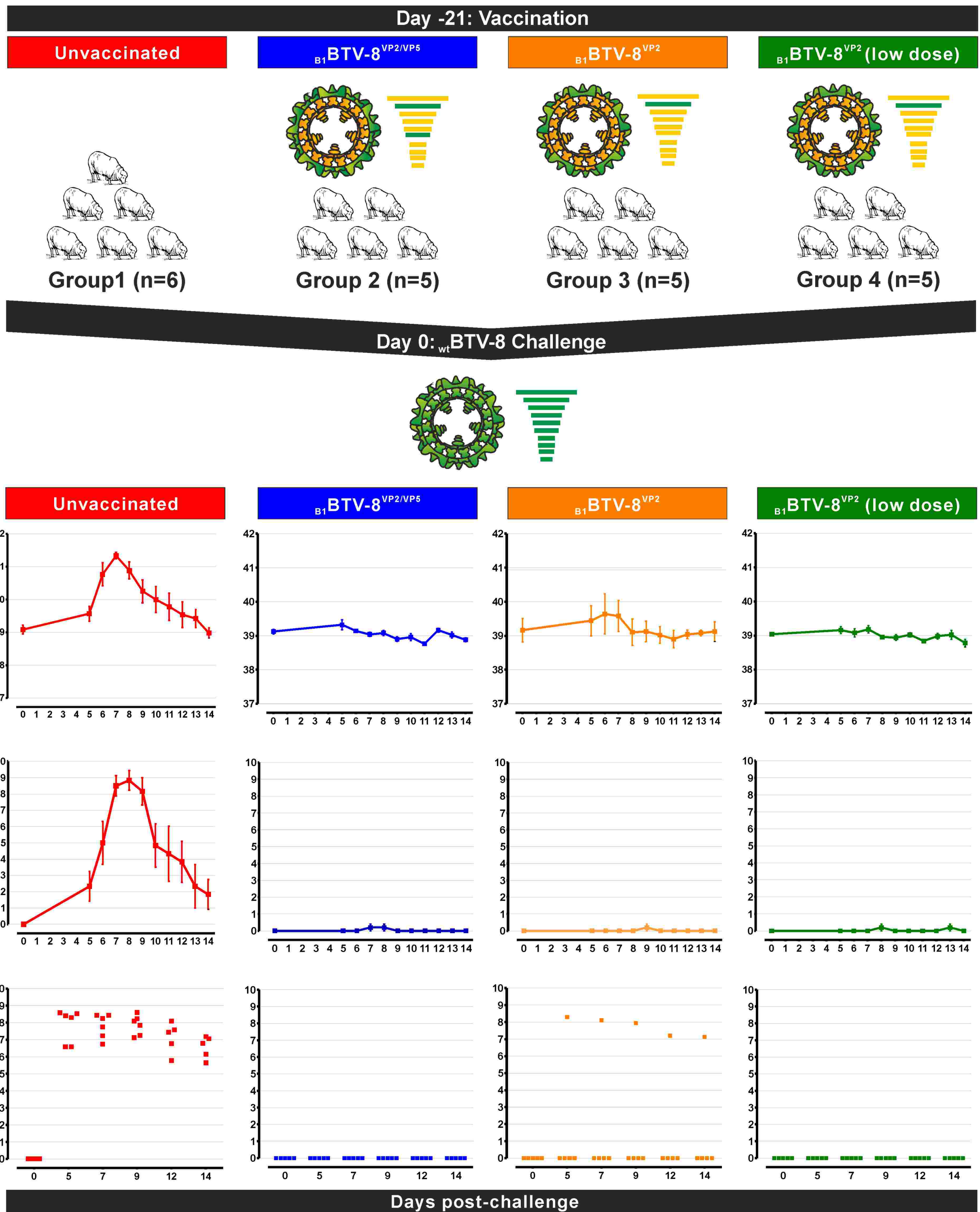
0 24 48 72

Time post-infection (Hours)

B

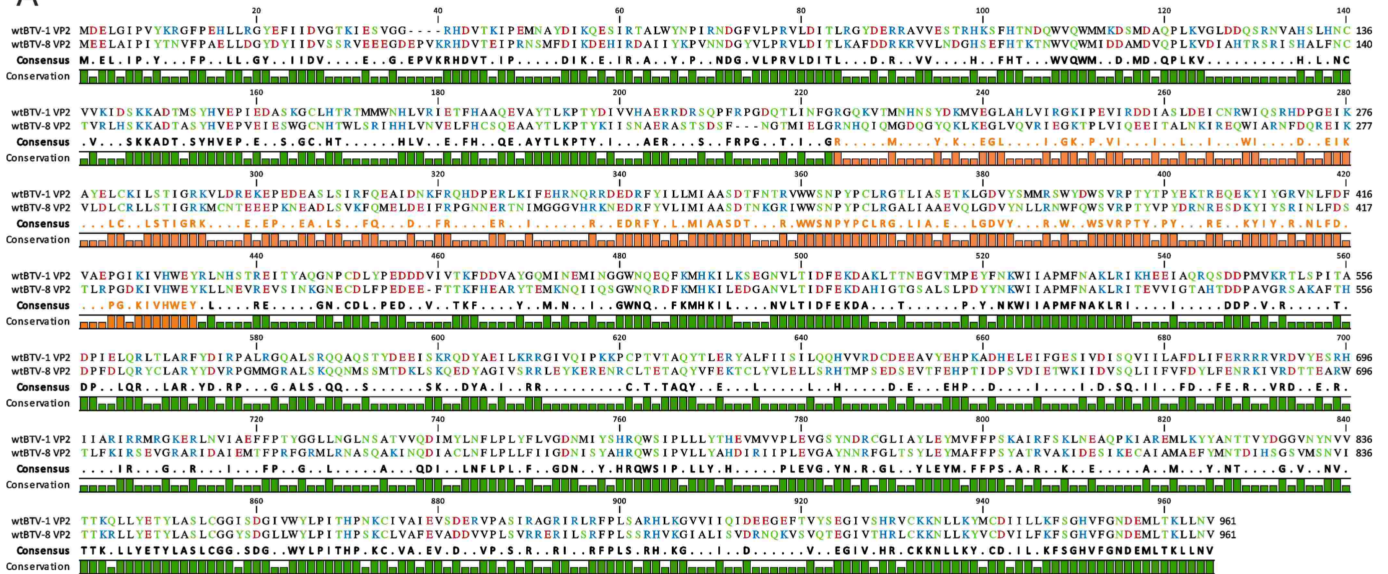


JVI Accepts published online ahead of print

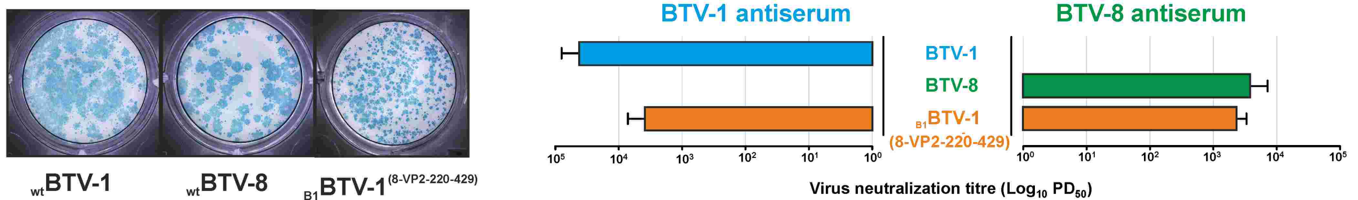


JVI Accepts published online ahead of print

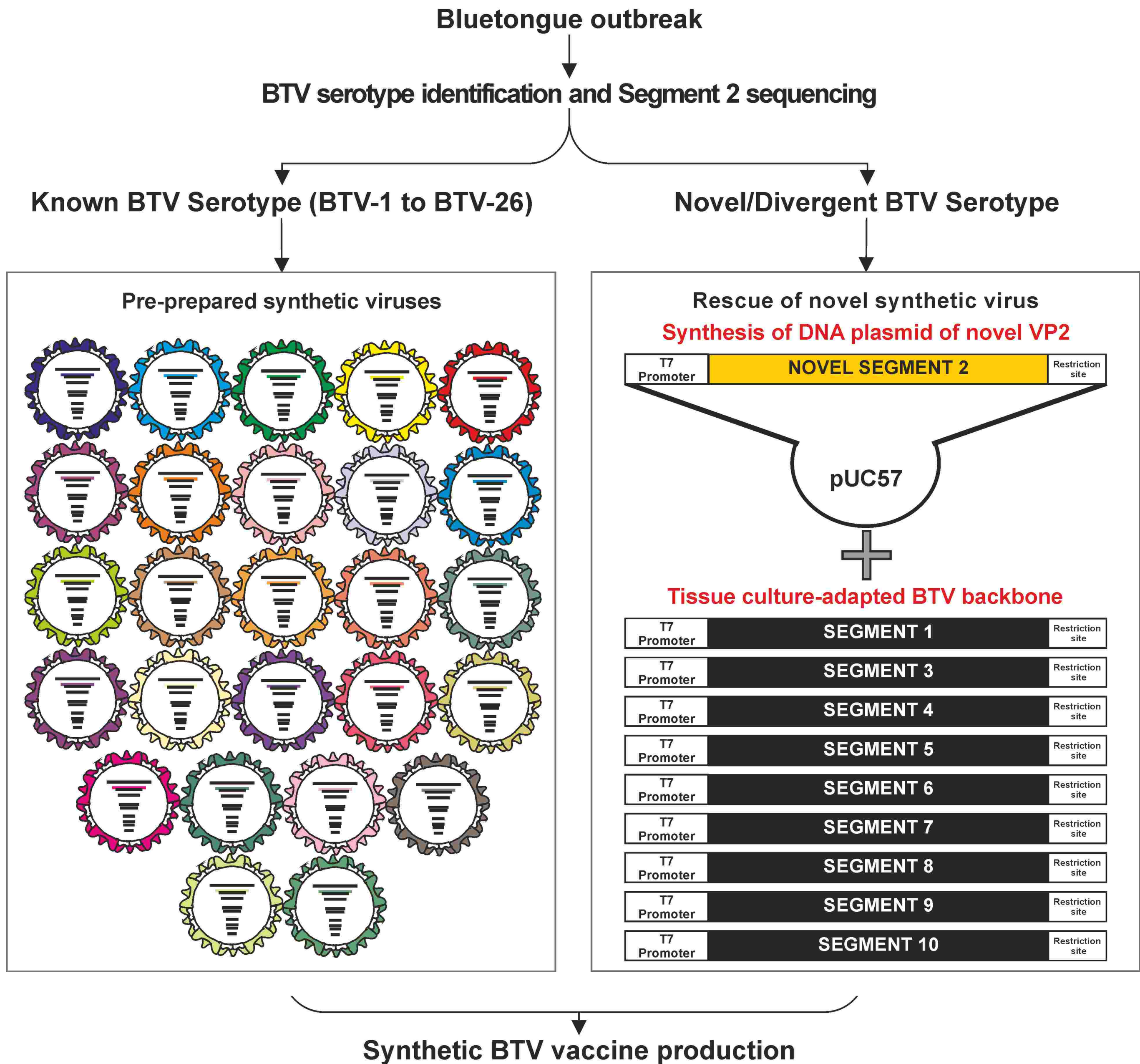
A



B

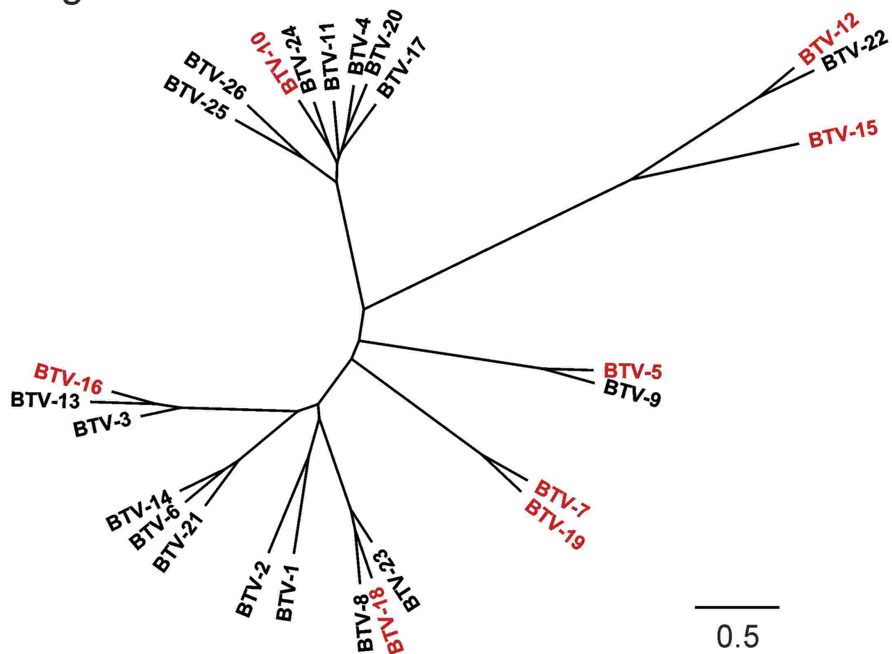


JVI Accepts published online ahead of print

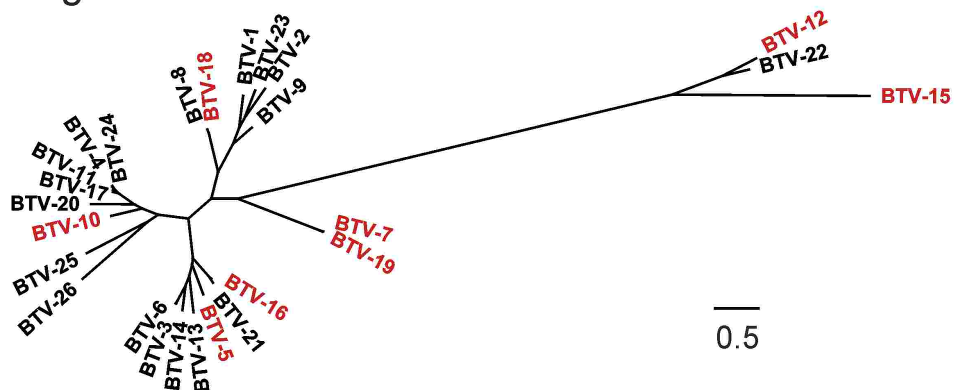


JVI Accepts published online ahead of print

Segment 2



Segment 6



Segment 7