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Subject: AchPV paper
Attachments: AchPV Paper CLEAN 08Nov2017.docx; Figure 1.tif; Figure 2.tif; Figure 3.tif

Hello all,
I hope you all had a nice break and are feeling refreshed for a new year!
I've attached the current version of our AchPV paper.
It would be great to get this published somewhere...any thoughts as to what journal we should target next?
Cheers,

enn

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Animal infection studies of two recently discovered African bat paramyxoviruses, Achimota 1 and Achimota 2

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Abstract

Novel emerging zoonotic viruses are one of the greatest threats facing public health. Viral spillover events originating from wildlife account for the majority of newly recognised diseases of people and have important impacts on agriculture and human health globally. Bats are implicated as the natural reservoirs for several highly pathogenic viruses that can infect other animal species, including man. Here, we investigate the potential for two recently discovered bat rubulaviruses, Achimota virus 1 (AchPV1) and Achimota virus 2 (AchPV2), which were isolated from urine collected under urban bat (*Eidolon helvum*) roosts in Ghana, West Africa, to infect small laboratory animals. AchPV1 and AchPV2 are classified in the family *Paramyxoviridae* (which includes the deadly zoonotic henipaviruses) and cluster with other bat derived zoonotic rubulaviruses (i.e. Sosuga, Menangle and Tioman viruses). To assess the susceptibility of AchPV1 and AchPV2 in animals, infection studies were conducted in ferrets, guinea pigs and mice. Seroconversion, immunohistological evidence of infection, and viral shedding were identified in ferrets and guinea pigs, but not in mice. Infection was associated with respiratory disease in ferrets. These results indicate that Achimota viruses have the ability to cross the species barrier and may infect domesticated animals and humans in areas of Africa where infected reservoir hosts are widely distributed. This study supports the strategy for surveillance-based viral discovery, contributes to the understanding of spillover dynamics and highlights the need for outbreak preparedness.

INTRODUCTION

New and emerging viral infections impose a significant burden on human health and on the world economy. The majority of emerging infectious diseases affecting humans today are of animal origin, with approximately three quarters arising from wildlife [1]. Bats have been shown to harbour more zoonotic viruses than other mammalian species [2 & 3] and are implicated in outbreaks of a number of highly pathogenic zoonotic viruses, including filoviruses, coronaviruses, paramyxoviruses and reoviruses. Ebola virus (EboV) RNA and antibodies have been discovered in African fruit bats [4]. The largest human outbreak of EboV occurred in West Africa in 2013-2014, resulting in nearly 30,000 infections and 11,000 deaths and took over a year to contain [5]. SARS coronavirus emerged in China in 2002 and infected over 8000 people causing 774 deaths [6] and the MERS coronavirus continues to infect people and cause death in the Middle East [7]. Bats have since been found to harbour a multitude of coronaviruses closely related to SARS and MERS [8 & 9]. Pteropid bats are the reservoir hosts for the deadly henipaviruses, Nipah virus (NiV) and Hendra virus (HeV) [10 & 11]. NiV continues to cause fatal encephalitis in humans almost annually in Bangladesh, while HeV has spilled-over into horses in Australia nearly every year since 2004 and has killed four people [12 & 13]. Orthoreoviruses have been isolated from bats and humans in Southeast Asia where they have caused flu-like illness in people [14]. Known zoonotic viruses of bat origin continue to be of concern for human and animal health, and active surveillance provides our best option for monitoring these agents as well as identifying novel pathogens of zoonotic potential.

70 The recently discovered rubulaviruses, Achimota virus 1 and Achimota virus 2 (AchPV1
71 and AchPV2), were isolated from bat (*Eidolon helvum)* urine samples collected beneath
72 urban bat roosts in Ghana, West Africa [15]. AchPV1 and AchPV2 are newly recognised
73 viral species in the family *Paramyxoviridae*, where they cluster with other bat
74 rubulaviruses. Despite being discovered in the same study, the AchPVs are not nearest-
75 phylogenetic relatives and share only 31 to 64% protein amino acid identities [15]. Their
76 relationship to each other is similar to their relationships with other bat rubulaviruses
77 such as Sosuga (SosPV), Menangle (MenPV) and Tioman (TioPV) viruses (sharing 58-
78 70% N protein amino acid sequence identities), which have been shown to cause human
79 infection. SosPV was isolated from a wildlife biologist studying bats and rodents in
80 Africa in 2012 and is believed to be the causative agent for a severe flu-like illness and
81 skin rash [16]. Follow up investigations revealed the presence of this virus in the spleen
82 of *Rousettus aegyptiacus* bats [17]. MenPV first emerged in a piggery in NSW, Australia,
83 in 1997 causing reproductive disease in pigs [18]. Two piggery workers had flu-like
84 illness during the outbreak and were later found to have MenPV neutralising antibodies.
85 Serological evidence of MenPV infection was also found in flying foxes roosting near the
86 piggery and the virus was later isolated from *Pteropus alecto* urine [18 & 19]. TioPV was
87 isolated from pteropid bat urine on Tioman Island in 2001 during the search for the
88 reservoir host of Nipah virus [20]. It was later found that humans on the island had
89 neutralising antibodies to TioPV, although no associated disease has been reported [21].
90
91 Based on what is known of these closely related bat rubulaviruses, the potential of
92 AchPV1 and AchPV2 to infect and cause disease in other species is worthy of further

investigation. Serological surveys of *Eidolon helvum* populations in Africa have been conducted for Achimota viruses and have shown a widespread presence of neutralising antibodies [15]. In addition, a survey of human sera collected from Ghana and Tanzania detected AchPV2 neutralising antibodies in three of 442 samples tested, however no neutralising antibodies to AchPV1 were detected in these sera. Two of the antibody positive samples were from healthy adults and one was from a febrile paediatric patient [15]. These data suggest that AchPV2 is zoonotic, but whether AchPV1 is zoonotic remains unknown.

To further investigate the infection potential of AchPV1 and AchPV2, we conducted studies in three species of small laboratory animal; ferret (*Mustela putorius furo*), guinea pig (*Cavia porcellus*) and mouse (*Mus musculus domesticus*). First, we conducted observational studies to determine the susceptibility of these animals to infection by AchPV1 or AchPV2. Second, time course studies were performed using AchPV2 to obtain data on viral replication sites and potential routes of transmission.

RESULTS

Observational study with AchPV1 and AchPV2 in ferrets

Two adult male ferrets aged 11 – 13 months were given 10^5 $TCID_{50}$ AchPV1 oronasally in 1 ml of inoculum and another two adult male ferrets aged 11 – 13 months were given 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum. The animals were observed daily for clinical signs and then electively euthanased at 21 days post challenge (pc).

One of the two ferrets exposed to AchPV1 remained clinically well and was electively euthanased at the end of experiment on day 21 pc. The other ferret showed signs of upper respiratory tract infection (sneezing, coughing) and weight loss from day 1 pc, and was euthanased at day 14 pc when it had reached a predetermined humane endpoint of 10 % bodyweight loss. Post mortem examination revealed a pleural effusion and bronchopneumonia of the right intermediate lung lobe. Each ferret developed neutralising antibody against AchPV1, with titres of 1:320 (healthy) and 1:80 (ill) (Table 1).

One of two ferrets exposed to AchPV2 remained clinically well and was electively euthanased at the end of the experiment on day 21 pc. The other ferret maintained normal play activity but showed signs of upper respiratory tract infection (sneezing, purulent nasal discharge) from day 3 pc and which resolved by day 11 pc. The animal was electively euthanased at the end of the experiment on day 21 pc. Each ferret developed neutralising antibody against AchPV2, with titres > 1:1280 (healthy) and 1:1280 (ill/recovered) (Table 1).

In summary, it is unclear whether the signs of respiratory tract disease in one of two ferrets given either AchPV1 or AchPV2 were attributable to infection by the challenge virus or by co-infection of an unknown pathogen. As higher neutralising antibody titres were observed in ferrets infected with AchPV2, and as there was prior serological evidence of this virus in people, AchPV2 was selected for a time-course study with ferrets.

Observational study with AchPV1 and AchPV2 in guinea pigs

Four adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV1 oronasally in 1 ml of inoculum and another four adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum. The animals were observed daily for clinical signs and then electively euthanased at 21 days pc.

All four guinea pigs exposed to AchPV1 remained clinically well and were electively euthanased on day 21 pc. Each guinea pig developed neutralising antibody against AchPV1, with titres of 1:320, 1:80, 1:80 and 1:40 (Table 1). Similarly, all four guinea pigs exposed to AchPV2 remained clinically well and were electively euthanased on day 21 pc. Each guinea pig developed neutralising antibody against AchPV2, with titres of 1:320, 1:160, 1:80 and 1:40 (Table 1).

Based on the same rationale as for ferrets, AchPV2 was selected for a time-course study with guinea pigs.

Observational study with AchPV1 and AchPV2 in mice

Ten mice (five female Balb-C mice aged 12 weeks and five female BalbC mice aged over 12 months) were given 10^3 $TCID_{50}$ AchPV1 intranasally in 50 µl of inoculum and another ten mice (five female Balb-C mice aged 12 weeks and five female BalbC mice aged over 12 months) were given 10^3 $TCID_{50}$ AchPV2 intranasally in 50 µl of inoculum. The animals were observed daily for clinical signs and then electively euthanased at 21 days pc.

All ten mice exposed to AchPV1 remained clinically well and were electively euthanased on day 21 pc. Neutralising antibody against AchPV1 was not detected in any mouse. Likewise, all ten mice exposed to AchPV2 remained clinically well and were electively euthanased on day 21 pc. Neutralising antibody against AchPV2 was not detected in any mouse. As mice had no detectable signs of disease and did not seroconvert to either AchPV1 or AchPV2, no further studies were conducted with mice.

Time course study with AchPV2 in ferrets

1. Clinical and Pathological findings

For this study, eight adult female ferrets were given 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum and then two animals were pre-allocated for euthanasia on each of days 6, 8, 10 and 21 pc. All eight ferrets in this study showed a mild but significant increase in rectal temperature over baseline on day 4 pc ($p = 0.02$), and a mild but significant loss of bodyweight compared to baseline on days 4 ($p = 0.03$) and 5 ($p = 0.0004$) pc. Otherwise, the animals remained clinically well until elective euthanasia, apart from one of two ferrets scheduled for euthanasia on day 8 pc. This ferret (#9) showed signs of upper respiratory tract infection (sneezing, serous and then purulent nasal discharge) between days 2 and 6 pc and was euthanased on humane grounds on day 6 pc following markedly decreased play activity. Other than ferret #9, no significant gross abnormalities were observed at post mortem examination in any of the ferrets.

The three ferrets euthanased on day 6 pc had minor histopathological changes associated with viral replication. Ferret #12 (day 6 pc) showed very mild acute bronchiolitis. Immunohistochemistry revealed viral antigen in germinal centres of the retropharyngeal lymph node but not in other tissues. In ferret #14 (day 6 pc), significant histopathological changes were confined to mild acute tonsillitis, and viral antigen was detected in the tonsillar and pharyngeal epithelium, retropharyngeal lymph node (particularly the parafollicular areas), bronchial epithelial cells, bronchus-associated lymphoid tissues (BALT), perivascular spindle cells in lung, and germinal centres and periarteriolar lymphoid sheaths of the spleen. In ferret #9, euthanased on day 6 pc with respiratory disease, there was moderately severe acute bronchiolitis, hyperplasia of the BALT, excess mucus production by bronchial glands, and focal lipoid pneumonia consistent with chronic bronchial disease. Post mortem examination of ferret #9 also revealed marked nodular hyperplasia of the liver with hepatic steatosis, but this lesion was considered to be unrelated to virus exposure as no AchPV2 viral antigen was detected in the liver.

In ferret #9, AchPV2 viral antigen was identified in tonsillar and pharyngeal epithelium, germinal centres, parafollicular area and medulla of the retropharyngeal lymph node, tracheal epithelium, bronchial and bronchiolar epithelium (Fig. 1), BALT and perivascular connective tissues of the lung, bronchial and mediastinal lymph node, periarteriolar lymphoid sheaths and red pulp of the spleen, mononuclear cells in the intestinal lamina propria and cells either within or lining the hepatic sinusoids.

206 In the single ferret euthanased on day 8 pc (ferret #16), there was mild focal acute
207 tracheitis and bronchiolitis. The distribution of viral antigen was similar to ferret #9, with
208 the addition of occasional bile duct epithelial cells and mononuclear cells of the portal
209 triads, gut associated lymphoid tissue (GALT), and transitional epithelial cells in the
210 bladder.
211
212 The two ferrets killed on day 10 pc showed only small amounts of viral detection by
213 immunohistochemistry. Ferret #11 (day 10 pc) had mild focal acute bronchiolitis, and
214 detection of viral antigen was limited to small amounts in tonsillar lymphoid tissue,
215 bronchiolar epithelial cells, periarteriolar lymphoid sheaths of the spleen, and a diffuse
216 scattering throughout the retropharyngeal lymph node. Ferret #13 (day 10 pc) also
217 showed very mild acute bronchiolitis, with AchPV2 viral antigen confined to scattered
218 gastric epithelial cells, GALT, and sparse deposits throughout tonsillar lymphoid tissue,
219 bronchial and retropharyngeal lymph nodes.
220
221 Of the two ferrets killed on day 21 pc, one (ferret #15) had very mild acute bronchiolitis
222 and tracheitis and one had no detectable lesions. AchPV2 viral antigen was not detected
223 in any tissue from either of these two ferrets.
224
225 ***2. Detection of viral genomes***
226 AchPV2 RNA was detected by RT-qPCR in the oral swabs of 4 ferrets on day 2 pc and in
227 all ferrets by day 4 pc until euthanasia: the highest levels were typically recorded on day
228 6 or 8 pc (Table 2). Similar results were seen for the nasal washes: viral RNA was

229 detected in 3 ferrets on day 2 pc, and in all ferrets by day 4 pc until euthanasia, the
230 highest levels typically were recorded on day 6 or 8 pc. Rectal swabs first detected
231 AchPV2 on day 4 pc, when all ferrets were positive, the highest levels occurred on day 6
232 or 8 pc. Viral RNA was commonly detected in blood samples from days 2 to 21 pc.
233 Where viral RNA was found in successive blood samples from individual animals,
234 highest levels were recorded on day 6 or 8 pc (Table 2).
235
236 All tissue samples analysed from ferrets #9, #12 and #14 euthanased on day 6 pc were
237 positive for viral RNA (data summarised in Fig. 2), with the highest levels in bronchial
238 and retropharyngeal lymph nodes and the lowest levels in heart, kidney and brain. Each
239 tissue sample tested from ferret #16 (euthanased 8 days pc) was also positive for viral
240 RNA, with the highest reading in retropharyngeal lymph node. On day 10 pc, ferrets #11
241 and #13 exhibited generally similar distribution and quantities of viral RNA to the
242 animals above. However, on day 21 pc the levels of viral RNA in ferrets #10 and #15
243 were substantially lower, and largely limited to the retropharyngeal and bronchial lymph
244 nodes and the spleen.
245
246 ***3. Virus isolation***
247 Virus was reisolated from the nasal wash and from the oral and rectal swabs of ferret #9
248 on day 6 pc, the nasal wash of ferret #10 on day 6 pc, the oral swab of ferret #11 on day 6
249 pc, and from the oral swabs of ferret #16 on days 6 and 8 pc, plus the nasal wash of this
250 ferret on day 8 pc (Table 2).
251

252 Virus was reisolated from ferrets killed on day 6 pc from tonsil, bronchial and
253 retropharyngeal lymph nodes, lung and brain (ferret #9); trachea, tonsil, bronchial and
254 retropharyngeal lymph nodes, and bladder (ferret #12); and tonsil and bronchial and
255 retropharyngeal lymph nodes (ferret #14) (Fig. 2). Virus was reisolated from tonsil and
256 trachea of ferret #16 (which was killed on day 8 pc) and from the retropharyngeal lymph
257 node and kidney of ferret #13 (day 10 pc), but not from ferret #11 (day 10 pc). Virus re-
258 isolation from ferrets killed on day 21 pc was limited to the retropharyngeal lymph node
259 of ferret #15.
260
261 ***4. Serology***
262 No neutralising antibodies were observed at day 2 or 4 pc but by day 6 low levels of
263 neutralising antibodies were detected in the ferrets (Table 4). They showed an increase in
264 neutralising antibody titre across the time points, with the animals bled at day 21 pc
265 having neutralising antibody titres > 1:1280.
266
267 **Time course study with AchPV2 in guinea pigs**
268 ***1. Clinical and Pathological findings***
269 For this study, eight adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV2 oronasally
270 in 1 ml of inoculum and then two animals were pre-allocated for euthanasia on each of
271 days 6, 8, 10 and 21 pc. In guinea pigs, there were no significant differences in
272 temperature or bodyweight over baseline up to day 6 pc, and the animals remained
273 clinically healthy until elective euthanasia. No significant gross abnormalities were
274 observed at post mortem examination, apart from enlarged bronchial lymph nodes in one

guinea pig euthanased on day 6 pc. The only histopathological changes observed were: mild acute tracheitis in all animals, two animals with mild acute bronchitis and/or bronchiolitis, and four with mild chronic interstitial pneumonia attributable to inhalation of plant material. In contrast to the observations in ferrets, the pattern of respiratory tract lesions did not correlate with the time post-exposure to AchPV2; very few histopathological changes were observed and all sections of tissues from all guinea pigs were negative for AchPV2 antigen by immunohistochemistry.

2. Detection of viral genomes

Low levels of AchPV2 were detected in the oral swab of one guinea pig on day 6 pc and of another on day 8 pc (Table 3). Rectal swabs were positive in four of six guinea pigs on day 8 pc, and from one guinea pig on day 10 pc. Viral RNA was found in the blood of one guinea pig on day 6 pc and of another on day 8 pc (both of which had viral RNA-positive oral swabs at these times).

Most tissue samples analysed from guinea pigs euthanased on day 6 and 8 pc were positive for viral RNA (data summarised in Fig. 3), with highest levels present in nasal turbinates, bronchial and retropharyngeal lymph nodes, and spleen, and lower levels in trachea, lung, and liver. Detection was lowest and inconsistent from heart, kidney and brain. By day 10 pc, viral genome detection was limited to bronchial and/or retropharyngeal lymph nodes, lung, spleen, and nasal turbinates (one of two animals). On day 21 pc, one guinea pig was negative by RT-qPCR for all tissues; in the other, viral genome was detected only in bronchial lymph node and spleen.

3. Virus isolation

Virus was not reisolated from any of the clinical samples, including those that were positive by AchPV2-specific RT-qPCR (Table 3). In addition, virus was not reisolated from any tissue sample, including those that were positive by AchPV2-specific RT-qPCR (Fig. 3).

4. Serology

No neutralising antibodies were observed at day 2 or 4 pc but by day 6 low levels of neutralising antibodies were detected in the guinea pigs (Table 4). There was a slight increase in neutralising antibody titre across the time points with a titre of 1:160 by day 21 pc.

DISCUSSION

We investigated the potential of the two recently discovered bat rubulaviruses, AchPV1 and AchPV2, to infect laboratory animals representing three species: ferret, guinea pig and mouse. Seroconversion to both Achimota viruses in ferrets and guinea pigs indicated these animals were susceptible to infection, however mice did not seroconvert to either virus. Due to their body size, mice were given a lower dose of inoculum, and were challenged intranasally, rather than via the oronasal route used for the ferrets and guinea pigs. The difference in volume and inoculation route may account for the lack of seroconversion seen in the mice, however it is probably more likely that this species is resistant to infection. Higher levels of neutralising antibodies were observed in the ferrets

321 and guinea pigs infected with AchPV2 compared to those infected with AchPV1. This
322 result reflected *in vitro* data where it was observed previously that AchPV2 consistently
323 grows to a higher titre than AchPV1 in vero and PaKi cell lines [15]. Additionally,
324 respiratory tract disease in one of two ferrets given either AchPV1 or AchPV2 was seen
325 in the observational studies, although it remains unclear if this was related to Achimota
326 virus infection or was entirely due to co-infection by an unknown pathogen. AchPV2 was
327 chosen for a time-course study based on two criteria: higher neutralising antibodies
328 observed in ferrets and guinea pigs compared to AchPV1, and previous evidence of
329 human infection (AchPV2 neutralising antibodies). Given more time and resources, it
330 would be worthwhile to do an additional time course study with AchPV1, to further
331 investigate the differences between these two viruses.
332
333 The AchPV2 time-course studies provided additional evidence that this virus can infect
334 ferrets and guinea pigs and revealed viral replication sites and potential routes of
335 transmission. Evidence of infection was supported by virus re-isolation from clinical
336 specimens and post-mortem tissue samples, and viral antigen detection in tissues by
337 quantitative real-time PCR and immunohistochemistry. Although there was evidence of
338 mild malaise in infected ferrets (raised body temperature and weight loss), a distinct
339 clinical syndrome with specific clinical signs was not identified in either ferrets or guinea
340 pigs. Moreover, no histological lesions were attributed with confidence to infection by
341 AchPV2. Mild tonsillitis, tracheitis and bronchiolitis were recorded in ferrets, but in some
342 animals the lesions were identified without evidence of specific association with AchPV2
343 antigen. The ferrets were sourced from a colony free of influenza and canine distemper

344 virus, were clinically healthy at the time of exposure to AchPV2, and were not
345 maintained on a particulate substrate. The pathogenesis of these legions remains
346 uncertain, although an opportunistic bacterial or other viral aetiology could not be
347 excluded. In other tissues, such as bile duct epithelium and transitional epithelial cells of
348 the bladder, viral antigen was seen without substantial inflammatory reaction or tissue
349 injury.
350
351 For ferrets, following an incubation period of 5 to 6 days, AchPV2 was shed in oral and
352 nasal secretions and the development of virus neutralising antibody was generally
353 associated with virus clearance. The results of quantitative real-time PCR, virus re-
354 isolation and immunohistochemistry taken together identified the major sites of AchPV2
355 replication in ferrets to be respiratory tract epithelium and associated lymphoid tissues.
356 Although virus was not re-isolated from blood, the development of viremia may be
357 inferred by confirmation of infection within spleen and urinary tract epithelium. In the
358 absence of other clinico-pathological support for CNS infection, virus in blood may also
359 account for the re-isolation of AchPV2 from one sample of ferret brain tissue.
360
361 Although exposure to AchPV2 resulted in production of neutralising antibodies in guinea
362 pigs, antibody titres were much lower than for the ferrets. Patterns of detection of viral
363 RNA from guinea pigs were generally similar to those in ferrets, but virus was not
364 recovered from guinea pigs and viral antigen was not demonstrated in their tissues. The
365 sites of AchPV2 replication in guinea pigs, therefore, could not be determined with

366 confidence. Our observations suggest that guinea pigs are less permissive to AchPV2
367 infection than ferrets.
368
369 When assessing the spill-over potential and working up an animal model for a novel
370 virus, it is important to use animals from more than one species. There is no reliable
371 method to determine the best species simply by characterising the virus, which is a big
372 limitation of using virus discovery as a stand-alone surveillance strategy for zoonotic
373 pathogen discovery. Therefore, it is only possible to do what is practical and feasible in
374 terms of assessing potential spill-over hosts. Small laboratory animals such as ferrets,
375 guinea pigs and mice, representing different mammalian orders or families, offer the most
376 practical advantages for testing spill-over potential and, in this study, were a panel that
377 demonstrated discriminatory power for the infection potential of the novel viruses tested.
378
379 The continued search for novel viruses in wildlife species, particularly in regions of the
380 world where encroachment of humans and livestock into wildlife habitats is increasing,
381 such as sub-Saharan Africa, is imperative if we are going to be able to identify disease in
382 these regions caused by novel pathogens. New discoveries of wildlife viruses alone,
383 however, will not inform risks to livestock or public health. Viral phylogeny and other
384 signals of spill-over potential, such as the serosurveillance results that guided this study,
385 are required to identify potential new health threats [22]. The Achimota viruses described
386 in this paper demonstrate ability to cross the species barrier and may be causing
387 undiagnosed disease in domesticated animals and humans within the wide geographical
388 range of the bat reservoir species, *Eidolon helvum*.

389

390 **MATERIALS AND METHODS**

391 **Animals, accommodation, handling and biosafety**

392 Ferrets were acquired from a colony free of infection by influenza H1 and H3 subtypes.
393 Two male ferrets aged 11 13 months, four female guinea pigs, five female Balb-C mice
394 aged 12 wks, and five female BalbC mice aged over 12 months were used in each of the
395 AchPV1 and AchPV2 observational studies. Eight female ferrets and eight female guinea
396 pigs were used for the AchPV2 time course study. The animal husbandry methods and
397 experimental design were endorsed by the CSIRO Australian Animal Health
398 Laboratory's Animal Ethics Committee (approvals AEC 1608 and AEC 1621). Animals
399 were housed at BSL-3 in conventional caging systems to facilitate the expression and
400 monitoring of natural behaviours, given complete premium dry food appropriate to the
401 species, dietary treats, and provided with water *ad libitum*. Room temperature was
402 maintained at 22°C with 15 air changes per hour; and humidity varied between 40 and
403 60%. Before manipulation such as exposure to virus, collection of clinical samples, or
404 euthanasia, animals were immobilised with a mixture of ketamine HCl (Ketamil®: 5
405 mg/kg in ferrets, 16mg/kg in guinea pigs, 75mg/kg in mice) and medetomidine
406 (Domitor®: 50 μg/kg in ferrets, 20 μg/kg in guinea pigs, 1mg/kg in mice) by
407 intramuscular or intraperitoneal (mice) injection. Where indicated, reversal was achieved
408 with atipamazole (Antisedan®) administered by intramuscular (ferrets) or intraperitoneal
409 (guinea pigs and mice) injection at 50 % of the medetomidine volume. All animals were
410 implanted subcutaneously with temperature-sensing microchips (Lifechip®). Staff wore

411 powered air purifying respirators, coveralls, impervious gloves and boots while in animal
412 rooms.
413
414 **Animal infections and sampling**
415 For the observational studies, animals were exposed to either AchPV1 or AchPV2,
416 isolated, grown and titrated in vero cells. After initial virus isolation, a parent stock of
417 each virus was grown in vero cells. These parent stocks were then purified by three
418 rounds of limiting dilution in vero cells. Finally, an animal inoculation stock was
419 prepared from the third limiting dilution, resulting in a passage number of 6 times in vero
420 cells from original isolation. The sequence of the animal inoculation stock was not
421 compared to the original sequence of the isolated virus. Ferrets and guinea pigs were
422 given 10^5 $TCID_{50}$ oronasally in 1 ml of inoculum (500 µl oral and 500 µl nasal), and mice
423 were given 10^3 $TCID_{50}$ intranasally in 50 µl of inoculum. General clinical observations
424 were documented daily prior to as well as post challenge (pc). Animals were weighed and
425 their temperatures recorded daily. Animals were euthanased at either a predetermined
426 humane endpoint or 21 days pc. Blood was collected for serology prior to virus exposure
427 and at euthanasia. Tissues were not collected for the observational studies.
428
429 For the subsequent time course studies, ferrets and guinea pigs were exposed oronasally
430 to 10^5 $TCID_{50}$ AchPV2, prepared as described above, in 1 ml of inoculum (500 µl oral
431 and 500 µl nasal). Two animals were pre-allocated for euthanasia on each of days 6, 8, 10
432 and 21 pc. Nasal washes (ferrets only), oral and rectal swabs and blood samples, both in
433 EDTA and for serum preparation, were collected from all available animals at days 2, 4,

6, 8, 10 and 21 pc. Clinical samples were collected into tubes containing PBS with antibiotic-antimycotic (Invitrogen) for virus isolation and into tubes containing MagMAX viral lysis buffer (Ambion) for RNA extraction. While under anaesthesia, rectal temperatures of ferrets were recorded by digital thermometer.

At post mortem examination of animals used for the AchPV2 time course study, the following tissues were collected for histology, immunohistochemistry, viral genome detection and virus isolation: nasal turbinates, tonsil, retropharyngeal lymph node, trachea, lung, hilar lymph node, bronchial lymph node, spleen, heart, kidney, liver, bladder and brain. Stomach, small and large intestine, pancreas, adrenal gland, ovary and uterus were also collected for histology and immunohistochemistry. Tissues were collected into tubes containing either neutral buffered 10% formalin (for histology and immunohistochemistry) or PBS plus antibiotic-antimycotic (Invitrogen) and homogenisation beads, homogenised using a bead beater, and clarified by centrifugation (for virus isolation or viral RNA detection).

RNA extraction and Reverse Transcriptase-quantitative Polymerase Chain Reaction

For viral genome detection, RNA was extracted from tissue, blood and swab samples using the MagMAX viral RNA isolation kit (Ambion) following the manufacturers guidelines. A novel Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR), was designed that specifically targets the nucleoprotein gene (N-gene) of AchPV2. For the design process, the N-gene sequence of AchPV2 (JX051320), as well as

457 other closely related paramyxoviruses including SosPV, MenPV and TioPV, was
458 retrieved from GenBank. Subsequently, sequence alignments were performed using
459 Geneious software (Version 8.1, Biomatters). Potential primer and probe regions
460 distinctive of AchPV2 were identified from these alignments and candidate primers and
461 probes assessed using the Primer Express 3.0.1 program (Thermofisher-Applied
462 Biosystems). An assay targeting the 625-700 bp region of AchPV2 (JX051320), consists
463 of forward primer: D-715 (5'-GCAGGTCTGGATCACAGTATGC -3'), reverse primer
464 D-716 (5'-TGCCAGTCGCCTCTCATCT -3'), and probe
465 D-717 (5' [FAM]-TGCATGACAGCATATGATCAGCCCACT-[BHQ-1]-3'. The
466 optimized primer and probe concentrations and assay conditions were as follows: forward
467 primer (D-715) and reverse primer (D-716): 300 nM, probe (D-716): 200 nM. Reactions
468 were performed using AgPath-ID One-Step RT-PCR Kit (Thermofisher-Ambion) on an
469 AB7500 Fast instrument using the thermal cycle: 1 cycle of 45 °C 10 min, 95 °C 10 min
470 followed by 45 cycles of 95 °C 15 sec, 60 °C 45 sec. For interpretation of results
471 duplicate samples producing an average cycle threshold (Ct) less than 38 were considered
472 positive.
473
474 **Virus isolation**
475 Vero cell monolayers were grown in 96 well tissue culture plates to 80 % confluency in
476 cell media (Minimal Essential Medium containing Earle's salts and supplemented with 2
477 mM glutamine, antibiotic-antimycotic and 10 % fetal calf serum).
478 Swab media and blood were serially diluted 10 fold and 50 µl added to each well.
479 Supernatant from centrifuged tissue homogenate was serially diluted 10 fold and 50 µl

added to each well. Vero cell monolayers were observed for viral CPE seven days post infection.

Serology

Serum was collected prior to viral challenge and again at euthanasia, and tested using a standard virus neutralisation test. Serial two-fold dilutions of test sera were prepared in duplicate in a 96-well tissue culture plate in 50 µL cell media (Minimal Essential Medium containing Earle's salts and supplemented with 2 mM glutamine, antibiotic-antimycotic and 10 % fetal calf serum). An equal volume of either AchPV1 or AchPV2 working stock containing 200 $TCID_{50}$ was added and the virus-sera mix incubated for 30 min at 37 °C in a humidified 5 % CO_2 incubator. 100 µL of Vero cell suspension containing 2 x 10^5 cells/mL was added and the plate incubated at 37 °C in a humidified 5 % CO_2 incubator. The plate was observed for viral CPE after seven days and the serum neutralisation titre determined.

Histology and immunohistochemistry

Formalin-fixed tissues were processed into paraffin wax and prepared into 4 µm thick sections using routine histological methods. For immunohistochemistry, antigen retrieval was performed using the DAKO PT LINK machine (Dako, Glostrup, Denmark) by heating the tissue sections to 97 °C for 30 minutes and then cooling to 70 °C in the Envision Flex Target high pH retrieval solution (DAKO) and washing for 5 minutes in Tris Buffer. After this, endogenous peroxidases were quenched by the addition of 3 % H_2O_2 solution. Tissue sections were then incubated with the primary antibody, polyclonal

503 rabbit antisera raised against AchPV2, at a dilution of 1:2000. The visualization system
504 used was Envision FLEX /horseradish peroxidase (HRP) conjugated with 3-Amino-9-
505 Ethylcarbazole (AEC) chromogen (DAKO AEC + substrate chromagen K3469). Slides
506 were then counterstained with Lillie-Mayer haematoxylin (Australian Biostain,
507 Traralgon, Australia) and Scotts tap water before mounting. A duplicate set of tissue
508 sections were stained with hematoxylin and eosin stain for histological examination using
509 routine methods.
510
511 **Statistical analysis**
512 In the time-course study, bodyweights and rectal temperatures of ferrets, and
513 bodyweights and microchip temperatures of guinea pigs, up to and including day 6 pc
514 were compared using a repeated measures ANOVA followed by Dunnett's multiple
515 comparisons test (GraphPad Prism 7.02).
516
517 **Funding Information**
518 KSB is funded by a Wellcome Trust Clinical Career Development Fellowship
519 (106690/A/14/Z). AAC was part-funded by a Royal Society Wolfson research merit
520 award. L-FW is funded in part by the NRF-CRP grant (NRF2012NRF-CRP001-056).
521
522 **Acknowledgments**
523 We thank Jessica Haining, Sarah Riddell and Rachel Arkinstall from the AAHL Animal
524 Studies Team for their help with the animal trials.
525

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The animal husbandry methods and experimental design were endorsed by the CSIRO Australian Animal Health Laboratory's Animal Ethics Committee (approvals AEC 1608 and AEC 1621).

Abbreviations

AchPV1: Achimota paramyxovirus 1

AchPV2: Achimota paramyxovirus 2

BALT: Bronchus-associated lymphoid tissue

BSL: Biosafety Level

CNS: Central nervous system

CSIRO: Commonwealth Scientific and Industrial Research Organisation

Ct: Cycle threshold

CPE: Cytopathic effect

EboV: Ebola virus

GALT: Gut associated lymphoid tissue

HeV: Hendra virus

MERS: Middle East Respiratory Syndrome

MenPV: Menangle paramyxovirus

NA: Not available

549 NiV: Nipah virus

550 NSW: New South Wales

551 Pc: Post challenge

552 PaKi: *Pteropus alecto* kidney

553 SARS: Severe Acute Respiratory Syndrome

554 SosPV: Sosuga paramyxovirus

555 SNT: Serum neutralisation test

556 TioPV: Tioman paramyxovirus

557

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635
636 **Figure legends**
637

Figure 1. Viral antigen in bronchiolar epithelial cells and BALT in ferret #9 (polyclonal rabbit anti-AchPV2): note also intraluminal acute inflammatory infiltrate, of uncertain pathogenic significance.

Figure 2. Analysis of virus infection in ferrets by RNA detection and virus isolation. Average cycle threshold (Ct) values were obtained from testing tissues from AchPV2 ferrets using RT-qPCR. Stars indicate samples that AchPV2 was re-isolated from.

Figure 3. Analysis of virus infection in guinea pigs by RNA detection and virus isolation. Average cycle threshold (Ct) values were obtained from testing tissues from AchPV2 guinea pigs using RT-qPCR. AchPV2 was unable to be re-isolated from any sample.

Table 1. The serum neutralisation titres against AchPV1 and AchPV2 for ferret and guinea pig serum collected 21 days pc. The serum collected from the animals pre-challenge (day 0) were all negative. Mouse sera were also tested but the data is not shown as they didn't seroconvert.

AchPV1		AchPV2	
Animal	SNT Titre	Animal	SNT Titre
Ferret 1	1:320	Ferret 1	>1:1280
Ferret 2*	1:80	Ferret 2	1:1280
Guinea Pig 1	1:40	Guinea Pig 1	1:160
Guinea Pig 2	1:80	Guinea Pig 2	1:40
Guinea Pig 3	1:320	Guinea Pig 3	1:80
Guinea Pig 4	1:80	Guinea Pig 4	1:320

*This animal became ill and was euthanized at day 14 pc instead of day 21 pc.

657

658 **Table 2.** Analysis of viral shedding and viraemia in ferrets by RNA detection and virus
659 isolation. Average cycle threshold (Ct) values were obtained from testing oral and rectal
660 swabs, nasal washes and blood from AchPV2 ferrets using RT-qPCR.
661 KEY: - indicates sample was negative (Av Ct >38); NA indicates sample was unavailable
662 for testing; * indicates virus re-isolated at neat dilution; ** indicates virus re-isolated at
663 1:5 dilution and *** indicates virus re-isolated at 1:50 dilution

664

		Days Post Challenge						
	Sample	**0**	**2**	**4**	**6**	**8**	**10**	**21**
Ferret 9	Oral Swab	-	-	32.9	24.4 ***			
	Rectal swab	-	-	34.1	24.9 **			
	Nasal wash	-	36.8	33	26 ***			
	Blood	-	-	NA	31.5			
Ferret 12	Oral Swab	-	-	32.10	21.8			
	Rectal swab	-	-	29.50	22.6			
	Nasal wash	-	-	31.40	25.1			
	Blood	-	35.9	26.50	NA			
Ferret 14	Oral Swab	-	36.9	31.90	33			
	Rectal swab	-	-	32.20	24.8			
	Nasal wash	-	-	31.40	27.1			
	Blood	-	37.8	29.10	28.7			
Ferret 16	Oral Swab	-	34.5	32.50	22.2 *	24 *		
	Rectal swab	-	-	32.00	22.6	23		
	Nasal wash	-	-	34.10	28.3	27 **		
	Blood	-	-	31.30	30.1	20.9		
Ferret 13	Oral Swab	-	36	31.50	23.5	24	23.60	
	Rectal swab	-	-	29.50	22.8	23.4	23.70	
	Nasal wash	-	-	33.60	25.9	22.3	24.50	
	Blood	-	-	29.40	29.2	30.8	34.00	
Ferret 11	Oral Swab	-	-	31.9	22.9 ***	22	25.30	
	Rectal swab	-	-	30.9	22	19.6	25.50	
	Nasal wash	-	-	32.00	24.8	22.8	25.00	
	Blood	-	-	28.90	NA	27.3	36.10	

Ferret 15	Oral Swab	-	35	32.90	25.8	20.9	26.50	30
	Rectal swab	-	-	31.30	23.2	22.5	23.70	29
	Nasal wash	-	34.3	34.20	26.3	25	25.30	30.5
	Blood	-	-	24.70	NA	24	36.00	30
Ferret 10	Oral Swab	-	-	30.5	30.3	23.3	27.00	31.2
	Rectal swab	-	-	29	23.6	22.4	27.00	32.5
	Nasal wash	-	37	31.9	25.3 **	24.6	26.30	28.5
	Blood	-	-	-	NA	NA	33.80	-

665

666

667 **Table 3.** Analysis of viral shedding and viraemia in guinea pigs by RNA detection and

668 virus isolation. Average cycle threshold (Ct) values were obtained from testing oral and

669 rectal swabs and blood from AchPV2 guinea pigs using RT-qPCR. AchPV2 was unable

670 to be re-isolated from any sample.

671 KEY: - indicates sample was negative (Av Ct >38)

		Days Post Challenge						
	Sample	**0**	**2**	**4**	**6**	**8**	**10**	**21**
Guinea Pig 1	Oral Swab	-	-	-	-			
	Rectal swab	-	-	-	-			
	Blood	-	-	-	-			
Guinea Pig 2	Oral Swab	-	-	-	37.74			
	Rectal swab	-	-	-	-			
	Blood	-	-	-	35.25			
Guinea Pig 3	Oral Swab	-	-	-	-	36.44		
	Rectal swab	-	-	-	-	35.09		
	Blood	-	-	-	-	34.71		
Guinea Pig 4	Oral Swab	-	-	-	-	-		
	Rectal swab	-	-	-	-	37.31		
	Blood	-	-	-	-	-		
Guinea Pig 5	Oral Swab	-	-	-	-	-	-	
	Rectal swab	-	-	-	-	-	-	
	Blood	-	-	-	-	-	-	
Guinea Pig 6	Oral Swab	-	-	-	-	-	-	
	Rectal swab	-	-	-	-	-	-	

	Blood	-	-	-	-	-		
Guinea Pig 7	Oral Swab	-	-	-	-	-	-	-
	Rectal swab	-	-	-	-	35.39	34.97	-
	Blood	-	-	-	-	-	-	-
Guinea Pig 8	Oral Swab	-	-	-	-	-	-	-
	Rectal swab	-	-	-	-	33.98	-	-
	Blood	-	-	-	-	-	-	-

672

673

674

675 **Table 4.** The serum neutralisation titres against AchPV2 for ferret and guinea pig serum

676 collected on days 6, 8, 10 and 21 pc. Sera collected on day 2 and 4 pc were also tested but

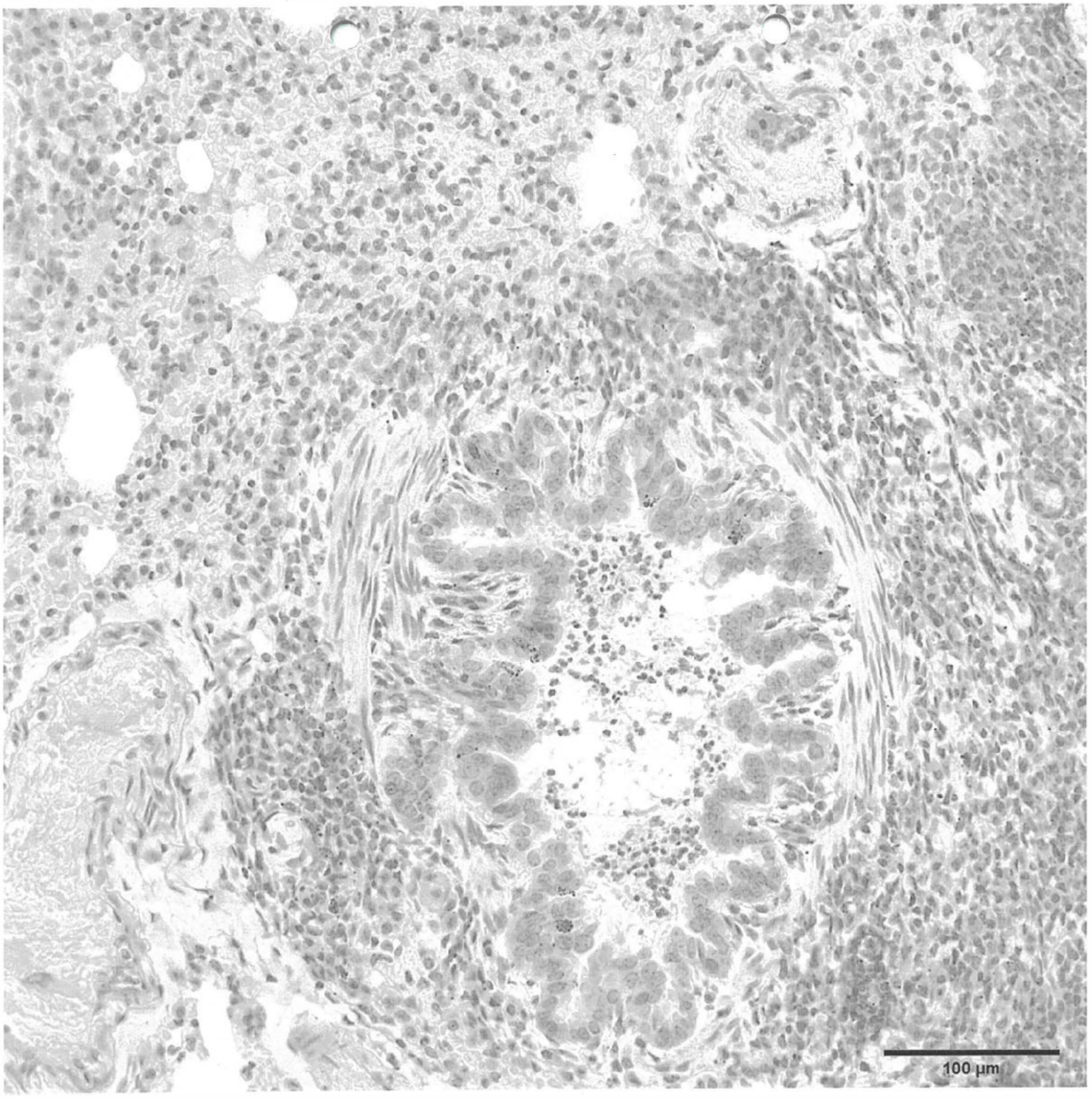
677 were negative for neutralising antibodies (data not shown).

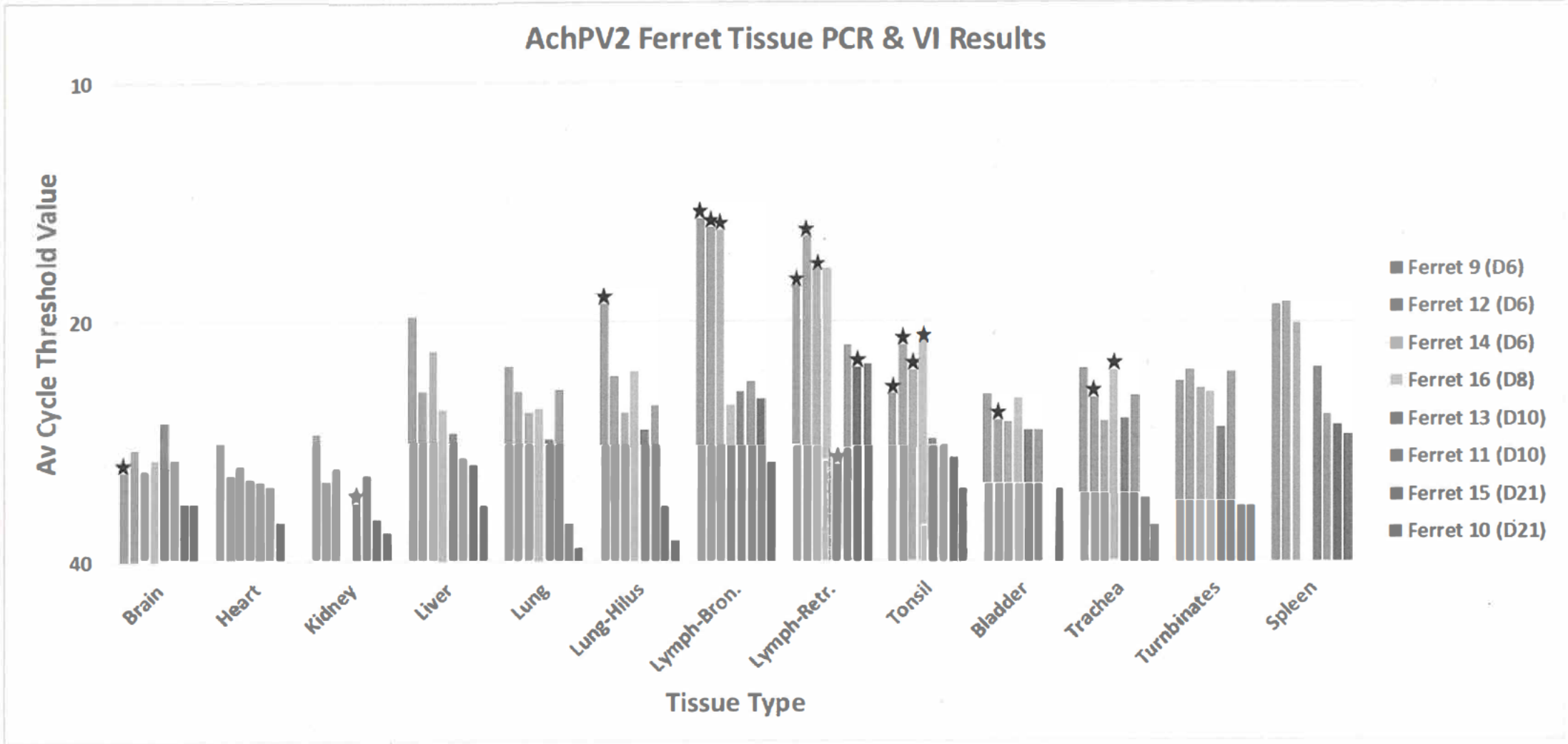
Animal	SNT Titre	Animal	SNT Titre
Ferret 9* (Day6)	1:20	G.Pig 1 (Day6)	1:20
Ferret 12 (Day6)	1:40	G.pig 2 (Day6)	1:20
Ferret 14 (Day6)	1:640	G.Pig 3 (Day8)	1:20
Ferret 16 (Day8)	1:640	G.pig 4 (Day8)	1:20
Ferret 11 (Day10)	1:640	G.Pig 5 (Day10)	1:40
Ferret 13 (Day10)	>1:1280	G.pig 6 (Day10)	1:40
Ferret 10 (Day21)	>1:1280	G.Pig 7 (Day21)	1:160
Ferret 15 (Day21)	>1:1280	G.pig 8 (Day21)	1:160

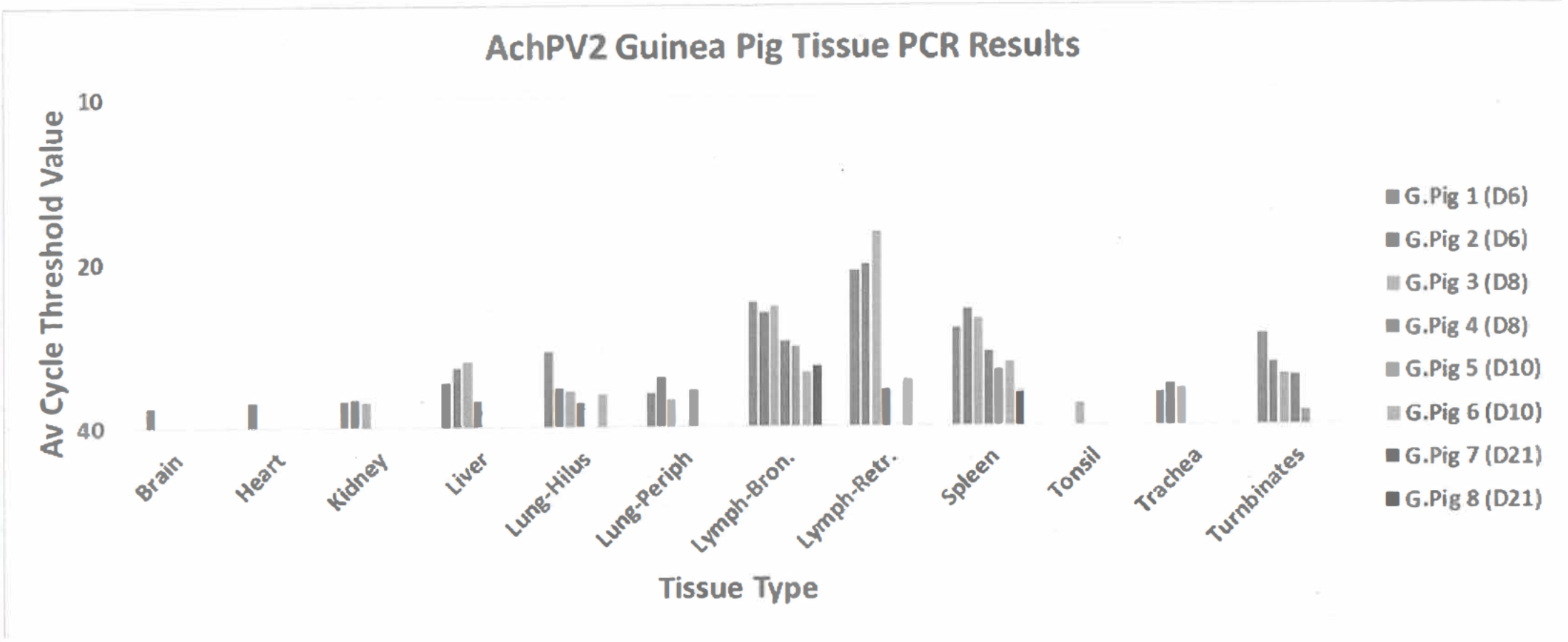
678 *This animal became ill and was euthanized at day 6 pc instead of day 8 pc

679

680


100 µm


AchPV2 Ferret Tissue PCR & VI Results
Av Cycle Threshold Value
10
20
40
Brain
Heart
Kidney
Liver
Lung
Lung-Hilus
Lymph-Bron.
Lymph-Retr.
Tonsil
Bladder
Trachea
Turnbinates
Spleen
Tissue Type
Ferret 9 (D6)
Ferret 12 (D6)
Ferret 14 (D6)
Ferret 16 (D8)
Ferret 13 (D10)
Ferret 11 (D10)
Ferret 15 (D21)
Ferret 10 (D21)


AchPV2 Guinea Pig Tissue PCR Results
Av Cycle Threshold Value
10
20
40
Brain
Heart
Kidney
Liver
Lung-Hilus
Lung-Periph
Lymph-Bron.
Lymph-Retr.
Spleen
Tonsil
Trachea
Turnbinates
Tissue Type
G.Pig 1 (D6)
G.Pig 2 (D6)
G.Pig 3 (D8)
G.Pig 4 (D8)
G.Pig 5 (D10)
G.Pig 6 (D10)
G.Pig 7 (D21)
G.Pig 8 (D21)

[redacted]

From: Andrew Cunningham [redacted]
Sent: Wednesday, 17 January 2018 7:12 PM
To: Jennifer.Bar[redacted]
Cc: linfa.wang[redacted] jlnw2[redacted] Shawn.Todd[redacted]
garycrameri1[redacted] Adam.Foord[redacted] Glenn.Marsh[redacted]
Kate.Baker[redacted] middled35[redacted]
Subject: Re: [EXT]: AchPV paper

Thanks, Jenn

I'm very happy with your proposed way forward.

Cheers

Andrew

Sent from my iPhone

> On 17 Jan 2018, at 00:25, "Jennifer.Barr[redacted]
>
> Thanks everyone for your support and guidance.
> I also agree with James and believe the paper should stand on its own merits!
> If everyone is in agreeance, let's aim for Scientific Reports. I'll have a look at what we need to change as far as the formatting and get back to you with the final draft for submission to this journal.
> Cheers,
>
> Jenn
>
>
> Jennifer Barr BSc (Biotech) (Hons)
> Research Assistant
> Dangerous Pathogens Team
> CSIRO Australian Animal Health Lab
[redacted]

> I work part time: Tues/Wed/Thurs
>
>
>
> -----Original Message---
> From: Wang Linf[redacted]
> Sent: Tuesday, 16 January 2018 9:06 PM
> To: James Wood[redacted] 'Andrew Cunningham'
[redacted]
> Cc: Barr, Jenn (H&B, Geelong AAHL)[redacted] Todd,
> Shawn (H&B, Geelong AAHL)[redacted]
> garycrameri1[redacted] oord, Adam (AAHL, Geelong AAHL)
[redacted] Marsh, Glenn (H&B, Geelong AAHL)
leah.frazer14[redacted] Payne, Jean (AAHL,
> Geelong AAHL)[redacted] Harper, Jenni (AAHL, Geelong

> AAHL) [redacted]; Kate.Baker [redacted];
> middled35 [redacted]
> Subject: RE: [EXT]: AchPV paper
>
> I am supportive.
>
> Thanks
>
> Linfa (Lin-Fa) WANG, PhD FTSE
> Professor & Director
> Programme in Emerging Infectious Disease Duke-NUS Medical School,
[redacted]
>
>
> -----Original Message-----
> From: James Wood [redacted]
> Sent: Tuesday, 16 January, 2018 5:23 PM
> To: 'Andrew Cunningham'
> Cc: Jennifer.Bar [redacted] Shawn.Todd [redacted]
> garycrameri [redacted] Adam.Foord [redacted] Glenn.Marsh [redacted]
> leah.frazer1 [redacted] Jean.Payne [redacted] Jenni.Rooke [redacted]
> Kate.Baker [redacted] middled35 [redacted]; Wang Linfa
> Subject: RE: [EXT]: AchPV paper
>
> Far better if we can get in to them, agreed!!
>
> -----Original Message-----
> From: Andrew Cunningham [redacted]
> Sent: 16 January 2018 08:52
> To: James Wood
> Cc: Jennifer.Bar [redacted] Shawn.Todd [redacted];
> garycrameri1 [redacted] Adam.Foord [redacted] Glenn.Marsh [redacted]
> leah.frazer14 [redacted] Jean.Payne [redacted] Jenni.Rooke [redacted]
> Kate.Baker [redacted] middled35 [redacted]
> linfa.wang [redacted]
> Subject: Re: [EXT]: AchPV paper
>
> Royal Society Open Science or Scientific Reports might be a better bet?
> Cheers
> Andrew
>
> Sent from my iPhone
>
>> On 16 Jan 2018, at 08:39, James Wood [redacted] wrote:
>>
>> I am still disappointed by the previous rejection!
>> I think that the paper will stand on its own merits and so think that
>> we could do a lot worse than PLoS ONE Best wishes James
>>
>> **********
>> James Wood
>> Head of Department of Veterinary Medicine and Alborada Professor of
>> Equine and Farm Animal Science Disease Dynamics Unit

>>
>> University of Cambridge
>> Madingley Road Cambridge
>> CB3 0ES
[redacted]
>> Honorary Research Fellow, Institute of Zoology
>> http://www.research.vet.cam.ac.uk/research staff directory/principal-
>> i
>> nvesti
>> gators/disease dynamics/james wood
>> http://www.infectiousdisease.cam.ac.uk/directory/jlnw2@cam.ac.uk
>> http://www.wolfson.cam.ac.uk/people/professor james wood
>> http://www.vet.cam.ac.uk/ddu/
>> http://www.cambridge africa.cam.ac.uk
>> http://ethicobots.com
>>
>> Original Message -
>> From: Andrew Cunningham [redacted]
>> Sent: 16 January 2018 08:30
>> To: Jennifer.Barr [redacted]
>> Cc: Shawn.Todd [redacted] garycrameri1 [redacted] Adam.Foord [redacted]
>> Glenn.Marsh [redacted] leah.frazer14 [redacted] Jean.Payne [redacted]
>> Jenni.Rookes [redacted] Kate.Baker [redacted] jlnw2 [redacted]
>> middled35 [redacted] linfa.wang [redacted]
>> Subject: Re: [EXT]: AchPV paper
>>
>> Thanks, Jenn
>>
>> Further to Deborah's last email, perhaps we should make more explicit
>> the environmental conditions (substrate) that might have led to
>> respiratory disease? This would help address some of the previous
>> reviewer
> comments.
>>
>> Cheers
>>
>> Andrew
>>
>> Sent from my iPhone

[redacted]
Duplicate Email - Removed

[redacted]

From: [redacted] behalf of PLOS ONE

Sent: Wednesday, 24 January 2018 4:48 AM
To: glenn.marsh[redacted]
Subject: Notification of Formal Acceptance for PONE D 17 23529R1 - [EMID:5cf3e016944e6edb]

You are being carbon copied ("cc:'d") on an e mail "To" "Ina Smith" [redacted]
CC: kohlc [redacted] mary.tachedjian [redacted] shawn.tod [redacted] monaghan [redacted]
vicky.boyd [redacted] glenn.marsh [redacted] garycrameri1 [redacted], hume.field [redacted]
kurth [redacted] linfa.wang [redacted]

PONE-D-17-23529R1
Hervey virus: Study on co-circulation with Henipaviruses in Pteropid bats within their distribution range from Australia to Africa

Dear Dr. Smith:

I am pleased to inform you that your manuscript has been deemed suitable for publication in PLOS ONE. Congratulations! Your manuscript is now with our production department.

If your institution or institutions have a press office, please notify them about your upcoming paper at this point, to enable them to help maximize its impact. If they will be preparing press materials for this manuscript, please inform our press team within the next 48 hours. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information please contact onepress@plos.org.

For any other questions or concerns, please email plosone@plos.org.

Thank you for submitting your work to PLOS ONE.

With kind regards,

PLOS ONE Editorial Office Staff
on behalf of

Prof Wanda Markotter
Academic Editor
PLOS ONE

[redacted]

From: Kohl, Claudia [redacted]
Sent: Friday, 26 January 2018 2:24 AM
To: Wang Linfa; Hume Field
Cc: mary.tachedjia [redacted] shawn.todd [redacted] monaghan [redacted]
vicky.boyd [redacted] glenn.marsh [redacted] garycrameri1 [redacted]
ina.smith [redacted] Kurth, Andreas
Subject: AW: PONE D 17 23529R1: Final Decision Being Processed - [EMID:762e1e51bcf8239b]

Yes, finally there! Thanks and congrats to everone!

Cheers,
Claudia

Gesendet von meinem BlackBerry 10-Smartphone.
Von: Wang Linfa
Gesendet: Sonntag, 21. Januar 2018 02:23
An: Hume Field
Cc: Kohl, Claudia; mary.tachedjian [redacted] shawn.todd [redacted] monaghan [redacted]
vicky.boyd [redacted] glenn.marsh [redacted] garycrameri [redacted] ina.smith [redacted] urth, Andreas
Betreff: Re: PONE-D 17 23529R1: Final Decision Being Processed [EMID:762e1e51bcf8239b]

Great news indeed after a long journey!

Congrats to all!

Sent from my iPhone

On 21 Jan 2018, at 9:21 AM, Hume Field
[redacted] wrote:

Great news Claudia and all. It will be good to see this finally published!

All the best for 2018

Hume

From:
[redacted]] On Behalf Of PLOS ONE
Sent: Thursday, 18 January 2018 4:53 AM
To: hume.field [redacted]
Subject: PONE-D-17-23529R1: Final Decision Being Processed - [EMID:762e1e51bcf8239b]

You are being carbon copied ("cc:'d") on an e-mail "To" "Ina Smith" [redacted]
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Hervey virus: Study on co-circulation with Henipaviruses in Pteropid bats within their distribution range from Australia to Africa
PONE-D-17-23529R1

Dear Dr. Smith,

We are pleased to inform you that your manuscript has been judged scientifically suitable for publication and will be formally accepted for publication once it complies with all outstanding technical requirements.Please also note the small comment from reviewer 2 that you must please address.

Within one week, you will receive an e-mail containing information on the amendments required prior to publication. When all required modifications have been addressed, you will receive a formal acceptance letter and your manuscript will proceed to our production department and be scheduled for publication.

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With kind regards,

Wanda Markotter
Academic Editor
PLOS ONE

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Reviewer's Responses to Questions

Comments to the Author

1. If the authors have adequately addressed your comments raised in a previous round of review and you feel that this manuscript is now acceptable for publication, you may indicate that here to bypass the "Comments to the Author" section, enter your conflict of interest statement in the "Confidential to Editor" section, and submit your "Accept" recommendation.

Reviewer #1: All comments have been addressed

Reviewer #2: (No Response)

2. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

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3. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: Yes

Reviewer #2: Yes

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4. Have the authors made all data underlying the findings in their manuscript fully available?

The PLOS Data policy<http://www.plosone.org/static/policies.action#sharing> requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

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6. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: (No Response)

Reviewer #2: The authors have addressed all issues in a satisfying manner, and I recommend this mansucript for publication.
One small comment: in figure legend 1, line 478, the scale bar for the confocal images has the unit of "uM" (so: micromolar) instead of "um" (micrometer).

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7. If you would like your identity to be revealed to the authors, please include your name here (optional).

Your name and review will not be published with the manuscript.

Reviewer #1: (No Response)

Reviewer #2: (No Response)

Document 5

[redacted] s22

From: Barr, Jenn (H&B, Geelong AAHL) [redacted] s22
Sent: Wednesday, 7 February 2018 11:47 AM
To: Todd, Shawn (H&B, Geelong AAHL); Gary Crameri; Foord, Adam (AAHL, Geelong AAHL); Marsh, Glenn (H&B, Geelong AAHL); 'Leah Frazer'; Payne, Jean (AAHL, Geelong AAHL); Harper, Jenni (AAHL, Geelong AAHL); Baker, Kate; Andrew Cunningham; James Wood (jlnw2@cam.ac.uk); Deborah Middleton; Wang Linfa
Subject: RE: AchPV paper
Attachments: AchPV Paper 7Feb2018.docx; Cover Letter 7Feb2018.docx

Hi all,
I have re formatted the AchPV paper to suit submission to Scientific Reports.
Only a few changes/additions were required and the abstract needed to be under 200 words, so take a look and let me know if you think it still reads ok (I removed the last sentence of the abstract).
Also, they need a cover letter so I've also attached that for your approval.
Let me know if you are happy for me to submit.
Cheers,

Jenn

[redacted] Duplicate Email - Removed

Animal infection studies of two recently discovered African bat paramyxoviruses, Achimota 1 and Achimota 2

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Key words: Achimota virus, bat paramyxovirus, zoonotic, Africa, animal infection study

Main text word count: 3860

Abstract

Novel emerging zoonotic viruses pose a great threat to public health and viral spillover events originating from wildlife account for many newly recognised diseases of people. Bats are implicated as the natural reservoirs for several highly pathogenic viruses that can infect other animal species, including man. Here, we investigate the potential for two recently discovered bat rubulaviruses, Achimota virus 1 (AchPV1) and Achimota virus 2 (AchPV2), isolated from urine collected under urban bat (*Eidolon helvum*) roosts in Ghana, West Africa, to infect small laboratory animals. AchPV1 and AchPV2 are classified in the family *Paramyxoviridae* and cluster with other bat derived zoonotic rubulaviruses (i.e. Sosuga, Menangle and Tioman viruses). To assess the susceptibility of AchPV1 and AchPV2 in animals, infection studies were conducted in ferrets, guinea pigs and mice. Seroconversion, immunohistological evidence of infection, and viral shedding were identified in ferrets and guinea pigs, but not in mice. Infection was associated with respiratory disease in ferrets. These results indicate Achimota viruses are able to cross the species barrier and may infect domesticated animals and humans in areas of Africa where infected reservoir hosts are widely distributed.

Word count: 185

INTRODUCTION

New and emerging viral infections impose a significant burden on human health and on the world economy. The majority of emerging infectious diseases affecting humans today are of animal origin, with approximately three quarters arising from wildlife [1]. Bats have been shown to harbour more zoonotic viruses than other mammalian species [2 & 3] and are implicated in outbreaks of a number of highly pathogenic zoonotic viruses, including filoviruses, coronaviruses, paramyxoviruses and reoviruses. Ebola virus (EboV) RNA and antibodies have been discovered in African fruit bats [4]. The largest human outbreak of EboV occurred in West Africa in 2013-2014, resulting in nearly 30,000 infections and 11,000 deaths and took over a year to contain [5]. SARS coronavirus emerged in China in 2002 and infected over 8000 people causing 774 deaths [6] and the MERS coronavirus continues to infect people and cause death in the Middle East [7]. Bats have since been found to harbour a multitude of coronaviruses closely related to SARS and MERS [8 & 9]. Pteropid bats are the reservoir hosts for the deadly henipaviruses, Nipah virus (NiV) and Hendra virus (HeV) [10 & 11]. NiV continues to cause fatal encephalitis in humans almost annually in Bangladesh, while HeV has spilled-over into horses in Australia nearly every year since 2004 and has killed four people [12 & 13]. Orthoreoviruses have been isolated from bats and humans in Southeast Asia where they have caused flu-like illness in people [14]. Known zoonotic viruses of bat origin continue to be of concern for human and animal health, and active surveillance provides our best option for monitoring these agents as well as identifying novel pathogens of zoonotic potential.

70 The recently discovered rubulaviruses, Achimota virus 1 and Achimota virus 2 (AchPV1
71 and AchPV2), were isolated from bat (*Eidolon helvum)* urine samples collected beneath
72 urban bat roosts in Ghana, West Africa [15]. AchPV1 and AchPV2 are newly recognised
73 viral species in the family *Paramyxoviridae*, where they cluster with other bat
74 rubulaviruses. Despite being discovered in the same study, the AchPVs are not nearest-
75 phylogenetic relatives and share only 31 to 64% protein amino acid identities [15]. Their
76 relationship to each other is similar to their relationships with other bat rubulaviruses
77 such as Sosuga (SosPV), Menangle (MenPV) and Tioman (TioPV) viruses (sharing 58-
78 70% N protein amino acid sequence identities), which have been shown to cause human
79 infection. SosPV was isolated from a wildlife biologist studying bats and rodents in
80 Africa in 2012 and is believed to be the causative agent for a severe flu-like illness and
81 skin rash [16]. Follow up investigations revealed the presence of this virus in the spleen
82 of *Rousettus aegyptiacus* bats [17]. MenPV first emerged in a piggery in NSW, Australia,
83 in 1997 causing reproductive disease in pigs [18]. Two piggery workers had flu-like
84 illness during the outbreak and were later found to have MenPV neutralising antibodies.
85 Serological evidence of MenPV infection was also found in flying foxes roosting near the
86 piggery and the virus was later isolated from *Pteropus alecto* urine [18 & 19]. TioPV was
87 isolated from pteropid bat urine on Tioman Island in 2001 during the search for the
88 reservoir host of Nipah virus [20]. It was later found that humans on the island had
89 neutralising antibodies to TioPV, although no associated disease has been reported [21].
90
91 Based on what is known of these closely related bat rubulaviruses, the potential of
92 AchPV1 and AchPV2 to infect and cause disease in other species is worthy of further

93 investigation. Serological surveys of *Eidolon helvum* populations in Africa have been
94 conducted for Achimota viruses and have shown a widespread presence of neutralising
95 antibodies [15]. In addition, a survey of human sera collected from Ghana and Tanzania
96 detected AchPV2 neutralising antibodies in three of 442 samples tested, however no
97 neutralising antibodies to AchPV1 were detected in these sera. Two of the antibody
98 positive samples were from healthy adults and one was from a febrile paediatric patient
99 [15]. These data suggest that AchPV2 is zoonotic, but whether AchPV1 is zoonotic
100 remains unknown.
101
102 To further investigate the infection potential of AchPV1 and AchPV2, we conducted
103 studies in three species of small laboratory animal; ferret (*Mustela putorius furo*), guinea
104 pig (*Cavia porcellus*) and mouse (*Mus musculus domesticus*). First, we conducted
105 observational studies to determine the susceptibility of these animals to infection by
106 AchPV1 or AchPV2. Second, time course studies were performed using AchPV2 to
107 obtain data on viral replication sites and potential routes of transmission.
108
109 **RESULTS**
110 **Observational study with AchPV1 and AchPV2 in ferrets**
111 Two adult male ferrets aged 11 13 months were given 10^5 $TCID_{50}$ AchPV1 oronasally
112 in 1 ml of inoculum and another two adult male ferrets aged 11 13 months were given
113 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum. The animals were observed daily for
114 clinical signs and then electively euthanased at 21 days post challenge (pc).
115

One of the two ferrets exposed to AchPV1 remained clinically well and was electively euthanased at the end of experiment on day 21 pc. The other ferret showed signs of upper respiratory tract infection (sneezing, coughing) and weight loss from day 1 pc, and was euthanased at day 14 pc when it had reached a predetermined humane endpoint of 10 % bodyweight loss. Post mortem examination revealed a pleural effusion and bronchopneumonia of the right intermediate lung lobe. Each ferret developed neutralising antibody against AchPV1, with titres of 1:320 (healthy) and 1:80 (ill) (Table 1).

One of two ferrets exposed to AchPV2 remained clinically well and was electively euthanased at the end of the experiment on day 21 pc. The other ferret maintained normal play activity but showed signs of upper respiratory tract infection (sneezing, purulent nasal discharge) from day 3 pc and which resolved by day 11 pc. The animal was electively euthanased at the end of the experiment on day 21 pc. Each ferret developed neutralising antibody against AchPV2, with titres > 1:1280 (healthy) and 1:1280 (ill/recovered) (Table 1).

In summary, it is unclear whether the signs of respiratory tract disease in one of two ferrets given either AchPV1 or AchPV2 were attributable to infection by the challenge virus or by co-infection of an unknown pathogen. As higher neutralising antibody titres were observed in ferrets infected with AchPV2, and as there was prior serological evidence of this virus in people, AchPV2 was selected for a time-course study with ferrets.

Observational study with AchPV1 and AchPV2 in guinea pigs

Four adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV1 oronasally in 1 ml of inoculum and another four adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum. The animals were observed daily for clinical signs and then electively euthanased at 21 days pc.

All four guinea pigs exposed to AchPV1 remained clinically well and were electively euthanased on day 21 pc. Each guinea pig developed neutralising antibody against AchPV1, with titres of 1:320, 1:80, 1:80 and 1:40 (Table 1). Similarly, all four guinea pigs exposed to AchPV2 remained clinically well and were electively euthanased on day 21 pc. Each guinea pig developed neutralising antibody against AchPV2, with titres of 1:320, 1:160, 1:80 and 1:40 (Table 1).

Based on the same rationale as for ferrets, AchPV2 was selected for a time-course study with guinea pigs.

Observational study with AchPV1 and AchPV2 in mice

Ten mice (five female Balb-C mice aged 12 weeks and five female BalbC mice aged over 12 months) were given 10^3 $TCID_{50}$ AchPV1 intranasally in 50 µl of inoculum and another ten mice (five female Balb-C mice aged 12 weeks and five female BalbC mice aged over 12 months) were given 10^3 $TCID_{50}$ AchPV2 intranasally in 50 µl of inoculum. The animals were observed daily for clinical signs and then electively euthanased at 21 days pc.

All ten mice exposed to AchPV1 remained clinically well and were electively euthanased on day 21 pc. Neutralising antibody against AchPV1 was not detected in any mouse. Likewise, all ten mice exposed to AchPV2 remained clinically well and were electively euthanased on day 21 pc. Neutralising antibody against AchPV2 was not detected in any mouse. As mice had no detectable signs of disease and did not seroconvert to either AchPV1 or AchPV2, no further studies were conducted with mice.

Time course study with AchPV2 in ferrets

1. Clinical and Pathological findings

For this study, eight adult female ferrets were given 10^5 $TCID_{50}$ AchPV2 oronasally in 1 ml of inoculum and then two animals were pre-allocated for euthanasia on each of days 6, 8, 10 and 21 pc. All eight ferrets in this study showed a mild but significant increase in rectal temperature over baseline on day 4 pc (p 0.02), and a mild but significant loss of bodyweight compared to baseline on days 4 (p 0.03) and 5 (p = 0.0004) pc. Otherwise, the animals remained clinically well until elective euthanasia, apart from one of two ferrets scheduled for euthanasia on day 8 pc. This ferret (#9) showed signs of upper respiratory tract infection (sneezing, serous and then purulent nasal discharge) between days 2 and 6 pc and was euthanased on humane grounds on day 6 pc following markedly decreased play activity. Other than ferret #9, no significant gross abnormalities were observed at post mortem examination in any of the ferrets.

184 The three ferrets euthanased on day 6 pc had minor histopathological changes associated
185 with viral replication. Ferret #12 (day 6 pc) showed very mild acute bronchiolitis.
186 Immunohistochemistry revealed viral antigen in germinal centres of the retropharyngeal
187 lymph node but not in other tissues. In ferret #14 (day 6 pc), significant histopathological
188 changes were confined to mild acute tonsillitis, and viral antigen was detected in the
189 tonsillar and pharyngeal epithelium, retropharyngeal lymph node (particularly the
190 parafollicular areas), bronchial epithelial cells, bronchus-associated lymphoid tissues
191 (BALT), perivascular spindle cells in lung, and germinal centres and periarteriolar
192 lymphoid sheaths of the spleen. In ferret #9, euthanased on day 6 pc with respiratory
193 disease, there was moderately severe acute bronchiolitis, hyperplasia of the BALT,
194 excess mucus production by bronchial glands, and focal lipoid pneumonia consistent with
195 chronic bronchial disease. Post mortem examination of ferret #9 also revealed marked
196 nodular hyperplasia of the liver with hepatic steatosis, but this lesion was considered to
197 be unrelated to virus exposure as no AchPV2 viral antigen was detected in the liver.
198
199 In ferret #9, AchPV2 viral antigen was identified in tonsillar and pharyngeal epithelium,
200 germinal centres, parafollicular area and medulla of the retropharyngeal lymph node,
201 tracheal epithelium, bronchial and bronchiolar epithelium (Fig. I), BALT and
202 perivascular connective tissues of the lung, bronchial and mediastinal lymph node,
203 periarteriolar lymphoid sheaths and red pulp of the spleen, mononuclear cells in the
204 intestinal lamina propria and cells either within or lining the hepatic sinusoids.
205

206 In the single ferret euthanased on day 8 pc (ferret #16), there was mild focal acute
207 tracheitis and bronchiolitis. The distribution of viral antigen was similar to ferret #9, with
208 the addition of occasional bile duct epithelial cells and mononuclear cells of the portal
209 triads, gut associated lymphoid tissue (GALT), and transitional epithelial cells in the
210 bladder.
211
212 The two ferrets killed on day 10 pc showed only small amounts of viral detection by
213 immunohistochemistry. Ferret #11 (day 10 pc) had mild focal acute bronchiolitis, and
214 detection of viral antigen was limited to small amounts in tonsillar lymphoid tissue,
215 bronchiolar epithelial cells, periarteriolar lymphoid sheaths of the spleen, and a diffuse
216 scattering throughout the retropharyngeal lymph node. Ferret #13 (day 10 pc) also
217 showed very mild acute bronchiolitis, with AchPV2 viral antigen confined to scattered
218 gastric epithelial cells, GALT, and sparse deposits throughout tonsillar lymphoid tissue,
219 bronchial and retropharyngeal lymph nodes.
220
221 Of the two ferrets killed on day 21 pc, one (ferret #15) had very mild acute bronchiolitis
222 and tracheitis and one had no detectable lesions. AchPV2 viral antigen was not detected
223 in any tissue from either of these two ferrets.
224
225 ***2. Detection of viral genomes***
226 AchPV2 RNA was detected by RT-qPCR in the oral swabs of 4 ferrets on day 2 pc and in
227 all ferrets by day 4 pc until euthanasia: the highest levels were typically recorded on day
228 6 or 8 pc (Table 2). Similar results were seen for the nasal washes: viral RNA was

229 detected in 3 ferrets on day 2 pc, and in all ferrets by day 4 pc until euthanasia, the
230 highest levels typically were recorded on day 6 or 8 pc. Rectal swabs first detected
231 AchPV2 on day 4 pc, when all ferrets were positive, the highest levels occurred on day 6
232 or 8 pc. Viral RNA was commonly detected in blood samples from days 2 to 21 pc.
233 Where viral RNA was found in successive blood samples from individual animals,
234 highest levels were recorded on day 6 or 8 pc (Table 2).
235
236 All tissue samples analysed from ferrets #9, #12 and #14 euthanased on day 6 pc were
237 positive for viral RNA (data summarised in Fig. II), with the highest levels in bronchial
238 and retropharyngeal lymph nodes and the lowest levels in heart, kidney and brain. Each
239 tissue sample tested from ferret #16 (euthanased 8 days pc) was also positive for viral
240 RNA, with the highest reading in retropharyngeal lymph node. On day 10 pc, ferrets #11
241 and #13 exhibited generally similar distribution and quantities of viral RNA to the
242 animals above. However, on day 21 pc the levels of viral RNA in ferrets #10 and #15
243 were substantially lower, and largely limited to the retropharyngeal and bronchial lymph
244 nodes and the spleen.
245
246 ***3. Virus isolation***
247 Virus was reisolated from the nasal wash and from the oral and rectal swabs of ferret #9
248 on day 6 pc, the nasal wash of ferret #10 on day 6 pc, the oral swab of ferret #11 on day 6
249 pc, and from the oral swabs of ferret #16 on days 6 and 8 pc, plus the nasal wash of this
250 ferret on day 8 pc (Table 2).
251

252 Virus was reisolated from ferrets killed on day 6 pc from tonsil, bronchial and
253 retropharyngeal lymph nodes, lung and brain (ferret #9); trachea, tonsil, bronchial and
254 retropharyngeal lymph nodes, and bladder (ferret #12); and tonsil and bronchial and
255 retropharyngeal lymph nodes (ferret #14) (Fig. II). Virus was reisolated from tonsil and
256 trachea of ferret #16 (which was killed on day 8 pc) and from the retropharyngeal lymph
257 node and kidney of ferret #13 (day 10 pc), but not from ferret #11 (day 10 pc). Virus re-
258 isolation from ferrets killed on day 21 pc was limited to the retropharyngeal lymph node
259 of ferret #15.
260
261 ***4. Serology***
262 No neutralising antibodies were observed at day 2 or 4 pc but by day 6 low levels of
263 neutralising antibodies were detected in the ferrets (Table 4). They showed an increase in
264 neutralising antibody titre across the time points, with the animals bled at day 21 pc
265 having neutralising antibody titres > 1:1280.
266
267 **Time course study with AchPV2 in guinea pigs**
268 ***1. Clinical and Pathological findings***
269 For this study, eight adult female guinea pigs were given 10^5 $TCID_{50}$ AchPV2 oronasally
270 in 1 ml of inoculum and then two animals were pre-allocated for euthanasia on each of
271 days 6, 8, 10 and 21 pc. In guinea pigs, there were no significant differences in
272 temperature or bodyweight over baseline up to day 6 pc, and the animals remained
273 clinically healthy until elective euthanasia. No significant gross abnormalities were
274 observed at post mortem examination, apart from enlarged bronchial lymph nodes in one

guinea pig euthanased on day 6 pc. The only histopathological changes observed were: mild acute tracheitis in all animals, two animals with mild acute bronchitis and/or bronchiolitis, and four with mild chronic interstitial pneumonia attributable to inhalation of plant material. In contrast to the observations in ferrets, the pattern of respiratory tract lesions did not correlate with the time post-exposure to AchPV2; very few histopathological changes were observed and all sections of tissues from all guinea pigs were negative for AchPV2 antigen by immunohistochemistry.

2. Detection of viral genomes

Low levels of AchPV2 were detected in the oral swab of one guinea pig on day 6 pc and of another on day 8 pc (Table 3). Rectal swabs were positive in four of six guinea pigs on day 8 pc, and from one guinea pig on day 10 pc. Viral RNA was found in the blood of one guinea pig on day 6 pc and of another on day 8 pc (both of which had viral RNA-positive oral swabs at these times).

Most tissue samples analysed from guinea pigs euthanased on day 6 and 8 pc were positive for viral RNA (data summarised in Fig. III), with highest levels present in nasal turbinates, bronchial and retropharyngeal lymph nodes, and spleen, and lower levels in trachea, lung, and liver. Detection was lowest and inconsistent from heart, kidney and brain. By day 10 pc, viral genome detection was limited to bronchial and/or retropharyngeal lymph nodes, lung, spleen, and nasal turbinates (one of two animals). On day 21 pc, one guinea pig was negative by RT-qPCR for all tissues; in the other, viral genome was detected only in bronchial lymph node and spleen.

298

299 ***3. Virus isolation***

300 Virus was not reisolated from any of the clinical samples, including those that were
301 positive by AchPV2-specific RT-qPCR (Table 3). In addition, virus was not reisolated
302 from any tissue sample, including those that were positive by AchPV2-specific RT-qPCR
303 (Fig. III).

304

305 ***4. Serology***

306 No neutralising antibodies were observed at day 2 or 4 pc but by day 6 low levels of
307 neutralising antibodies were detected in the guinea pigs (Table 4). There was a slight
308 increase in neutralising antibody titre across the time points with a titre of 1:160 by day
309 21 pc.

310

311 **DISCUSSION**

312 We investigated the potential of the two recently discovered bat rubulaviruses, AchPV1
313 and AchPV2, to infect laboratory animals representing three species: ferret, guinea pig
314 and mouse. Seroconversion to both Achimota viruses in ferrets and guinea pigs indicated
315 these animals were susceptible to infection, however mice did not seroconvert to either
316 virus. Due to their body size, mice were given a lower dose of inoculum, and were
317 challenged intranasally, rather than via the oronasal route used for the ferrets and guinea
318 pigs. The difference in volume and inoculation route may account for the lack of
319 seroconversion seen in the mice, however it is probably more likely that this species is
320 resistant to infection. Higher levels of neutralising antibodies were observed in the ferrets

and guinea pigs infected with AchPV2 compared to those infected with AchPV1. This result reflected *in vitro* data where it was observed previously that AchPV2 consistently grows to a higher titre than AchPV1 in vero and PaKi cell lines [15]. Additionally, respiratory tract disease in one of two ferrets given either AchPV1 or AchPV2 was seen in the observational studies, although it remains unclear if this was related to Achimota virus infection or was entirely due to co-infection by an unknown pathogen. AchPV2 was chosen for a time-course study based on two criteria: higher neutralising antibodies observed in ferrets and guinea pigs compared to AchPV1, and previous evidence of human infection (AchPV2 neutralising antibodies). Given more time and resources, it would be worthwhile to do an additional time course study with AchPV1, to further investigate the differences between these two viruses.

The AchPV2 time-course studies provided additional evidence that this virus can infect ferrets and guinea pigs and revealed viral replication sites and potential routes of transmission. Evidence of infection was supported by virus re-isolation from clinical specimens and post-mortem tissue samples, and viral antigen detection in tissues by quantitative real-time PCR and immunohistochemistry. Although there was evidence of mild malaise in infected ferrets (raised body temperature and weight loss), a distinct clinical syndrome with specific clinical signs was not identified in either ferrets or guinea pigs. Moreover, no histological lesions were attributed with confidence to infection by AchPV2. Mild tonsillitis, tracheitis and bronchiolitis were recorded in ferrets, but in some animals the lesions were identified without evidence of specific association with AchPV2 antigen. The ferrets were sourced from a colony free of influenza and canine distemper

344 virus, were clinically healthy at the time of exposure to AchPV2, and were not
345 maintained on a particulate substrate. The pathogenesis of these legions remains
346 uncertain, although an opportunistic bacterial or other viral aetiology could not be
347 excluded. In other tissues, such as bile duct epithelium and transitional epithelial cells of
348 the bladder, viral antigen was seen without substantial inflammatory reaction or tissue
349 injury.
350
351 For ferrets, following an incubation period of 5 to 6 days, AchPV2 was shed in oral and
352 nasal secretions and the development of virus neutralising antibody was generally
353 associated with virus clearance. The results of quantitative real-time PCR, virus re-
354 isolation and immunohistochemistry taken together identified the major sites of AchPV2
355 replication in ferrets to be respiratory tract epithelium and associated lymphoid tissues.
356 Although virus was not re-isolated from blood, the development of viremia may be
357 inferred by confirmation of infection within spleen and urinary tract epithelium. In the
358 absence of other clinico-pathological support for CNS infection, virus in blood may also
359 account for the re-isolation of AchPV2 from one sample of ferret brain tissue.
360
361 Although exposure to AchPV2 resulted in production of neutralising antibodies in guinea
362 pigs, antibody titres were much lower than for the ferrets. Patterns of detection of viral
363 RNA from guinea pigs were generally similar to those in ferrets, but virus was not
364 recovered from guinea pigs and viral antigen was not demonstrated in their tissues. The
365 sites of AchPV2 replication in guinea pigs, therefore, could not be determined with

366 confidence. Our observations suggest that guinea pigs are less permissive to AchPV2
367 infection than ferrets.
368
369 When assessing the spill-over potential and working up an animal model for a novel
370 virus, it is important to use animals from more than one species. There is no reliable
371 method to determine the best species simply by characterising the virus, which is a big
372 limitation of using virus discovery as a stand-alone surveillance strategy for zoonotic
373 pathogen discovery. Therefore, it is only possible to do what is practical and feasible in
374 terms of assessing potential spill-over hosts. Small laboratory animals such as ferrets,
375 guinea pigs and mice, representing different mammalian orders or families, offer the most
376 practical advantages for testing spill-over potential and, in this study, were a panel that
377 demonstrated discriminatory power for the infection potential of the novel viruses tested.
378
379 The continued search for novel viruses in wildlife species, particularly in regions of the
380 world where encroachment of humans and livestock into wildlife habitats is increasing,
381 such as sub-Saharan Africa, is imperative if we are going to be able to identify disease in
382 these regions caused by novel pathogens. New discoveries of wildlife viruses alone,
383 however, will not inform risks to livestock or public health. Viral phylogeny and other
384 signals of spill-over potential, such as the serosurveillance results that guided this study,
385 are required to identify potential new health threats [22]. The Achimota viruses described
386 in this paper demonstrate ability to cross the species barrier and may be causing
387 undiagnosed disease in domesticated animals and humans within the wide geographical
388 range of the bat reservoir species, *Eidolon helvum*.

389

390 **MATERIALS AND METHODS**

391 **Animals, accommodation, handling and biosafety**

392 Ferrets were acquired from a colony free of infection by influenza H1 and H3 subtypes.
393 Two male ferrets aged 11 – 13 months, four female guinea pigs, five female Balb-C mice
394 aged 12 wks, and five female BalbC mice aged over 12 months were used in each of the
395 AchPV1 and AchPV2 observational studies. Eight female ferrets and eight female guinea
396 pigs were used for the AchPV2 time course study. The animal husbandry methods and
397 experimental design were endorsed by the CSIRO Australian Animal Health
398 Laboratory's Animal Ethics Committee (approvals AEC 1608 and AEC 1621). Animals
399 were housed at Biosafety Level 3 (BSL-3) in conventional caging systems to facilitate the
400 expression and monitoring of natural behaviours, given complete premium dry food
401 appropriate to the species, dietary treats, and provided with water *ad libitum*. Room
402 temperature was maintained at 22°C with 15 air changes per hour; and humidity varied
403 between 40 and 60%. Before manipulation such as exposure to virus, collection of
404 clinical samples, or euthanasia, animals were immobilised with a mixture of ketamine
405 HCl (Ketamil®: 5 mg/kg in ferrets, 16mg/kg in guinea pigs, 75mg/kg in mice) and
406 medetomidine (Domitor®: 50 μg/kg in ferrets, 20 μg/kg in guinea pigs, 1mg/kg in mice)
407 by intramuscular or intraperitoneal (mice) injection. Where indicated, reversal was
408 achieved with atipamazole (Antisedan®) administered by intramuscular (ferrets) or
409 intraperitoneal (guinea pigs and mice) injection at 50 % of the medetomidine volume. All
410 animals were implanted subcutaneously with temperature-sensing microchips

411 (Lifechip®). Staff wore powered air purifying respirators, coveralls, impervious gloves
412 and boots while in animal rooms.
413
414 **Animal infections and sampling**
415 For the observational studies, animals were exposed to either AchPV1 or AchPV2,
416 isolated, grown and titrated in vero cells. After initial virus isolation, a parent stock of
417 each virus was grown in vero cells. These parent stocks were then purified by three
418 rounds of limiting dilution in vero cells. Finally, an animal inoculation stock was
419 prepared from the third limiting dilution, resulting in a passage number of 6 times in vero
420 cells from original isolation. The sequence of the animal inoculation stock was not
421 compared to the original sequence of the isolated virus. Ferrets and guinea pigs were
422 given 10^5 $TCID_{50}$ oronasally in 1 ml of inoculum (500 µl oral and 500 µl nasal), and mice
423 were given 10^3 $TCID_{50}$ intranasally in 50 µl of inoculum. General clinical observations
424 were documented daily prior to as well as post challenge (pc). Animals were weighed and
425 their temperatures recorded daily. Animals were euthanased at either a predetermined
426 humane endpoint or 21 days pc. Blood was collected for serology prior to virus exposure
427 and at euthanasia. Tissues were not collected for the observational studies.
428
429 For the subsequent time course studies, ferrets and guinea pigs were exposed oronasally
430 to 10^5 $TCID_{50}$ AchPV2, prepared as described above, in 1 ml of inoculum (500 µl oral
431 and 500 µl nasal). Two animals were pre-allocated for euthanasia on each of days 6, 8, 10
432 and 21 pc. Nasal washes (ferrets only), oral and rectal swabs and blood samples, both in
433 EDTA and for serum preparation, were collected from all available animals at days 2, 4,

6, 8, 10 and 21 pc. Clinical samples were collected into tubes containing PBS with antibiotic-antimycotic (Invitrogen) for virus isolation and into tubes containing MagMAX viral lysis buffer (Ambion) for RNA extraction. While under anaesthesia, rectal temperatures of ferrets were recorded by digital thermometer.

At post mortem examination of animals used for the AchPV2 time course study, the following tissues were collected for histology, immunohistochemistry, viral genome detection and virus isolation: nasal turbinates, tonsil, retropharyngeal lymph node, trachea, lung, hilar lymph node, bronchial lymph node, spleen, heart, kidney, liver, bladder and brain. Stomach, small and large intestine, pancreas, adrenal gland, ovary and uterus were also collected for histology and immunohistochemistry. Tissues were collected into tubes containing either neutral buffered 10% formalin (for histology and immunohistochemistry) or PBS plus antibiotic-antimycotic (Invitrogen) and homogenisation beads, homogenised using a bead beater, and clarified by centrifugation (for virus isolation or viral RNA detection).

RNA extraction and Reverse Transcriptase-quantitative Polymerase Chain Reaction

For viral genome detection, RNA was extracted from tissue, blood and swab samples using the MagMAX viral RNA isolation kit (Ambion) following the manufacturers guidelines. A novel Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR), was designed that specifically targets the nucleoprotein gene (N-gene) of AchPV2. For the design process, the N-gene sequence of AchPV2 (JX051320), as well as other closely related paramyxoviruses including SosPV, MenPV and TioPV, was

457 retrieved from GenBank. Subsequently, sequence alignments were performed using
458 Geneious software (Version 8.1, Biomatters). Potential primer and probe regions
459 distinctive of AchPV2 were identified from these alignments and candidate primers and
460 probes assessed using the Primer Express 3.0.1 program (Thermofisher-Applied
461 Biosystems). An assay targeting the 625-700 bp region of AchPV2 (JX051320), consists
462 of forward primer: D-715 (5'-GCAGGTCTGGATCACAGTATGC -3'), reverse primer
463 D-716 (5'-TGCCAGTCGCCTCTCATCT -3'), and probe
464 D-717 (5' [FAM]-TGCATGACAGCATATGATCAGCCCACT-[BHQ-1]-3'. The
465 optimized primer and probe concentrations and assay conditions were as follows: forward
466 primer (D-715) and reverse primer (D-716): 300 nM, probe (D-716): 200 nM. Reactions
467 were performed using AgPath-ID One-Step RT-PCR Kit (Thermofisher-Ambion) on an
468 AB7500 Fast instrument using the thermal cycle: 1 cycle of 45 °C 10 min, 95 °C 10 min
469 followed by 45 cycles of 95 °C 15 sec, 60 °C 45 sec. For interpretation of results
470 duplicate samples producing an average cycle threshold (Ct) less than 38 were considered
471 positive.
472
473 **Virus isolation**
474 Vero cell monolayers were grown in 96 well tissue culture plates to 80 % confluency in
475 cell media (Minimal Essential Medium containing Earle's salts and supplemented with 2
476 mM glutamine, antibiotic-antimycotic and 10 % fetal calf serum).
477 Swab media and blood were serially diluted 10 fold and 50 µl added to each well.
478 Supernatant from centrifuged tissue homogenate was serially diluted 10 fold and 50 µl

added to each well. Vero cell monolayers were observed for viral CPE seven days post infection.

Serology

Serum was collected prior to viral challenge and again at euthanasia, and tested using a standard virus neutralisation test. Serial two-fold dilutions of test sera were prepared in duplicate in a 96-well tissue culture plate in 50 µL cell media (Minimal Essential Medium containing Earle's salts and supplemented with 2 mM glutamine, antibiotic-antimycotic and 10 % fetal calf serum). An equal volume of either AchPV1 or AchPV2 working stock containing 200 $TCID_{50}$ was added and the virus-sera mix incubated for 30 min at 37 °C in a humidified 5 % CO_2 incubator. 100 µL of Vero cell suspension containing 2 x 10^5 cells/mL was added and the plate incubated at 37 °C in a humidified 5 % CO_2 incubator. The plate was observed for viral CPE after seven days and the serum neutralisation titre determined.

Histology and immunohistochemistry

Formalin-fixed tissues were processed into paraffin wax and prepared into 4 µm thick sections using routine histological methods. For immunohistochemistry, antigen retrieval was performed using the DAKO PT LINK machine (Dako, Glostrup, Denmark) by heating the tissue sections to 97 °C for 30 minutes and then cooling to 70 °C in the Envision Flex Target high pH retrieval solution (DAKO) and washing for 5 minutes in Tris Buffer. After this, endogenous peroxidases were quenched by the addition of 3 % H_2O_2 solution. Tissue sections were then incubated with the primary antibody, polyclonal

rabbit antisera raised against AchPV2, at a dilution of 1:2000. The visualization system used was Envision FLEX /horseradish peroxidase (HRP) conjugated with 3-Amino-9-Ethylcarbazole (AEC) chromogen (DAKO AEC + substrate chromagen K3469). Slides were then counterstained with Lillie-Mayer haematoxylin (Australian Biostain, Traralgon, Australia) and Scotts tap water before mounting. A duplicate set of tissue sections were stained with hematoxylin and eosin stain for histological examination using routine methods.

Statistical analysis

In the time-course study, bodyweights and rectal temperatures of ferrets, and bodyweights and microchip temperatures of guinea pigs, up to and including day 6 pc were compared using a repeated measures ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 7.02).

Methods word count: 1328

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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We thank Jessica Haining, Sarah Riddell and Rachel Arkinstall from the AAHL Animal Studies Team for their help with the animal trials.

Author Contributions

D.M and J.B wrote the main manuscript text. J.B, S.T, G.C, A.F, L.F, J.P & J.H performed the experiments. J.P and J.H prepared figure I and J.B prepared figures II & III. All authors reviewed the manuscript and contributed to content and layout.

Additional Information

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Figures

Figure I. Viral antigen in bronchiolar epithelial cells and BALT in ferret #9 (polyclonal rabbit anti-AchPV2): note also intraluminal acute inflammatory infiltrate, of uncertain pathogenic significance.

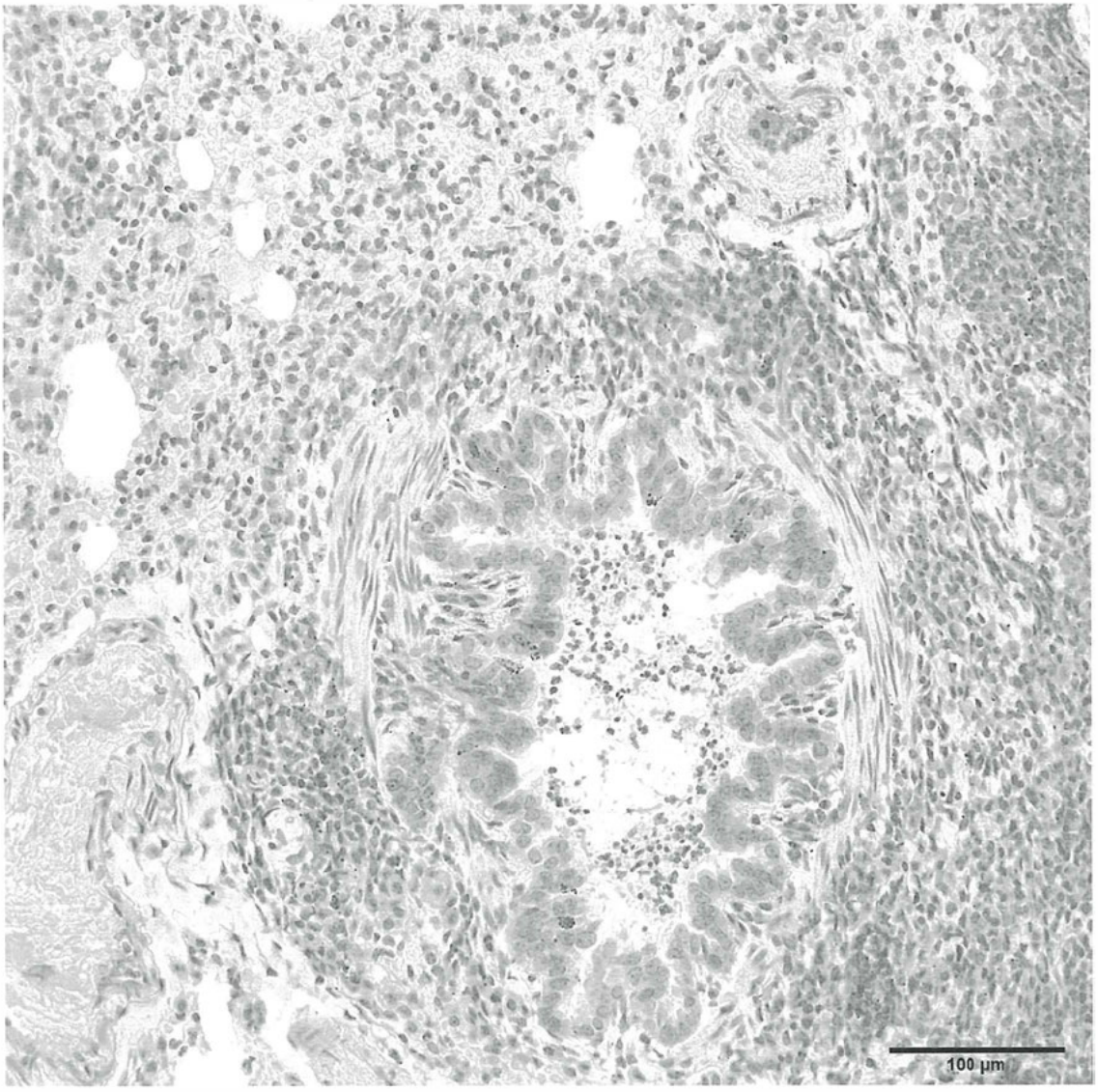
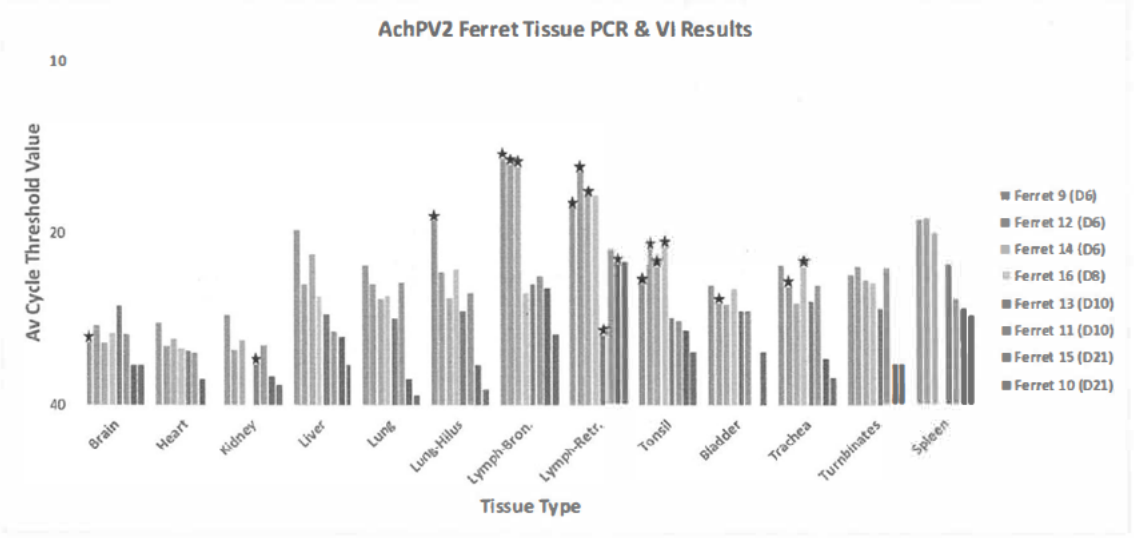
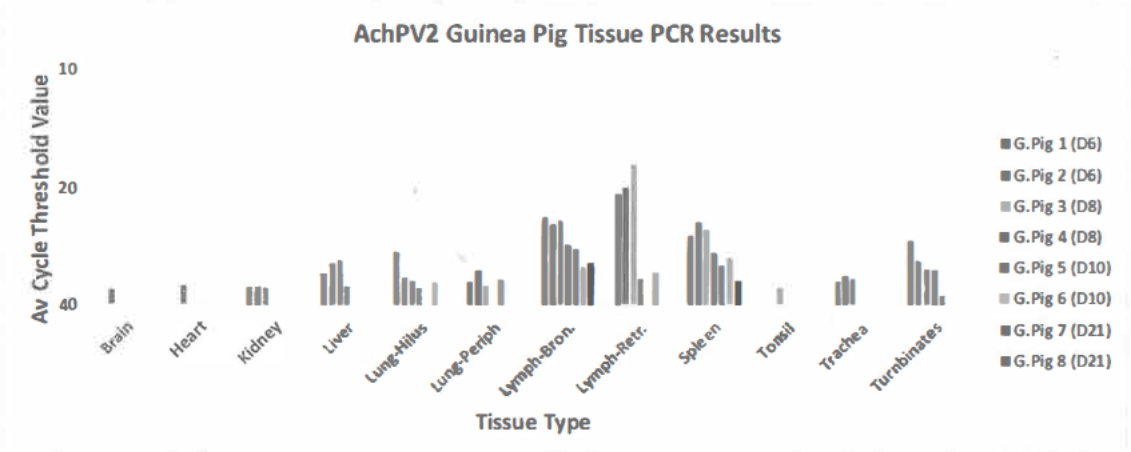


Figure II. Analysis of virus infection in ferrets by RNA detection and virus isolation. Average cycle threshold (Ct) values were obtained from testing tissues from AchPV2 ferrets using RT-qPCR. Stars indicate samples that AchPV2 was re-isolated from.



635 **Figure III.** Analysis of virus infection in guinea pigs by RNA detection and virus
636 isolation. Average cycle threshold (Ct) values were obtained from testing tissues from
637 AchPV2 guinea pigs using RT-qPCR. AchPV2 was unable to be re-isolated from any
638 sample.
639



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Tables

Table 1. The serum neutralisation titres against AchPV1 and AchPV2 for ferret and guinea pig serum collected 21 days pc. The serum collected from the animals pre-challenge (day 0) were all negative. Mouse sera were also tested but the data is not shown as they didn't seroconvert.

AchPV1		AchPV2	
Animal	SNT Titre	Animal	SNT Titre
Ferret 1	1:320	Ferret 1	>1:1280
Ferret 2*	1:80	Ferret 2	1:1280
Guinea Pig 1	1:40	Guinea Pig 1	1:160
Guinea Pig 2	1:80	Guinea Pig 2	1:40
Guinea Pig 3	1:320	Guinea Pig 3	1:80
Guinea Pig 4	1:80	Guinea Pig 4	1:320

*This animal became ill and was euthanized at day 14 pc instead of day 21 pc.

Table 2. Analysis of viral shedding and viraemia in ferrets by RNA detection and virus isolation. Average cycle threshold (Ct) values were obtained from testing oral and rectal swabs, nasal washes and blood from AchPV2 ferrets using RT-qPCR.

KEY: - indicates sample was negative (Av Ct >38); NA indicates sample was unavailable for testing; * indicates virus re-isolated at neat dilution; ** indicates virus re-isolated at 1:5 dilution and *** indicates virus re-isolated at 1:50 dilution

		Days Post Challenge						
	Sample	**0**	**2**	**4**	**6**	**8**	**10**	**21**
Ferret 9	Oral Swab	-	-	32.9	24.4 ***			
	Rectal swab	-	-	34.1	24.9 **			
	Nasal wash	-	36.8	33	26 ***			
	Blood	-	-	NA	31.5			
Ferret 12	Oral Swab	-	-	32.10	21.8			
	Rectal swab	-	-	29.50	22.6			
	Nasal wash	-	-	31.40	25.1			
	Blood	-	35.9	26.50	NA			
Ferret 14	Oral Swab	-	36.9	31.90	33			
	Rectal swab	-	-	32.20	24.8			
	Nasal wash	-	-	31.40	27.1			
	Blood	-	37.8	29.10	28.7			
Ferret 16	Oral Swab	-	34.5	32.50	22.2 *	24 *		
	Rectal swab	-	-	32.00	22.6	23		
	Nasal wash	-	-	34.10	28.3	27 **		
	Blood	-	-	31.30	30.1	20.9		
Ferret 13	Oral Swab	-	36	31.50	23.5	24	23.60	
	Rectal swab	-	-	29.50	22.8	23.4	23.70	
	Nasal wash	-	-	33.60	25.9	22.3	24.50	
	Blood	-	-	29.40	29.2	30.8	34.00	
Ferret 11	Oral Swab	-	-	31.9	22.9 ***	22	25.30	
	Rectal swab	-	-	30.9	22	19.6	25.50	
	Nasal wash	-	-	32.00	24.8	22.8	25.00	
	Blood	-	-	28.90	NA	27.3	36.10	
Ferret 15	Oral Swab	-	35	32.90	25.8	20.9	26.50	30
	Rectal swab	-	-	31.30	23.2	22.5	23.70	29
	Nasal wash	-	34.3	34.20	26.3	25	25.30	30.5
	Blood	-	-	24.70	NA	24	36.00	30
Ferret 10	Oral Swab	-	-	30.5	30.3	23.3	27.00	31.2
	Rectal swab	-	-	29	23.6	22.4	27.00	32.5
	Nasal wash	-	37	31.9	25.3 **	24.6	26.30	28.5
	Blood	-	-	-	NA	NA	33.80	-

672

673

Table 3. Analysis of viral shedding and viraemia in guinea pigs by RNA detection and virus isolation. Average cycle threshold (Ct) values were obtained from testing oral and rectal swabs and blood from AchPV2 guinea pigs using RT-qPCR. AchPV2 was unable to be re-isolated from any sample.

KEY: - indicates sample was negative (Av Ct >38)

		Days Post Challenge						
	Sample	**0**	**2**	**4**	**6**	**8**	**10**	**21**
Guinea Pig 1	Oral Swab	-	-	-	-			
	Rectal swab	-	-	-	-			
	Blood	-	-	-	-			
Guinea Pig 2	Oral Swab	-	-	-	37.74			
	Rectal swab	-	-	-	-			
	Blood	-	-	-	35.25			
Guinea Pig 3	Oral Swab	-	-	-	-	36.44		
	Rectal swab	-	-	-	-	35.09		
	Blood	-	-	-	-	34.71		
Guinea Pig 4	Oral Swab	-	-	-	-	-		
	Rectal swab	-	-	-	-	37.31		
	Blood	-	-	-	-	-		
Guinea Pig 5	Oral Swab	-	-	-	-	-	-	
	Rectal swab	-	-	-	-	-	-	
	Blood	-	-	-	-	-	-	
Guinea Pig 6	Oral Swab	-	-	-	-	-	-	
	Rectal swab	-	-	-	-	-	-	
	Blood	-	-	-	-	-		
Guinea Pig 7	Oral Swab	-	-	-	-	-	-	-
	Rectal swab	-	-	-	-	35.39	34.97	-
	Blood	-	-	-	-	-	-	-
Guinea Pig 8	Oral Swab	-	-	-	-	-	-	-
	Rectal swab	-	-	-	-	33.98	-	-
	Blood	-	-	-	-	-	-	-

681 **Table 4.** The serum neutralisation titres against AchPV2 for ferret and guinea pig serum
682 collected on days 6, 8, 10 and 21 pc. Sera collected on day 2 and 4 pc were also tested but
683 were negative for neutralising antibodies (data not shown).

Animal	SNT Titre	Animal	SNT Titre
Ferret 9* (Day6)	1:20	G.Pig 1 (Day6)	1:20
Ferret 12 (Day6)	1:40	G.pig 2 (Day6)	1:20
Ferret 14 (Day6)	1:640	G.Pig 3 (Day8)	1:20
Ferret 16 (Day8)	1:640	G.pig 4 (Day8)	1:20
Ferret 11 (Day10)	1:640	G.Pig 5 (Day10)	1:40
Ferret 13 (Day10)	>1:1280	G.pig 6 (Day10)	1:40
Ferret 10 (Day21)	>1:1280	G.Pig 7 (Day21)	1:160
Ferret 15 (Day21)	>1:1280	G.pig 8 (Day21)	1:160

684 *This animal became ill and was euthanized at day 6 pc instead of day 8 pc

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s22

From: Andrew Cunningham <s22
Sent: Wednesday, 7 February 2018 12:26 PM
To: 'Jennifer.Barr s22 Shawn.Todd s22 garycrameri s22 Adam.Foord s22 Glenn.Marsh s22 leah.frazer s22 Jean.Payne s22 enni.Rooke s22 Kate.Baker s22 s22 middled s22 linfa.wang s22
Subject: RE: [EXT]: RE: AchPV paper

Dear Jenn

Many thanks for doing this. It all looks fine to me, apart from one sentence in the Abstract and cover letter which I think should be amended from:

"The(se) results indicate AchPV's are able to cross the species barrier and may infect domesticated animals and humans in areas of Africa where their reservoir hosts are present."

to:

"The(se) results indicate AchPV's are able to cross the species barrier, therefore vigilance for infection with, and disease caused by, these viruses in people and domesticated animals is warranted in sub Saharan Africa and the Arabian peninsula where the reservoir hosts are present."

Cheers

Andrew

Andrew A. Cunningham BVMS PhD Dip. ECZM (Wildlife Population Health) FRCVS
Professor of Wildlife Epidemiology & Deputy Director, Institute of Zoology
Honorary Professor, University College London
Visiting Professor, Royal Veterinary College
Visiting Professor, Universidad Andres Bello
Visiting Professor, University of Leeds

Institute of Zoology
Zoological Society of London
Regent's Park
London NW1 4RY

s22

Duplicate Email - Removed

[redacted]

From: Barr, Jenn (H&B, Geelong AAHL [redacted]
Sent: Tuesday, 29 May 2018 1:50 PM
To: Wang Linfa; Deborah Middleton; Andrew Cunningham; James Wood [redacted] Baker, Kate; Gary Crameri; Todd, Shawn (H&B, Geelong AAHL); Marsh, Glenn (H&B, Geelong AAHL); Foord, Adam (AAHL, Geelong AAHL); 'Leah Frazer'; Payne, Jean (AAHL, Geelong AAHL); Harper, Jenni (AAHL, Geelong AAHL)
Subject: FW: Scientific Reports: Decision letter for SREP-18-06200

Hi all,
We've finally heard from Scientific Reports re the AchPV paper (I'm sure you've nearly forgotten about it, I know I had).
The Reviewers comments are below. They don't look too bad.
Hopefully I'll get a chance to look at this over the next week or two and will let you know where I need your input.
Cheers,

Jenn

Jennifer Barr BSc (Biotech) (Hons)
Research Assistant
Dangerous Pathogens Team
CSIRO Australian Animal Health Lab
[redacted]

I work part time: Tues/Wed/Thurs

Reviewer comments:

Reviewer #1 (Remarks to the Author):

The authors performed infection studies of bat paramyxovirus, AchPV1 and AchPV2, in ferrets, guinea pigs and BALB/c mice and provided valuable information on the cross-species transmission and the development of animal models for AchPV1 and AchPV2. The information is critical for the risk assessment of novel bat viruses. There are several questions, concerns, and comments for the manuscript though.
1. English editing is recommended for some sentences with fragmentations and unclear punctuations. Ex. Line 38, line 50, line 211, line 292, line 312, line 361-362, line 398, line 403, line 412, and line 416.
2. Some words used by the authors are wrong or unclear.
EX. 2.1 BALB/c (Balb/s) mice is not Balb-c.
2.2 It is not clear why the authors used "elective or electively" before euthanasia. Are there any criteria to "choose" which animal to sacrifice at each time point?
2.3 If the authors already used "euthanased", it would be better not to use "killed" in line 211 and 220.
3. Abstract: No quantitative data was listed in the abstract and the authors should summarize and present the data in the abstract. The background part can be shortened to meet the limitation of word counts in abstract.

4. Introduction:
4-1 The reference [1] cited in line 50 is about bacterial zoonosis. Maybe it would be better to cite a reference about viral zoonosis.
4-2 It is recommended to provide N protein amino acid sequence identities between AchPV1 and AchPV2 so the readers can compare the data from the same protein amino acid identities from reference [15] and [16].
5. Experimental Design:
5-1 Please explain why there were no animals without viral infection for negative control.
5-2 Please explain the reason that the length of RT-qPCR amplicon is about 625-700bp, which is much longer than the suggested amplicon length of 100-200bp. Can the authors provide any verified data or controls or previous reference for their RT-qPCR, including standard curve with the proper concentration range and the controls of non-template, efficiency, sensitivity and reproducibility.
5-3 Please explain the definition of negative VN results. It is no neutralization found in the tests of undiluted serum samples?
5-4 It would be better if the authors can put the data of body weights and temperature with statistical analysis in the supplementary information.
5-5 Please explain the reason for the termination dates at 6, 8, and 10 dpc because the dates are so close.

Reviewer #2 (Remarks to the Author):

Barr and colleagues present data on the potential zoonotic viruses Achimotavirus virus 1 (AchPV1) and Achimotavirus 2 (AchPV2) of laboratory infections in mice, guinea pigs, and ferrets. Clinical signs, viral load, serum neutralizing titers, and immunohistochemistry results are presented with the overall description being more focused on AchPV2 in guinea pigs and ferrets. The authors present important data on AchPV2 as it replicated in two separate species though much better in the ferrets. These data suggest that AchPV2 has zoonotic potential and should be monitored closely considering the close proximity of the bat (Eidolon helvum) roosts in urban areas as this virus was isolated from bat urine.

General comments:

In looking closely at the data from the second ferret experiment, it appears that virus was isolated from all of the ferrets in one sample at least (Swab or tissue). These are strong data and it was not apparent from the text but would make the manuscript stronger if these data are connected in the text.

For the serum neutralization dilutions reported: Are these 100% neutralization titers, 80%, 50%, etc? This was not clear from the Methods, Results, or Table Legends.

Discussion, Lines 355-358: There's viremia by viral RNA detection at least. The last lines are in reference to Ferret 9 which did not have virus isolated from blood; how does this explanation of virus in the blood of the brain being responsible for isolation but there not being virus isolated straight from blood make sense? Is it not possible that the virus is cell associated in the blood or tissues; like Nipah virus as an example? Infectious Nipah is difficult to isolate from blood and tissue compared to the high RT-qPCR loads detected.

s22

From: Wang Linfa s22
Sent: Tuesday, 29 May 2018 2:42 PM
To: Jennifer.Barr s22 middled35 s22 A.Cunningham s22 jlnw2 s22 Kate.Baker s22 garycrameri1 s22 s22 G h s22 Adam.Foord s22 leah.fraze s22 s22 Jean.Payne s22 Jenni.Rookes s22
Subject: RE: Scientific Reports: Decision letter for SREP 18-06200

Hi Jenn,

Well done!

I agree that the comments are all reasonable.

I am happy to go through it once you have a revised version and a draft rebuttal letter.

Cheers,

LF

Linfa (Lin-Fa) WANG, PhD FTSE
Professor & Director
Programme in Emerging Infectious Disease
s22

Duplicate Email - Removed

s22

From: James Wood s22
Sent: Tuesday, 29 May 2018 4:59 PM
To: Jennifer.Barr s22 linfa.wang s22 ; middled s22 A.Cunninghan s22 Kate.Ba s22 garycra s22 Shawn.Tod s22 Glenn.Marsh s22 Adam.Foord s22 leah.frazer s22 s22 ; Jean.Payne s22 Jenni.Rookes s22
Subject: RE: Scientific Reports: Decision letter for SREP 18 06200

That's great Jen! Well done
Cheers
James

From: Jennifer.Barr s22
Sent: 29 May 2018 0
To: linfa.wang s22 ; middled s22 A.Cunningham s22 James Wood s22 Kate.Baker s22 k; garycrameri s22 ; Shawn.Tod s22 Glenn.Marsh s22 Adam.Foor s22 leah.frazer s22 Jean.Payn s22 Jenni.Rookes s22
Subject: FW: Scientific Reports: Decision letter for SREP-18 06200

Hi all,
We've finally heard from Scientific Reports re the AchPV paper (I'm sure you've nearly forgotten about it, I know I had).
The Reviewers comments are below. They don't look too bad.
Hopefully I'll get a chance to look at this over the next week or two and will let you know where I need your input.
Cheers,

Jenn

Jennifer Barr BSc (Biotech) (Hons)
Research Assistant
Dangerous Pathogens Team
s22

I work part time: Tues/Wed/Thurs

Reviewer comments:

Reviewer #1 (Remarks to the Author):

The authors performed infection studies of bat paramyxovirus, AchPV1 and AchPV2, in ferrets, guinea pigs

and BALB/c mice and provided valuable information on the cross-species transmission and the development of animal models for AchPV1 and AchPV2. The information is critical for the risk assessment of novel bat viruses. There are several questions, concerns, and comments for the manuscript though.
1. English editing is recommended for some sentences with fragmentations and unclear punctuations. Ex. Line 38, line 50, line 211, line 292, line 312, line 361-362, line 398, line 403, line 412, and line 416.
2. Some words used by the authors are wrong or unclear.
EX. 2.1 BALB/c (Balb/s) mice is not Balb-c.
2.2 It is not clear why the authors used "elective or electively" before euthanasia. Are there any criteria to "choose" which animal to sacrifice at each time point?
2.3 If the authors already used "euthanased", it would be better not to use "killed" in line 211 and 220.
3. Abstract: No quantitative data was listed in the abstract and the authors should summarize and present the data in the abstract. The background part can be shortened to meet the limitation of word counts in abstract.
4. Introduction:
4-1 The reference [1] cited in line 50 is about bacterial zoonosis. Maybe it would be better to cite a reference about viral zoonosis.
4-2 It is recommended to provide N protein amino acid sequence identities between AchPV1 and AchPV2 so the readers can compare the data from the same protein amino acid identities from reference [15] and [16].
5. Experimental Design:
5-1 Please explain why there were no animals without viral infection for negative control.
5-2 Please explain the reason that the length of RT-qPCR amplicon is about 625-700bp, which is much longer than the suggested amplicon length of 100-200bp. Can the authors provide any verified data or controls or previous reference for their RT-qPCR, including standard curve with the proper concentration range and the controls of non-template, efficiency, sensitivity and reproducibility.
5-3 Please explain the definition of negative VN results. It is no neutralization found in the tests of undiluted serum samples?
5-4 It would be better if the authors can put the data of body weights and temperature with statistical analysis in the supplementary information.
5-5 Please explain the reason for the termination dates at 6, 8, and 10 dpc because the dates are so close.

Reviewer #2 (Remarks to the Author):

Barr and colleagues present data on the potential zoonotic viruses Achimotavirus virus 1 (AchPV1) and Achimotavirus 2 (AchPV2) of laboratory infections in mice, guinea pigs, and ferrets. Clinical signs, viral load, serum neutralizing titers, and immunohistochemistry results are presented with the overall description being more focused on AchPV2 in guinea pigs and ferrets. The authors present important data on AchPV2 as it replicated in two separate species though much better in the ferrets. These data suggest that AchPV2 has zoonotic potential and should be monitored closely considering the close proximity of the bat (Eidolon helvum) roosts in urban areas as this virus was isolated from bat urine.

General comments:

In looking closely at the data from the second ferret experiment, it appears that virus was isolated from all of the ferrets in one sample at least (Swab or tissue). These are strong data and it was not apparent from the text but would make the manuscript stronger if these data are connected in the text.

For the serum neutralization dilutions reported: Are these 100% neutralization titers, 80%, 50%, etc? This was not clear from the Methods, Results, or Table Legends.

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not possible that the virus is cell associated in the blood or tissues; like Nipah virus as an example? Infectious Nipah is difficult to isolate from blood and tissue compared to the high RT-qPCR loads detected.

[redacted]

From: Baker, Kate [redacted]
Sent: Wednesday, 15 August 2018 5:55 PM
To: James Wood; 'Wang Linfa'; Jennifer.Barr [redacted]
Cc: middled[redacted]A.Cunningham[redacted] Kate.Baker[redacted]
garycrameri[redacted] Shawn.Todd[redacted] Glenn.Mars[redacted]
Adam.Foord[redacted] leah.frazer14[redacted] Jean.Payne[redacted]
Jenni.Rookes[redacted]
Subject: Re: Scientific Reports: Decision letter for SREP-18-06200A

Great news, congrats! And a massive thanks to Jenn for continuing to push this along!

From: James Wood [redacted]
Sent: 15 August 2018 07:35:32
To: 'Wang Linfa'; Jennifer.Barr [redacted]
Cc: middled[redacted].Cunningham[redacted] Kate.Baker[redacted] garycrameri[redacted]
Shawn.Todd[redacted] Glenn.Mars[redacted] dam.Foord[redacted] eah.frazer[redacted]
Jean.Payne[redacted] Jenni.Rookes[redacted]
Subject: RE: Scientific Reports: Decision letter for SREP 18-06200A

Great news Jen!!
Thanks
James

From: Wang Linfa [redacted]
Sent: 15 August 2018 02:15
To: Jennifer.Barr[redacted]
Cc: middled35[redacted] A.Cunningham[redacted] James Wood[redacted]
Kate.Baker@liverpool.ac.uk; garycrameri1@gmail.com; Shawn.Todd@csiro.au; Glenn.Marsh[redacted]
Adam.Foord[redacted] leah.frazer[redacted]; Jean.Payne[redacted] Jenni.Rooke[redacted]
Subject: Re: Scientific Reports: Decision letter for SREP 18 06200A

Great!

Thanks and congrats to all!!

LF

Sent from my iPhone

On 15 Aug 2018, at 9:12 AM, [redacted] wrote:

> Finally!!!!!!!!!
>
> Thanks everyone for your help to get this published.
> Cheers,
>
> Jenn

From: scientificreports[redacted]
Sent: Monday, 13 August 2018 7:11 PM

To: Barr, Jenn (H&B, Geelong AAHL) <J[redacted] s22
Subject: Scientific Reports: Decision letter for SREP 18-06200A

Dear Mrs Barr,

We are delighted to accept your manuscript entitled "Animal infection studies of two recently discovered African bat paramyxoviruses, Achimota 1 and Achimota 2" for publication in Scientific Reports. Thank you for choosing to publish your work with us.

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A form to order reprints of your article is available at http://www.nature.com/reprints/author-reprints.html. To obtain the special author reprint rate, orders must be made within a month of the publication date. After that, reprints are charged at the normal (commercial) rate.

We look forward to publishing your article.

Best regards,

Muhammad Munir
Editorial Board Member
Scientific Reports

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s22

From: SMITH Craig s22

Sent: Wednesday, 24 April 2019 11:03 AM

To: hume.field s22 David.jordan s22 Edson, Daniel; Kerryn Parry Jones s22 s22 debra.melville s22 Alice Broos; Lee Anne McMichael; KUNG Nina; peter.kirkland s22 MAYER David; Alison Peel (s22 Alison Höger; Miranda Vidgen; Jo Kristoffersen; DE JONG Carol; BARRETT Janine; David.jordan s22 Plowright, Raina; Jon Epstein; LEE Jonathan; Andrew Breed; GORDON Anita N; abgawkin s22 Adam.Mckeown s22 Alyssa Pyke s22 David Warrilow s22 ; Billie Roberts s22 s22 CASSIDY Robert; Craig McLaughlin DNR; Wang Linfa; Peter Daszak; DIALLO Ibrahim; UNDERWOOD Darren; Gary Crameri; gerardommc s22 Glenn.Marsh s22 ; Ina.Smith s22 Michelle.Baker s22 Hamish McCallum (s22 ; Joanne Meers; Kim.Halpin s22 Jaewoon Jeong s22 John Giles; Justin Welbergen s22 AGNIHOTRI Kalpana; Mel Taylor s22 JORGENSEN Wayne; Woods, Rupert s22 Tiggy Grillo; Keren Cox Witton

Subject: OPEN DATA Hendra virus test results: under roost flying fox urine

Dear all,

An outcome of the National Hendra Virus Research Program was the collection of 14,988 flying fox pooled urine samples collected from 50 roosts across 20° of latitude from Cairns in northern QLD (latitude 16.9° S) to Bateman's Bay in southern NSW (latitude 35.7° S).

This project was the result of a collaboration between the Queensland Department of Agriculture and Fisheries and the New South Wales Department of Primary Industries. The findings from this project can be found in the scientific manuscript Spatiotemporal Aspects of Hendra Virus Infection in Pteropid Bats (Flying-Foxes) in Eastern Australia.

The raw data from this manuscript is now available for use by any person under a Creative Commons Attribution 4.0 license and can be accessed on the Queensland Government data portal.

Please share this link with any interest person.

Thanks,

Craig Smith PhD
Senior Scientist, Biosecurity Queensland
Department of Agriculture and Fisheries
s22
W www.daf.qld.gov.au/biosecurity
s22
PO Box 156, Archerfield BC QLD 4108
Queensland Government

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s22

From: Gary Crameri s22
Sent: Saturday, 13 July 2019 12:06 PM
To: James Wood
Cc: Kate.Baker s22 Glenn.Marsh s22 Jennifer.Barr s22
Ina.Smith s22 Michelle.Baker s22 linfa.wang s22
Shawn.Todd s22 A.Cunningha s22 mary.tachejian s22
aferlasvet s22 suuire s22 Sandra.Crameri s22
claire.holmes s22 Pablo.Murcia s22
Subject: Re: AchPV3 paper

Great to hear from the UK crew we were talking a couple of days ago about our African adventure as we watched the bats in Outback Qld. I am currently in Birdsville waiting to see Midnight Oil at the most remote concert on earth. Unfortunately we are waiting with the wind and dust and flies but thats the outback!!!
Kate if you have the energy I support James and I am sure Jenn will be able to furnish lots of ideas 😉
Stay in touch and good luck.
Yr friend G

On Thu, 4 Jul 2019, 9:26 PM James Wood s22 wrote:

> Gary cc'd here Kate – I have no idea how you found the time to do this, but do think that the quality and amount of work that you did on this merits more than just an un refereed announcement. Others will comment with more knowledge though.
>
> Well done!
>
> Best wishes
>
> James

From: Baker, Kate s22
Sent: 04 July 2019 12:01
To: Glenn.Marsh s22 Jennifer.Barr s22 Ina.Smith s22 Michelle.Baker s22
linfa.wang s22 ; Shawn.Todd s22 A.Cunningham s22 mary.tachejian s22
aferlasvet s22 suuire s22 Sandra.Crameri s22 ; claire.holmes s22
Pablo.Murci s22 James Wood s22
Subject: AchPV3 paper

Dear all,

Feel a bit like a ghost getting back in touch about this after nearly 6 years but I'm writing to resuscitate the publication of AchPV3!

I've attached a current draft which I'd appreciate your comments/feedback on. Particularly the classifications as there appears to have been a new genus defined since I stepped sideways from the world of virology – sad they didn't go for Chiropterulavirus in the end, so Pararubulavirus it is! I have tried to integrate this in the manuscript text and have added Teviot virus to the phylogenetic analyses (which I found as the only new genus member since I did the original one, but ICTV seems to list three further ones which I can't find data from on NCBI – Scoliodon, Hopichthys and Cynoglossus paramyxoviruses??

I've lost track of who did what a bit and who might be the most relevant co-corresponding author, so please feel free to suggest changes to author order, particularly from the antipodes.

Now that it's managed to grow on Veros there's not a huge amount to the paper, so I'm wondering what we should aim for in terms of journal or how to strengthen it. Are JGV, etc still interested in this kind of thing or if there's an isolated virus, or do we just count our losses and go for an ASM genome announcement (not peer reviewed, but indexed)? Let me know your thoughts.

If you could please get your comments to me by the **end of July**, I'd really appreciate it. If you have no comments/time to comment please at least make sure you check your affiliation and let me know that you're happy to be an author.

Best,

Kate

P.S. Could someone please reply-all to cc in Gary at a new email address and

P.P.S. It's really nice to have an excuse to be in touch with everyone again! I have missed you all in the bacterial world and I hope everyone is well. Things are good here – set up as a principal investigator at the University of Liverpool and have a small microbial genomics group (with a touch of lab work) looking mostly into *Shigella* bacteria. Personally, I'm living the happily ever after with my English beau and have two lovely kids who keep us busy 😊

--

Dr. Kate S Baker BVSc PhD MRCVS

Wellcome Trust Clinical Research Career Development Fellow

Tenure Track Fellow

Institute for Integrative Biology

University of Liverpool

s22

From: Barr, Jenn (H&B, Geelong AAHL) s22
Sent: Thursday, 25 July 2019 9:26 AM
To: Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Smith, Ina (H&B, Black Mountain); Baker, Michelle (H&B, Geelong AAHL); linfa.wang s22 Todd, Shawn (H&B, Geelong AAHL); A.Cunningham s22 Tachedjian, Mary (H&B, Geelong AAHL); aferlasvet s22 suuire s22 Crameri, Sandra (AAHL, Geelong AAHL); claire.holmes s22 Pablo.Murcia s22 .uk; James Wood; Gary Crameri
Subject: RE: AchPV3 paper
Attachments: AchPV3 Final JB24July19.docx

Hi Kate,
Great to see this coming together! And lovely to hear from you.
I've had a look and added in the growth in Vero-E6 to the results. I'm not sure of the best way to publish this, but there is enough there for a short communication at least. Depending on who we submit to, it might be better to combine the results and discussion. I'll leave that to others to comment who have more experience in that area...maybe Linfa has an idea?
So, as far as the discussion now and trying to bulk it out a little, AchPV3 was unable to be isolated in Vero cells but I was able to grow the PaKi x2 stock in Vero-E6 cells...there may be some discussion possible around the difference between Vero and VeroE6 cells? Or about isolation vs propagation? Also, the entire stock that I grew in Vero-E6 was used for sequencing and that is literally all we have done with this virus. So I'm wondering if I should do a further experiment to inoculate Vero E6 cells again, see if I get the same result, harvest the stock and pass again onto Vero-E6 and see if CPE appears more quickly...what do you think? Could provide some more discussion about adaption to veroE6 or something...what do others think? Obviously we would want to publish this without doing too much further work but I would be happy to do a couple of small experiments if it was helpful for the story.
Keep pushing this Kate, it will be great to get it out.
Cheers,

Jenn

Jennifer Barr BSc (Biotech) (Hons)
Dangerous Pathogens Team
CSIRO Australian Animal Health Lab
s22

I work part time: Tues/Wed/Thurs

From: Baker, Kate <s22
Sent: Thursday, 4 July 2019 9:01 PM
To: Marsh, Glenn (H&B, Geelong AAHL) <Glenn.Marsh s22 ; Barr, Jenn (H&B, Geelong AAHL) <Jennifer.Barr s22 Smith, Ina (H&B, Black Mountain) <Ina.Smith s22 Baker, Michelle (H&B, Geelong AAHL) <Michelle.Baker s22 linfa.wang s22 Todd, Shawn (H&B, Geelong AAHL) <Shawn.Todd s22 A.Cunningham s22 mary.tachejian s22 aferlasvet s22 suuire s22 Crameri, Sandra s22 claire.holmes s22

Pablo.Murcia [redacted] James Wood [redacted]
Subject: AchPV3 paper

Dear all,

Feel a bit like a ghost getting back in touch about this after nearly 6 years but I'm writing to resuscitate the publication of AchPV3!

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If you could please get your comments to me by the **end of July**, I'd really appreciate it. If you have no comments/time to comment please at least make sure you check your affiliation and let me know that you're happy to be an author.

Best,
Kate

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--
Dr. Kate S Baker BVSc PhD MRCVS
Wellcome Trust Clinical Research Career Development Fellow
Tenure Track Fellow
Institute for Integrative Biology
University of Liverpool

Office days: Monday Thursday

Title

Achimota virus 3: a new member of the pararubulavirus genus

Commented [BK1]: Add in Teviot virus
Redo L gene phylogenies, but maybe wait to hear back
Add in whole genome sequence

Authors

Kate S Baker* [a b c], Mary Tachedjian[d], Jennifer Barr[d], Glenn A Marsh [d], Shawn Todd [d] Gary Crameri [d], Sandra Crameri [d], Ina Smith, Michelle Baker, Claire EG Holmes [d], Richard Suu-Ire [e f], Andres Fernandez-Loras [b], Andrew A Cunningham [b], Pablo R Murcia [g], James LN Wood [a], Lin Fa Wang * [d,h]

Formatted: Superscript

Formatted: Superscript

Affiliations

a University of Cambridge, Department of Veterinary Medicine, Madingley Rd, Cambridge, Cambridgeshire, CB3 0ES, United Kingdom

b Institute of Zoology, Zoological Society of London, Regent's Park, NW1 4RY, United Kingdom

c University of Liverpool, Institute for Integrative Biology, Liverpool, L69 7ZB, United Kingdom

d CSIRO Australian Animal Health Laboratories, Portarlington Road, East Geelong, Victoria, 3219, Australia

e Wildlife Division of the Forestry Commission, Accra, Ghana

f University of Ghana, Noguchi Memorial Institute for Medical Research, Legon, Accra, Ghana

g MRC University of Glasgow Centre for Virus Research, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G61 1QH, United Kingdom

h Program in Emerging Infectious Diseases, Duke NUS Graduate Medical School, Singapore

* corresponding authors

Introduction

Paramyxoviruses are important pathogens of man and domestic animals, and bats are recognized as having an increasingly complex role with these viruses. Bats are recognised as reservoir hosts for a wide variety of viral zoonoses [1], and interest in the relationship between bats and paramyxoviruses was triggered by the finding that megabats in Australia and Asia were the reservoir species for the deadly zoonoses Hendra (HeV) and Nipah (NiV) viruses, which infect a wide variety of mammalian hosts causing significant morbidity and mortality [2,3,4]. Since those times, paramyxoviral prospecting in bats has revealed a breadth and diversity of paramyxoviruses [5,6,7,8,9] that is greater than that observed in other mammalian orders [10].

Within the phylogenetic diversity of paramyxoviruses, there exists a unique sub clade of viruses in the genus *Pararubulavirus* (subfamily *Rubulavirinae*), that are almost exclusively derived from bats. The first member of this rubulavirus sub clade was Menangle pararubulavirus (MenPV); first found as a disease agent of pigs and humans [11] and subsequently linked with bats [12] in Australia. This was followed by the discovery of Tioman pararubulavirus (TioPV) in Malaysia, where the virus was incidentally isolated during NiV investigations [13]. And finally, the Achimota pararubulaviruses 1 and 2 (AchPV1 and AchPV2) and Teviot virus that were isolated from pooled bat urine underneath roosts in Ghana and Australia respectively [14] [Add

Teviot]. Although no clinical illness in humans has been reported, low titre virus neutralising antibodies have been detected in humans living proximate to isolation sites for both TioPV and AchPV2 [13,14]. In addition to these five viruses, molecular evidence exists for many further paramyxoviruses in bats that belong to this genus (including the Tuhoko pararubulaviruses (ThkPVs) for which full genomic information exists) [7,9,10,15,16]. Collectively, these findings show pararubulaviruses exist across all four continents of the old world (namely Europe, Africa, Australia, Asia), and are frequently associated with bas, often from bat species that live in close proximity with humans.

In fact, the only pararubulavirus that not isolated from bats is Sosuga virus (SosV) that caused febrile systemic illness in a wildlife biologist shortly after handling a wide variety of wildlife species. Given this patient history and the phylogenetic clustering of this pathogen with viruses only previously described in bats, the authors who discovered SosV suggest that the infection was likely contracted from bats [17]. In conjunction with the known zoonotic nature of MenPV and human serological findings ~~suggestive~~indicative of AchPV2 and TioPV infection, these data suggest an increasing link between this group of viruses and human disease.

Here we report the isolation and whole genome sequence of a novel *Pararubulavirus* from the African straw-coloured fruit bat *Eidolon helvum*. The virus is phylogenetically unique so may be an important tool in the continued study of this important genus.

Commented [BK2]: If anyone can come up with something more inspired than this please do!

Materials and Methods

Cell culture conditions. Experiments described used either Vero cells (ATCC CCL 81), Vero E6 cells (ATCC CRL 1586) or *Pteropus alecto* primary kidney (PaKi) cells [18]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with F12

Ham (Sigma), 10% fetal calf serum, double strength antibiotic/ antimycotic (200 U/ml penicillin, 200 µg/ml streptomycin, and 0.5 µg/ml fungizone amphotericin B; Gibco), and ciprofloxacin (10 µg/ml; MP Bio medicals), at 37°C in 5% CO_2.

Urine samples. Urine samples were collected from underneath a colony of *E. helvum* in Accra Ghana [19], as previously described [20].

Isolation methods. Three passages of virus isolation were attempted on urine samples U34 U72 on *Pteropus alecto* primary kidney cells (PaKi) monolayers, in the same manner as, and in parallel with, attempts previously described on Vero cells [20]. The only exception to the previously described protocol, is that for some samples (U38, 42, 48 49, 58 62, 70, 72) flasks were frozen at 80°C and thawed at room temperature prior to passage (to synchronise experiments). Supernatants of cultures showing signs of cytopathic effect (CPE) were tested for the presence of paramyxoviral RNA using previously described RT-PCR [21]. PCR products were cloned (pGEM T Easy, Promega) and capillary sequenced for phylogenetic analysis.

Isolate propagation. Following the confirmation of a paramyxoviral isolate, the ~~isolation~~cell monolayer was scraped into the media and the material was frozen at 80°C. After thawing at room temperature, 350µL of the supernatant/cell mixture was added to 75cm^2 near confluent monolayers of Vero and PaKi cells in minimal media for 1 hr ~~under~~with gentle rocking at 37°C. Following the incubation, media was topped up and the cells were observed for cytopathic effect (CPE).

Genomic sequencing and bioinformatic analysis. Stocks for genomic sequencing were grown by inoculating PaKi cell monolayers and Vero E6 monolayers with 50ul of working stock virus and monitoring for viral CPE daily. Viral supernatant was

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AchPV3 had similar genomic organization to exiting pararubulaviruses. The genome was 15,600 bp in length, obeying the rule of six, and had the coding capacity to encode

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Discussion

Here, we have found another novel pararubulavirus from fruit bats. Genomic analysis of the virus, AchPV3, reveals it to be a typical representative of this species phylogenetically clustering with other members of the pararubulavirus genus, and using a conserved mRNA editing site. This further increases the evidence of the role for bats as reservoirs for this group of viruses.

The meta-analysis of paramyxovirus polymerase gene fragments from this and other studies [5,8,9,15] demonstrates the occurrence of these viruses throughout the Old World. These bat-associated pararubulaviruses are linked with human disease, including the known human pathogens MenPV and SosPV, as well as those that are suggested to be capable of infecting humans on the basis of serological evidence, AchPV2 and TioPV [11,13,14,17]. Owing to their distribution across a wide geographical area and their association with human disease, pararubulaviruses warrant further study. Specifically, in order to separate potential zoonoses from harmless viruses, more work must be done to understand the host restriction of paramyxoviral tropism and the mechanisms of viral infection in the reservoir.

With respect to determining mechanisms of host restriction, AchPV3 did not appear to grow in Vero cells. This is distinct from AchPV1 and AchPV2, which were isolated in parallel from the same sample set [14], from the other bat-derived rubulaviruses specific to this sub-clade (i.e. MenPV, TioPV both grow readily on Vero cells [13,25]), as well as other rubulaviruses (e.g. MprPV, PorPV [26,27]). Notably however, attempts to isolate the human pathogen SosPV and its nearest relative ThkPVs on Vero cells failed [16,17], with SosPV having to be sub-cultured in neonatal mice prior to being capable of non-syncitial CPE on Vero-E6 cells [17]. Further study of this restriction of *in vitro* host range will aid understanding of the barriers to zoonotic transmission for bat-associated rubulaviruses. MAYBE CITE THE ANIMAL STUDIES OF ACHPVs IN HERE AND RELATIONSHIP WITH ACHPV2.

Furthermore, AchPV3 has significant potential to provide insight into the behavior of paramyxoviruses in their bat hosts. AchPV3 was isolated, and subsequently had to be passaged, by freeze-thawing entire cell-culture flasks, possibly indicating a cell associated infection and/or control and sequestration of the virus. This possibility was

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supported by the low amounts of AchPV3 that was released into the media (not shown), complicating genome sequencing. There was also only subtle CPE observed in the cell culture flasks, but this may have resulted from the primary nature of the cell culture line being used rather than any inherent pathogen difference.

Acknowledgements

We would like to thank the Veterinary Services Directorate (Ghana). The authors acknowledge the support of the Australian Microscopy and Microanalysis Research Facility to the microscopy facilities of the Australian Animal Health Laboratory. We also thank Mary Tachedjian for technical assistance. This study was funded by a Wellcome Trust research-training fellowship (KB) and KB is funded by a Clinical Research Career Development Fellowship (xxxxxxxxx). PRM was also funded by the Wellcome Trust. JLNW is supported by the Alborada Trust and the RAPIDD program of the Science and Technology Directorate, Department of Homeland Security.

Figure legends

Figure 1. Growth of AchPV3 in culture. *Pteropus alecto* primary kidney cells uninfected (A), and infected (B) with AchPV3 showing multinucleate syncitial cells (B, arrows). Under electron microscopy (C) the characteristic *Paramyxovirus* ribonucleoprotein (RNP) was seen (indicated in C, inset)

Figure 2. Midpoint-rooted maximum likelihood phylogenetic trees based on a 585 amino acid alignment of the nucleoprotein of *Paramyxovirinae* members (main) and a 176 amino acid alignment of the polymerase protein for the same taxa, and rubulavirus and pararubulavirus fragments detected in bats (inset). Samples starting with U are from *Eidolon helvum* in Ghana [5]. AchPV3 is starred, scale bars represent expected number of substitutions per site, and bootstrap values (of 100) of relevant sites are shown.

Tables

Table 1. Pair-wise amino acid identities for AchPV3 nucleocapsid (N) and phosphoproteins (P) with other Paramyxovirinae.

		Achimota virus 3	
Genus		N	P
Pararubulavirus	AchPV1	66	43
	AchPV2	75	47
	ThkPV1	60	38
	ThkPV2	66	39
	ThkPV3	60	41
	MenPV	64	41
	TioPV	65	39
	SosV	58	42
	TevPV	77	29
Rubulavirus	MuV	51	24
	MapPV	47	26
	SimPV41	45	25
	hPIV2	44	25
	SimPV5	47	25
	PorPV	49	25
	hPIV4	42	24
Morbillivirus	RPV	22	9
	MeV	23	8
	CDV	23	10
Henipavirus	HeV	27	7
	NiV (M)	27	7
	NiV (B)	27	8
Avulavirus	NDV	31	20
Respirovirus	SeV	19	5
Unclassified	BeiV	25	10
	JPV	22	9
	MosV	25	8
	TPMV	23	8

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Document 14

s22 [redacted]

From: Smith, Ina (H&B, Black Mountain) s22 [redacted]
Sent: Thursday, 25 July 2019 9:38 AM
To: Barr, Jenn (H&B, Geelong AAHL); Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Baker, Michelle (H&B, Geelong AAHL); linfa.wang s22 [redacted] Todd, Shawn (H&B, Geelong AAHL); A.Cunningham s22 [redacted] Tachedjian, Mary (H&B, Geelong AAHL); aferlasve s22 [redacted] suuire s22 [redacted] Crameri, Sandra (AAHL, Geelong AAHL); claire.holmes s22 [redacted] ; Pablo.Murcia s22 [redacted] James Wood; Gary Crameri
Subject: RE: AchPV3 paper
Attachments: Achimota 3 paper Final.docx

Hi Kate

It's good to hear from you and hear that you are living the dream!

I've attached some edits and comments to your original manuscript.

Cheers
Ina

Ina Smith, PhD
Senior Research Scientist | Risk Evaluation and Preparedness Program | Health and Biosecurity| CSIRO, Clunies Ross St, Black Mountain ACT 2601 Ph. 02 6218 3579

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Title

Achimota virus 3: a new member of the pararubulavirus genus

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Redo L gene phylogenies, but maybe wait to hear back
Add in whole genome sequence

Authors

Kate S Baker* [a b c], Mary Tachedjian, Jenn Barr, Glenn A Marsh [d], Shawn Todd [d] Gary Crameri [d], Sandra Crameri [d], Ina Smith, Michelle Baker, Claire EG Holmes [d], Richard Suu-Ire [e f], Andres Fernandez-Loras [b], Andrew A Cunningham [b], Pablo R Murcia [g], James LN Wood [a], Lin-Fa Wang * [d,h]

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h Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore

* corresponding authors

Introduction

Paramyxoviruses are important pathogens of man and domestic animals, and bats have beenare recognized as having an increasingly complex role with these viruses (REFS). Bats are recognised as reservoir hosts for a wide variety of viral zoonoses [1]., and iInterest in the relationship between bats and paramyxoviruses was triggered instigated by the finding that megabats in Australia and Asia were the reservoir species for the deadly zoonoses Hendra (HeV) and Nipah (NiV) viruses, which infect a wide variety of mammalian hosts causing significant morbidity and mortality [2,3,4]. Since those timesthen, paramyxoviral prospecting in bats has revealed a breadth and diversity of paramyxoviruses [5,6,7,8,9] that is greater than that observed in other mammalian orders [10].

Within the phylogenetic diversity of paramyxoviruses, there exists a unique sub clade of viruses in the genus *Pararubulavirus* (subfamily *Rubulavirinae*), that are almost exclusively derived from bats. The first member of this rubulavirus sub-clade was Menangle pararubulavirus (MenPV); first foundwhich was discovered as a disease causing agent of pigs and humans [11] and subsequently linked with bats [12] in Australia. This was followed by theThe discovery of Tioman pararubulavirus (TioPV) in Malaysiafollowed, where when the virus was incidentally isolated during NiV investigations [13]. And finally, the Achimota pararubulaviruses 1 and 2 (AchPV1 and AchPV2) and Teviot virus that were isolated from pooled bat urine underneath roosts

in Ghana and Australia respectively [14] [Add Teviot]. Although no clinical illness in humans has been reported, low-titre virus neutralising antibodies have been detected in humans living proximate to isolation sites for both TioPV and AchPV2 [13,14]. In addition to these five viruses, molecular evidence exists for many further paramyxoviruses in bats that belong to this genus (including the Tuhoko pararubulaviruses (ThkPVs) for which full genomic information exists) [7,9,10,15,16]. Collectively, these findings show pararubulaviruses exist across all four continents of the old world (namely Europe, Africa, Australia, Asia), and are frequently associated with bas, often from bat species that live in close proximity with humans.

In fact, the only pararubulavirus that was not isolated from bats ~~is~~ was Sosuga virus (SosV) that caused a febrile systemic illness in a wildlife biologist shortly after handling a wide variety of wildlife species. Given this patient history and the phylogenetic clustering of this pathogen with viruses only previously described in bats, ~~the authors who discovered~~ it was suggested that SosV ~~suggest that the infection~~ was likely contracted from bats [17]. In conjunction with the known zoonotic nature of MenPV and human serological findings suggestive of AchPV2 and TioPV infection, these data suggest an increasing link between this group of viruses and human disease.

Here we report the isolation and whole genome sequence of a novel *Pararubulavirus* from the African straw-coloured fruit bat *Eidolon helvum*. The virus is phylogenetically unique so may be an important ~~tool~~ indicator virus in the continued study of this important genus.

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Materials and Methods

Cell culture conditions. Experiments described used either Vero cells (ATCC CCL-81) or *Pteropus alecto* primary kidney (PaKi) cells [18]. Cells were grown in Dulbecco's

modified Eagle's medium supplemented with F12-Ham (Sigma), 10% fetal calf serum, double strength antibiotic/ antimycotic (200 U/ml penicillin, 200 μg/ml streptomycin, and 0.5 μg/ml fungizone amphotericin B; Gibco), and ciprofloxacin (10 μg/ml; MP Bio medicals), at 37°C in 5% CO_2.

Urine samples. Urine samples were collected from underneath a colony of *E. helvum* in Accra Ghana [19], as previously described [20].

Isolation methods. Three passages of virus isolation were attempted on urine samples U34 U72 on *Pteropus alecto* primary kidney cells (PaKi) monolayers, in the same manner as, and in parallel with, attempts previously described on Vero cells [20]. The only exception to the previously described protocol, is that for some samples (U38, 42, 48 49, 58 62, 70, 72) flasks were frozen at -80°C and thawed at room temperature prior to passage (to synchronise experiments). Supernatants of cultures showing signs of cytopathic effect (CPE) were tested for the presence of paramyxoviral RNA using previously described RT-PCR [21]. PCR products were cloned (pGEM-T Easy, Promega) and ~~capillary sequenced~~Sanger sequenced for phylogenetic analysis.

Isolate propagation. Following the confirmation of a paramyxoviral isolate, the isolation monolayer was scraped into the media and the material was frozen at 80°C. After thawing at room temperature, 350μL of the supernatant/cell mixture was added to 75cm^2 near confluent monolayers of Vero and PaKi cells in minimal media for 1 hr under gentle rocking at 37°C. Following the incubation, media was topped-up and the cells were observed for cytopathic effect (CPE).

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Discussion

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The meta-analysis of paramyxovirus polymerase gene fragments from this and other studies [5,8,9,15] demonstrates the occurrence of these viruses throughout the Old World. These bat-associated pararubulaviruses ~~are~~ have been linked with human disease, including the known human pathogens MenPV and SosPV, as well as those that are suggested to be capable of infecting humans on the basis of serological evidence, AchPV2 and TioPV [11,13,14,17]. Owing to their distribution across a wide geographical area and their association with human disease, pararubulaviruses warrant further study. Specifically, in order to separate potential zoonoses from harmless viruses, more work must be done to understand the host restriction of paramyxoviral tropism and the mechanisms of viral infection in the reservoir.

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With respect to determining mechanisms of host restriction, AchPV3 did not appear to grow in Vero cells. This is distinct from AchPV1 and AchPV2, which were isolated in parallel from the same sample set [14], from the other bat-derived rubulaviruses specific to this sub-clade (i.e. MenPV, TioPV both grow readily on Vero cells [13,25]), as well as other rubulaviruses (e.g. MprPV, PorPV [26,27]). Notably however, attempts to isolate the human pathogen SosPV and its nearest relative ThkPVs on Vero cells failed [16,17], with SosPV ~~having to be~~being sub-cultured in neonatal mice prior to being capable of non-~~syncitial~~syncytial CPE on Vero-E6 cells [17]. Further study of this restriction of *in vitro* host range will aid understanding of the barriers to zoonotic transmission for bat-associated rubulaviruses. MAYBE CITE THE ANIMAL STUDIES OF ACHPVs IN HERE AND RELATIONSHIP WITH ACHPV2.

Commented [BK10]: Jenn can you please have a read of this and see if any of it is still relevant or should it just be deleted? I'm not sure how hard you had to work to get it growing in Vero cells?

Furthermore, AchPV3 has significant potential to provide insight into the behavior of paramyxoviruses in their bat hosts. AchPV3 was isolated, and subsequently had to be passaged, by freeze-thawing entire cell-culture flasks, possibly indicating a cell associated infection and/or control and sequestration of the virus. This possibility was supported by the low amounts of AchPV3 that was released into the media (not shown), complicating genome sequencing. There was also only subtle CPE observed in the cell culture flasks, but this may have resulted from the primary nature of the cell culture line being used rather than any inherent pathogen difference.

Commented [SI(BM11]: Maybe the virus didn't grow to very high titres

Acknowledgements

We would like to thank the Veterinary Services Directorate (Ghana). The authors acknowledge the support of the Australian Microscopy and Microanalysis Research Facility to the microscopy facilities of the Australian Animal Health Laboratory. ~~We also thank Mary Tachedjian for technical assistance.~~ This study was funded by a Wellcome Trust research-training fellowship (KB) and KB is funded by a Clinical Research Career Development Fellowship (xxxxxxxxx). PRM was also funded by the Wellcome Trust. JLNW is supported by the Alborada Trust and the RAPIDD program of the Science and Technology Directorate, Department of Homeland Security.

Figure legends

Figure 1. Growth of AchPV3 in culture. *Pteropus alecto* primary kidney cells uninfected (A), and infected (B) with AchPV3 showing multinucleate syncitial cells (B, arrows). Under electron microscopy (C) the characteristic *Paramyxovirus* ribonucleoprotein (RNP) was seen (indicated in C, inset)

Figure 2. Midpoint rooted maximum likelihood phylogenetic trees based on a 585 amino acid alignment of the nucleoprotein of *Paramyxovirinae* members (main) and a 176 amino acid alignment of the polymerase protein for the same taxa, and rubulavirus and pararubulavirus fragments detected in bats (inset). Samples starting with U are from *Eidolon helvum* in Ghana [5]. AchPV3 is starred, scale bars represent expected number of substitutions per site, and bootstrap values (of 100) of relevant sites are shown.

Tables

Table 1. Pair-wise amino acid identities for AchPV3 nucleocapsid (N) and phosphoproteins (P) with other Paramyxovirinae.

		Achimota virus 3	
Genus		N	P
Pararubulavirus	AchPV1	66	43
	AchPV2	75	47
	ThkPV1	60	38
	ThkPV2	66	39
	ThkPV3	60	41
	MenPV	64	41
	TioPV	65	39
	SosV	58	42
	TevPV	77	29
Rubulavirus	MuV	51	24
	MapPV	47	26
	SimPV41	45	25
	hPIV2	44	25
	SimPV5	47	25
	PorPV	49	25
	hPIV4	42	24
Morbillivirus	RPV	22	9
	MeV	23	8
	CDV	23	10
Henipavirus	HeV	27	7
	NiV (M)	27	7
	NiV (B)	27	8
Avulavirus	NDV	31	20
Respirovirus	SeV	19	5
Unclassified	BeiV	25	10
	JPV	22	9
	MosV	25	8
	TPMV	23	8

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[redacted]

From: Tachedjian, Mary (H&B, Geelong AAHL) <[redacted]
Sent: Wednesday, 31 July 2019 6:42 PM
To: Barr, Jenn (H&B, Geelong AAHL); Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Smith, Ina (H&B, Black Mountain); Baker, Michelle (H&B, Geelong AAHL); linfa.wang [redacted] Todd, Shawn (H&B, Geelong AAHL); A.Cunningham [redacted] aferlasvet [redacted] suuire [redacted] Crameri, Sandra (AAHL, [redacted] AHL); clair [redacted] Pablo.Murcia [redacted] James Wood; [redacted] i
Subject: RE: AchPV3 p
Attachments: AchPV3 Final JB24July19 MT.docx

Hi Kate,

Hope you are well!

I've just snuck my edits in before the end of July deadline (attached). Please note edits have been added to those provided by Jenn.

Not exactly sure what's going on with AchPV3 propagation in Vero vs Vero-E6 (originally passaged in PaKi x2) vs PaKi. Suffice to say material provided by Jenn for NGS resulted in *de novo* assembly of the complete AchPV3 genome for both PaKi and Vero E6 (originally PaKi x2 passaged) cultured viruses.
In addition, the genome sequences for both was identical as well as the percentage of **AchPV3 specific Illumina pair-end reads** which was ~35% of total Illumina PE reads for samples propagated in PaKi and Vero E6 (originally PaKi x2 passaged).

Jenn, it might be a good idea to perform the experiments you've suggested although I'll defer to the virologists on this paper for their expert opinion.

Glenn and Jenn, could you also please verify my edits in the Materials and Methods re: virus enrichment via sucrose gradient (didn't think you guys did this) and whether DNaseI was used for Total RNA extraction with the Zymo Direct zol RNA Miniprep kit.

Kate, please let me know if you need anything else for your manuscript.

Cheers

MT

Mary Tachedjian
Senior Experimental Scientist
Health and Biosecurity
CSIRO Australian Animal Health Laboratory (AAHL)
[redacted]

Duplicate Email - Removed

Title

Achimota virus 3: a new member of the pararubulavirus genus

Commented [BK1]: Add in Teviot virus
Redo L gene phylogenies, but maybe wait to hear back
Add in whole genome sequence

Authors

Kate S Baker* [a b c], Mary Tachedjian [d i], Jennifer Barr [d i], Glenn A Marsh [d i], Shawn Todd [d i] Gary Crameri [d], Sandra Crameri [d], Ina Smith [j], Michelle Baker [i], Claire EG Holmes [d], Richard Suu Ire [e f], Andres Fernandez-Loras [b], Andrew A Cunningham [b], Pablo R Murcia [g], James LN Wood [a], Lin Fa Wang * [d,h]

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* corresponding authors

Introduction

Paramyxoviruses are important pathogens of man and domestic animals, and bats are recognized as having an increasingly complex role with these viruses. Bats are recognised as reservoir hosts for a wide variety of viral zoonoses [1], and interest in the relationship between bats and paramyxoviruses was triggered by the finding that megabats in Australia and Asia were the reservoir species for the deadly zoonoses Hendra (HeV) and Nipah (NiV) viruses, which infect a wide variety of mammalian hosts causing significant morbidity and mortality [2,3,4]. Since those times, paramyxoviral prospecting in bats has revealed a breadth and diversity of paramyxoviruses [5,6,7,8,9] that is greater than that observed in other mammalian orders [10].

Within the phylogenetic diversity of paramyxoviruses, there exists a unique sub clade of viruses in the genus *Pararubulavirus* (subfamily *Rubulavirinae*), that are almost

exclusively derived from bats. The first member of this rubulavirus sub clade was Menangle pararubulavirus (MenPV); first found as a disease agent of pigs and humans [11] and subsequently linked with bats [12] in Australia. This was followed by the discovery of Tioman pararubulavirus (TioPV) in Malaysia, where the virus was incidentally isolated during NiV investigations [13]. And finally, the Achimota pararubulaviruses 1 and 2 (AchPV1 and AchPV2) and Teviot virus that were isolated from pooled bat urine underneath roosts in Ghana and Australia respectively [14] [Add Teviot]. Although no clinical illness in humans has been reported, low-titre virus neutralising antibodies have been detected in humans living proximate to isolation sites for both TioPV and AchPV2 [13,14]. In addition to these five viruses, molecular evidence exists for many further paramyxoviruses in bats that belong to this genus (including the Tuhoko pararubulaviruses (ThkPVs) for which full genomic information exists) [7,9,10,15,16]. Collectively, these findings show pararubulaviruses exist across all four continents of the old world (namely Europe, Africa, Australia, Asia), and are frequently associated with bats, often from bat species that live in close proximity with humans.

In fact, the only pararubulavirus that was not isolated from bats is Sosuga virus (SosV) that caused febrile systemic illness in a wildlife biologist shortly after handling a wide variety of wildlife species. Given this patient history and the phylogenetic clustering of this pathogen with viruses only previously described in bats, the authors who discovered SosV suggest that the infection was likely contracted from bats [17]. In conjunction with the known zoonotic nature of MenPV and human serological findings ~~suggestive~~indicative of AchPV2 and TioPV infection, these data suggest an increasing link between this group of viruses and human disease.

Here we report the isolation and whole genome sequence of a novel *Pararubulavirus* from the African straw-coloured fruit bat *Eidolon helvum*. The virus is phylogenetically unique so may be an important tool in the continued study of this important genus.

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Materials and Methods

Cell culture conditions. Experiments described used either Vero cells (ATCC CCL 81), Vero-E6 cells (ATCC CRL 1586) or *Pteropus alecto* primary kidney (PaKi) cells [18]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with F12 Ham (Sigma), 10% fetal calf serum, double strength antibiotic/ antimycotic (200 U/ml penicillin, 200 µg/ml streptomycin, and 0.5 µg/ml fungizone amphotericin B; Gibco), and ciprofloxacin (10 µg/ml; MP Bio medicals), at 37°C in 5% CO_2.

Urine samples. Urine samples were collected from underneath a colony of *E. helvum* in Accra Ghana [19], as previously described [20].

Isolation methods. Three passages of virus isolation were attempted on urine samples U34 – U72 on *Pteropus alecto* primary kidney cells (PaKi) monolayers, in the same manner as, and in parallel with, attempts previously described on Vero cells [20]. The only exception to the previously described protocol, is that for some samples (U38, 42, 48 49, 58 62, 70, 72) flasks were frozen at 80°C and thawed at room temperature prior to passage (to synchronise experiments). Supernatants of cultures showing signs of cytopathic effect (CPE) were tested for the presence of paramyxoviral RNA using previously described RT PCR [21]. PCR products were cloned (pGEM T Easy, Promega) and capillary sequenced for phylogenetic analysis.

Isolate propagation. Following the confirmation of a paramyxoviral isolate, the ~~isolation~~cell monolayer was scraped into the media and the material was frozen at

80°C. After thawing at room temperature, 350µL of the supernatant/cell mixture was added to 75cm² near-confluent monolayers of Vero and PaKi cells in minimal media for 1 hr ~~under~~with gentle rocking at 37°C. Following the incubation, media was topped-up and the cells were observed for cytopathic effect (CPE).

Genomic sequencing and bioinformatic analysis. Stocks for genomic sequencing were grown by inoculating PaKi cell monolayers and Vero-E6 monolayers with 50ul of working stock virus and monitoring for viral CPE daily. Viral supernatant was harvested from PaKi cells 6 days post inoculation and Vero-E6 cells 13 days post inoculation. Semi-purification of the propagated isolate by sucrose-cushion ~~and high-throughput metagenomic sequencing~~ was performed as previously described [20]. Whole-genome sequencing was performed as previously described (Johnson et al 2018) except total RNA was extracted from 100 uL culture supernatant with Zymo's Direct-zol RNA Mini kit without DNaseI digestion (Zymo Research, Irvine, CA, USA) and Nextera XT DNA libraries (Illumina, San Diego, CA, USA) were sequenced on the CSIRO AAHL Illumina MiniSeq Sequencing System and Mid Output Kit (300-cycles) generating 150 bp paired-end (PE) reads.

Complete genome sequences were obtained with a previously established *de novo* assembly pipeline (Hayward~~d and/or~~ Tachedjian et al 2019 unpublished) except host subtraction was omitted. *De novo* assembled contigs were verified by mapping back trimmed reads using default settings of the CLC Genomics Workbench ver 10.1.1 "Map Reads to Reference" tool in addition to 5' and 3' genome end determination and genome annotation. Predicted ORFs were verified by querying the NCBI Nucleotide BLAST non-redundant database. P gene RNA editing site was verified with the CLC Genomics Workbench v10.1.1 "Low Frequency Variant Detection" algorithm with minimum frequency percentage of 0.01%.

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Commented [TM(GA4]: Jenn, did you enrich virus samples with a sucrose-cushion? My records indicate AchPV3 samples as well as Glenn's samples that were extracted at the same time were purified with the Zymo Direct zol RNA kit from 100 uL of culture S/N? I have amended the methods to reflect this.

Commented [TM(GA5]: Refer to link for full reference details: https://www.mdpi.com/1999-4915/10/12/675

Commented [TM(GA6]: Glenn and Jenn, Do you remember if you used DNaseI for viral RNA extractions?

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Commented [TM(GA7]: Kate, detailed protocol will be published in another manuscript which we aim to submit by the end of August. I will provide reference details once they are available so you can include in your manuscript.

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~~Contiguous sequences were built from 454 pyrosequencing reads using CLC genomics workbench (v. 4.8).~~ The genome sequence has been deposited under accession number: xxxxxx. Other bioinformatic analyses of genome sequences, including ~~gene annotations,~~ phylogenetic analysis and protein sequence analysis were performed as previously described [14]. ~~Even though virus RNA was extracted from cell culture supernatant containing predominantly virus genome, presence of low levels of virus mRNA contamination allowed for the determination of the P gene RNA editing site via readmapping and variant detection using CLC Genomic Workbench v10.1.1 "Low Frequency variant detection" tool~~

Electron microscopy. Cells prepared as in isolate propagation were harvested for negative contrast electron microscopy (EM) as well as stained thin section EM. [More info here please, and please check pictures]

Commented [TM(GA8]: Hi Jenn and Kate. I was able to obtain full genome assemblies for AchPV3 propagated in both PaKi and Vero-E6 cells. The AchPV3 denovo assembled genome sequence were identical for isolates grown in Vero-E6 and PaKi cells.

In addition, although samples were harvested 1 week apar[illegible] amount of AchPV3 in both PaKi and Vero-E6 culture superi[illegible]t appears to be similar as determined by the NGS data. Specifically, total number of trimmed, paired-end reads that mapped to the complete AchPV3 genome sequence was basically identical at 35.7% and 36.0% for PaKi and Vero-E6 respectively (determined using default settings of the CLC Genomics Workbench ver 10.1.1 "Map Reads to Reference" tool).

Commented [BK9]: Please update is this is dated!

Commented [BJ(GA10R9]: I'm not sure if Mary T was able to get sequence from both Paki and VeroE6 stocks???

Hi Jenn and Kate – answer is Yes. Please refer to my comments above.

Results

Primary bat kidney cells yielded a novel paramyxovirus isolate. Two samples produced CPE on PaKi cell monolayers. An adenovirus was isolated from sample U69 (which gave rise to a paramyxovirus, Achimota virus 2, on Vero cells [20]). The adenovirus was called *Eidolon helvum adenovirus 1*, and is described elsewhere [23].

Additionally, a novel pararubulavirus isolate was obtained from sample U72. This sample had previously been shown to contain paramyxovirus RNA related to respiro-morbilli henipaviruses [24], but did not give rise to a paramyxovirus isolate in a parallel attempt on Vero cells [14]. Here, on the fifth day post infection of the second passage, subtle CPE of syncytia formation and multinucleate cells were noted for Sample U72 (Figure 1). Subsequent RT PCR on RNA extracted from the supernatant of this flask was positive by paramyxovirinae PCR, but not respiro morbilli henipavirus PCR. Sequencing revealed the virus to be a novel rubulavirus (see below), and the virus was called Achimota pararubulavirus 3 (AchPV3) after the local area in which the samples

were collected and to align it with other paramyxoviruses isolated from these samples [14].

AchPV3 behavior in cell culture. AchPV3 was isolated from sample U72 on PaKi cells but was not isolated on the parallel Vero cell monolayer. ~~following t~~The initial PaKi cell monolayer isolate ~~being~~was frozen and thawed before attempting to propagate further on both PaKi and Vero cell ~~passage. AchPV3 was not isolated on a Vero cell~~ monolayers [14]. On PaKi cells, subtle CPE typified by syncytia and multinucleate cell formation was observed from day three post-infection until harvesting on day six. Again, no CPE was observed in the parallel Vero cell monolayers and a working stock was generated from the PaKi cells only. ~~W~~When ~~propagating the isolate~~ generating a stock for full genome sequencing from the working stock, ~~attempts were made to infect~~PaKi cells were used and in addition, infection of VeroE6 cells was attempted~~, but no CPE was observed~~. ~~On PaKi cells however, subtle CPE typified by syncitia and multinucleate cell formation was observed from day three post infection until harvesting on day six.~~ Surprisingly, syncytial CPE was eventually observed on the VeroE6 cell monolayers after 12 days post infection on flasks that had been checked daily.

Commented [BK11]: Jenn, can you please add something about how you were able to eventually get this growing on Vero cells or whatever passage? Maybe it was just a higher MOI or something? And obviously update the methods too – thanks!

Commented [BK12]: Jenn, can you please add something about how you were able to eventually get this growing on Vero cells or whatever passage? Maybe it was just a higher MOI or something? And obviously update the methods too – thanks!

Electron microscopy. Electron micrograph pictures show … [More info here please] (Figure 1C).

Commented [BK13]: Sandy? Clare?

Genomic organisation.

AchPV3 had similar genomic organization to existing pararubulaviruses. The genome was 15,600 bp in length, obeying the rule-of-six, and had the coding capacity to encode eight proteins; the nucleocapsid (513 aa in length), matrix (376 aa), fusion (527 aa), attachment (587 aa), polymerase (2273 aa) proteins as well as the overlapping V protein (238 aa), W protein (168 aa) and phosphorprotein (395 aa). These overlapping reading

frames were facilitated by the existence of an RNA editing site at position 2410 of the genome. Although viral RNA was extracted from cell culture supernatant containing predominantly intact virons, presence of low levels of virus mRNA contamination allowed for the determination of the P gene RNA editing site.

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Relationship with other paramyxoviruses. AchPV3 is related to, but distinct from previously described pararubulaviruses. Phylogenetic analysis of the full length N protein of AchPV3 demonstrated that the virus clustered phylogenetically with AchPV2, and further expanded the pararubulavirus genus comprised of AchPVs, ThkPVs, TioPV, TevPV and the human pathogens MenPV and SosPV (Figure 2). Phylogenetic comparison of available partial polymerase gene sequences also showed AchPV3 to be distinct from viral sequence fragments previously detected using consensus PCRs in the same bat population [24] and from other bat populations elsewhere [8,9,15] (Figure 2, inset). Amino acid sequence identities of AchPV3 proteins were highest with other pararubulaviruses and SosPV, followed by other rubulaviruses when compared with members from other genera within the *Paramyxovirinae* (Table 1).

Discussion

Here, we have found another novel pararubulavirus from fruit bats. Genomic analysis of the virus, AchPV3, reveals it to be a typical representative of this species phylogenetically clustering with other members of the pararubulavirus genus, and using a conserved mRNA editing site. This further increases the evidence of the role for bats as reservoirs for this group of viruses.

The meta-analysis of paramyxovirus polymerase gene fragments from this and other studies [5,8,9,15] demonstrates the occurrence of these viruses throughout the Old World. These bat-associated pararubulaviruses are linked with human disease, including the known human pathogens MenPV and SosPV, as well as those that are suggested to be capable of infecting humans on the basis of serological evidence, AchPV2 and TioPV [11,13,14,17]. Owing to their distribution across a wide geographical area and their association with human disease, pararubulaviruses warrant further study. Specifically, in order to separate potential zoonoses from harmless viruses, more work must be done to understand the host restriction of paramyxoviral tropism and the mechanisms of viral infection in the reservoir.

With respect to determining mechanisms of host restriction, AchPV3 did not appear to grow in Vero cells. This is distinct from AchPV1 and AchPV2, which were isolated in parallel from the same sample set [14], from the other bat-derived rubulaviruses specific to this sub-clade (i.e. MenPV, TioPV both grow readily on Vero cells [13,25]), as well as other rubulaviruses (e.g. MprPV, PorPV [26,27]). Notably however, attempts to isolate the human pathogen SosPV and its nearest relative ThkPVs on Vero cells failed [16,17], with SosPV having to be sub-cultured in neonatal mice prior to being capable of non-syncitial CPE on Vero-E6 cells [17]. Further study of this restriction of *in vitro* host range will aid understanding of the barriers to zoonotic transmission for bat-associated rubulaviruses. MAYBE CITE THE ANIMAL STUDIES OF ACHPVs IN HERE AND RELATIONSHIP WITH ACHPV2.

Furthermore, AchPV3 has significant potential to provide insight into the behavior of paramyxoviruses in their bat hosts. AchPV3 was isolated, and subsequently had to be passaged, by freeze-thawing entire cell-culture flasks, possibly indicating a cell-associated infection and/or control and sequestration of the virus. This possibility was

Commented [BK14]: The paper will kind of have to end here if we don't come up with something to say – maybe we should aim for a different format. Could go for a genome announcement, which is a shame, but would get it out?

Commented [BK15]: Jenn can you please have a read of this and see if any of it is still relevant or should it just be deleted? I'm not sure how hard you had to work to get it growing in Vero cells?

Commented [BJ(GA16R15]: So, it was unable to be isolated in Vero cells but the PaKi x2 stock was eventually able to grow in VeroE6 cells...there may be some discussion possible around the difference between Vero and VeroE6 cells? Also, the entire stock that grew in veroE6 was used for sequencing and that is literally all we have done with this virus. So I'm wondering if I should do a further experiment to inoculate veroE6 cells again, see if I get the same result, harvest the stock and pass again onto veroE6 and see if CPE appears more quickly...what do you think? Could provide some more discussion about adaption to veroE6 or something...

supported by the low amounts of AchPV3 that was released into the media (not shown), complicating genome sequencing. There was also only subtle CPE observed in the cell culture flasks, but this may have resulted from the primary nature of the cell culture line being used rather than any inherent pathogen difference.

Commented [TM(GA17]: KThe NGS sequencing wet work workflow we have developed isn't impaired by low amounts of virus unlike previous methods used in the past. We can obtain full genome sequence from total RNA amounts of <100pg. Consequently, this comment should be removed IMO.

Acknowledgements

We would like to thank the Veterinary Services Directorate (Ghana). The authors acknowledge the support of the Australian Microscopy and Microanalysis Research Facility to the microscopy facilities of the Australian Animal Health Laboratory. ~~We also thank Mary Tachedjian for technical assistance.~~ This study was funded by a Wellcome Trust research-training fellowship (KB) and KB is funded by a Clinical Research Career Development Fellowship (xxxxxxxxx). PRM was also funded by the Wellcome Trust. JLNW is supported by the Alborada Trust and the RAPIDD program of the Science and Technology Directorate, Department of Homeland Security.

Figure legends

Figure 1. Growth of AchPV3 in culture. *Pteropus alecto* primary kidney cells uninfected (A), and infected (B) with AchPV3 showing multinucleate syncytial cells (B, arrows). Under electron microscopy (C) the characteristic *Paramyxovirus* ribonucleoprotein (RNP) was seen (indicated in C, inset)

Figure 2. Midpoint-rooted maximum likelihood phylogenetic trees based on a 585 amino acid alignment of the nucleoprotein of *Paramyxovirinae* members (main) and a 176 amino acid alignment of the polymerase protein for the same taxa, and rubulavirus and pararubulavirus fragments detected in bats (inset). Samples starting with U are from *Eidolon helvum* in Ghana [5]. AchPV3 is starred, scale bars represent expected number of substitutions per site, and bootstrap values (of 100) of relevant sites are shown.

Commented [TM(GA18]: Kate, unfortunately I can't see the star next to AchPV3. Probably not shown on my version of acrobat.

Tables

Table 1. Pair wise amino acid identities for AchPV3 nucleocapsid (N) and phosphoproteins (P) with other Paramyxovirinae.

		Achimota virus 3	
Genus		N	P
Pararubulavirus	AchPV1	66	43
	AchPV2	75	47
	ThkPV1	60	38
	ThkPV2	66	39
	ThkPV3	60	41
	MenPV	64	41
	TioPV	65	39
	SosV	58	42
	TevPV	77	29
Rubulavirus	MuV	51	24
	MapPV	47	26
	SimPV41	45	25
	hPIV2	44	25
	SimPV5	47	25
	PorPV	49	25
	hPIV4	42	24
Morbillivirus	RPV	22	9
	MeV	23	8
	CDV	23	10
Henipavirus	HeV	27	7
	NiV (M)	27	7
	NiV (B)	27	8
Avulavirus	NDV	31	20
Respirovirus	SeV	19	5
Unclassified	BeiV	25	10
	JPV	22	9
	MosV	25	8
	TPMV	23	8

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s22

From: Andrew Cunningham <s22
Sent: Thursday, 1 August 2019 9:05 PM
To: Tachedjian, Mary (H&B, Geelong AAHL); Barr, Jenn (H&B, Geelong AAHL); Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Smith, Ina (H&B, Black Mountain); Baker, Michelle (H&B, Geelong AAHL); linfa.wang s22 Todd, Shawn (H&B, Geelong AAHL); aferlasvet s22 ; suuire s22 Crameri, Sandra (AAHL, Geelong AAHL); cl s22 s22 James Wood; Gary Crameri
Subject: RE: [EXT]: RE: AchPV3 paper
Attachments: AchPV3 Final JB24July19 MT AAC.docx

Thanks for this, Kate.
My comments/edits using the version sent by Mary.
Cheers
Andrew

Duplicate Email - Removed

Title

Achimota virus 3: a new member of the pararubulavirus genus

Commented [BK1]: Add in Teviot virus
Redo L gene phylogenies, but maybe wait to hear back
Add in whole genome sequence

Authors

Kate S Baker* [a b c], Mary Tachedjian [d i], Jennifer Barr [d i], Glenn A Marsh [d i], Shawn Todd [d i] Gary Crameri [d], Sandra Crameri [d], Ina Smith [j], Michelle Baker [i], Claire EG Holmes [d], Richard Suu-Ire [e f], Andres Fernandez-Loras [b], Andrew A Cunningham [b], Pablo R Murcia [g], James LN Wood [a], Lin-Fa Wang * [d h]

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* corresponding authors

Introduction

Paramyxoviruses are important pathogens of man and domestic animals, and bats are recognized as having an increasingly complex role with these viruses. Bats are ~~recognised as~~ reservoir hosts for a wide variety of viral zoonoses [1], and interest in the relationship between bats and paramyxoviruses was triggered by the finding that megabats in Australia and Asia ~~were~~ are the reservoir ~~species~~ hosts for the deadly zoonoses Hendra (HeV) and Nipah (NiV) viruses, which can infect a wide variety of terrestrial mammalian hosts ~~causing~~ in which they cause significant morbidity and mortality [2,3,4]. ~~Since those times~~Subsequently, ~~paramyxoviral~~ virus prospecting in bats has revealed a breadth and diversity of paramyxoviruses [5,6,7,8,9] that is greater than that observed in any other mammalian orders [10].

Within the phylogenetic diversity of paramyxoviruses, there exists a unique sub-clade ~~of viruses~~ in the genus *Pararubulavirus* (subfamily *Rubulavirinae*), that are almost

Commented [AAC2]: "recognized" and "recognized" are used in this MS. Use only one spelling, depending on the journal.

Commented [AAC3]: Deleted to avoind having "..bats are recognized.....Bats are recognized..."

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Commented [AAC5]: Is it in the genus, or is it this genus?

exclusively derived from bats. The first member of this rubulavirus sub clade to be discovered was Menangle pararubulavirus (MenPV); first found as a disease agent of pigs and humans [11] and subsequently linked with bats [12] in Australia. This was followed by the discovery of Tioman pararubulavirus (TioPV) in fruit bats in Malaysia, where ~~the virus~~it was incidentally isolated during NiV investigations [13]. ~~And~~ ~~f~~Finally, the Achimota pararubulaviruses 1 and 2 (AchPV1 and AchPV2) and Teviot virus that were isolated from pooled bat urine underneath roosts in Ghana and Australia respectively [14] [Add Teviot]. Although no human clinical illness ~~in humans~~ has been reported, low titre virus neutralising antibodies have been detected in ~~humans~~ people living proximate to isolation sites for both TioPV and AchPV2 [13,14]. In addition to these five pararubulaviruses, molecular evidence ~~exists for~~indicates that there are many further paramyxoviruses in bats that belong to this genus (including the Tuhoko pararubulaviruses (ThkPVs) for which full genomic information exists) [7,9,10,15,16]. Collectively, these findings show pararubulaviruses exist in bats across all four continents of the old world (namely Europe, Africa, Australia~~,~~ and Asia), and are frequently associated with bats, ~~often from bat species~~ that live in close proximity ~~with~~ to humans.

Commented [AAC6]: It seems odd to pool the Achimotas with Teviot.

In fact, the only pararubulavirus that ~~was~~has not been isolated from bats is Sosuga virus (SosV), a virus that caused febrile systemic illness in a wildlife biologist shortly after handling a wide variety of wildlife species, including bats, in Africa. Given this patient history and the phylogenetic clustering of this pathogen with viruses only previously ~~described in~~known from bats, the authors who discovered SosV suggest that the infection was likely contracted from bats [17]. In conjunction with the known zoonotic nature of MenPV and human serological findings ~~suggestive~~indicative of AchPV2 and

TioPV infection, these data suggest an increasing link between this group of viruses and human disease.

Here we report the isolation and whole genome sequence of a novel *Pararubulavirus* from the African straw coloured fruit bat (*Eidolon helvum*). The virus is phylogenetically ~~unique~~ disparate from other pararubulaviruses, so ~~may~~ its study might be an important ~~tool in the continued study~~ addition to our understanding of this important genus.

Commented [AAC7]: I've changed this as, by default, each species of virus is phylogenetically unique. But I don't think this is what you meant either, as in the Results we say that AchV3 clusters phylogenetically with AchPV2.

Commented [BK8]: If anyone can come up with something more inspired than this please do!

Materials and Methods

Cell culture conditions. Experiments described used either Vero cells (ATCC CCL 81), Vero E6 cells (ATCC CRL 1586) or *Pteropus alecto* primary kidney (PaKi) cells [18]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with F12 Ham (Sigma), 10% fetal calf serum, double strength antibiotic/antimycotic (200 U/ml penicillin, 200 µg/ml streptomycin, and 0.5 µg/ml fungizone amphotericin B; Gibco), and ciprofloxacin (10 µg/ml; MP Bio medicals), at 37°C in 5% CO_2.

Urine samples. Urine samples were collected from underneath a colony of *E. helvum* in Accra Ghana [19], as previously described [20].

Isolation methods. Three passages of virus isolation were attempted on urine samples U34 U72 on *Pteropus alecto* primary kidney cells (PaKi) monolayers, in the same manner as, and in parallel with, attempts previously described on Vero cells [20]. The only exception to the previously described protocol, is that for some samples (U38, 42, 48 49, 58 62, 70, 72) flasks were frozen at 80°C and thawed at room temperature prior to passage (to synchronise experiments). Supernatants of cultures showing signs of cytopathic effect (CPE) were tested for the presence of paramyxoviral RNA using previously described RT PCR [21]. PCR products were cloned (pGEM T Easy,

Promega) and capillary-sequenced for phylogenetic analysis.

Isolate propagation. Following the confirmation of a paramyxoviral isolate, the ~~isolation~~cell monolayer was scraped into the media and the material was frozen at 80 °C. After thawing at room temperature, 350µL of the supernatant/cell mixture was added to 75cm^2 near confluent monolayers of Vero ~~and~~ or PaKi cells in minimal media for 1 h~~r~~ ~~under~~with gentle rocking at 37 °C. Following ~~the~~ incubation, media was topped-up and the cells were observed for cytopathic effect (CPE).

Genomic sequencing and bioinformatic analysis. Stocks for genomic sequencing were grown by inoculating PaKi cell monolayers and Vero E6 monolayers with 50 ul of working stock virus and monitoring for viral CPE daily. Viral supernatant was harvested from PaKi cells 6 days post inoculation and Vero E6 cells 13 days post inoculation. Semi purification of the propagated isolate by sucrose cushion ~~and high-throughput metagenomic sequencing~~ was performed as previously described [20]. Whole genome sequencing was performed –as previously described (Johnson et al 2018) except total RNA was extracted from 100 uL culture supernatant with Zymo's Direct zol RNA Mini kit without DNaseI digestion (Zymo Research, Irvine, CA, USA) and Nextera XT DNA libraries (Illumina, San Diego, CA, USA) were sequenced on the CSIRO AAHL Illumina MiniSeq Sequencing System and Mid Output Kit (300-cycles) generating 150 bp paired end (PE) reads.

Complete genome sequences were obtained with a previously established *de novo* assembly pipeline (Hayward~~d and/or~~ Tachedjian et al 2019 unpublished) except host subtraction was omitted. *De novo* assembled contigs were verified by mapping back trimmed reads using default settings of the CLC Genomics Workbench ver 10.1.1 "Map Reads to Reference" tool in addition to 5' and 3' genome end determination and

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Commented [TM(GA10]: Jenn, did you enrich virus samples with a sucrose-cushion? My records indicate AchPV3 samples as well as Glenn's samples that were extracted at the same time were purified with the Zymo Direct-zol RNA kit from 100 uL of culture S/N? I have amended the methods to reflect this.

Commented [TM(GA11]: Refer to link for full reference details: https://www.mdpi.com/1999-4915/10/12/675

Commented [TM(GA12]: Glenn and Jenn, Do you remember if you used DNaseI for viral RNA extractions?

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Commented [TM(GA13]: Kate, detailed protocol will be published in another manuscript which we aim to submit by the end of August. I will provide reference details once they are available so you can include in your manuscript.

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genome annotation. Predicted ORFs were verified by querying the NCBI Nucleotide BLAST non-redundant database. P gene RNA editing site was verified with the CLC Genomics Workbench v10.1.1 "Low Frequency Variant Detection" algorithm with minimum frequency percentage of 0.01%.

~~Contiguous sequences were built from 454 pyrosequencing reads using CLC genomics workbench (v. 4.8).~~ The genome sequence has been deposited under accession number: xxxxxx. Other bioinformatic analyses of genome sequences, including ~~gene annotations,~~ phylogenetic analysis and protein sequence analysis were performed as previously described [14]. ~~Even though virus RNA was extracted from cell culture supernatant containing predominantly virus genome, presence of low levels of virus-mRNA contamination allowed for the determination of the P gene RNA editing site via read mapping and variant detection using CLC Genomic Workbench v10.1.1 "Low Frequency variant detection" tool~~

Electron microscopy. Cells prepared as in isolate propagation were harvested for negative contrast electron microscopy (EM) as well as stained thin section EM. [More info here please, and please check pictures]

Commented [AAC14]: Where?

Commented [TM(GA15]: Hi Jenn and Kate. I was able to obtain full genome assemblies for AchPV3 propagated in both PaKi and Vero-E6 cells. The AchPV3 denovo assembled genome sequence were identical for isolates grown in Vero-E6 and PaKi cells.

In addition, although samples were harvested 1 week apart, the amount of AchPV3 in both PaKi and Vero-E6 culture supernatant appears to be similar as determined by the NGS data. Specifically, total number of trimmed, paired-end reads that mapped to the complete AchPV3 genome sequence was basically identical at 35.7% and 36.0% for PaKi and Vero-E6 respectively (determined using default settings of the CLC Genomics Workbench ver 10.1.1 "Map Reads to Reference" tool).

Commented [BK16]: Please update is this is dated!

Commented [BJ(GA17R16]: I'm not sure if Mary T was able to get sequence from both Paki and VeroE6 stocks???

Hi Jenn and Kate – answer is Yes. Please refer to my comments above.

Commented [AAC18]: What does this mean?

Results

Primary bat kidney cells yielded a novel paramyxovirus isolate. Two samples produced CPE on PaKi cell monolayers. An adenovirus was isolated from sample U69 (which also gave rise to a paramyxovirus, Achimota virus 2, on Vero cells [20]). The adenovirus was called *Eidolon helvum adenovirus 1*, and is described elsewhere [23]. Additionally, a novel pararubulavirus isolate was obtained from sample U72. This sample had previously been shown to contain paramyxovirus RNA related to respiro morbilli-henipaviruses [24], but did not give rise to a paramyxovirus isolate in a parallel attempt on Vero cells [14]. Here, on the fifth day post-infection of the second passage, a subtle CPE ~~of~~ comprising syncytia formation and multinucleate cells ~~were~~ was noted

for Sample U72 (Figure 1). Subsequent RT-PCR on RNA extracted from the supernatant of this flask was positive ~~by~~ using a generic paramyxovirinae PCR, but not using a respiro morbilli henipavirus PCR. Sequencing revealed the virus to be a novel rubulavirus (see below), and the virus was called Achimota pararubulavirus 3 (AchPV3) after the local area in which the samples were collected and to align it with other paramyxoviruses isolated from these urine samples [14].

AchPV3 behavior in cell culture. AchPV3 was isolated from sample U72 on PaKi cells but was not isolated on the parallel Vero cell monolayer. ~~following t~~The initial PaKi cell monolayer isolate ~~being~~was frozen and thawed before attempting to propagate further on both PaKi and Vero cell ~~passage. AchPV3 was not isolated on a Vero cell~~ monolayers [14]. On PaKi cells, subtle CPE typified by syncytia and multinucleate cell formation was observed from day three post infection until harvesting on day six. Again, no CPE was observed in the parallel Vero cell monolayers and a working stock was generated from the PaKi cells only. ~~W~~When ~~propagating the isolate~~ generating a stock for full genome sequencing from the working stock, ~~attempts were made to infect~~PaKi cells were used and, in addition, infection of VeroE6 cells was attempted~~, but no CPE was observed. On PaKi cells however, subtle CPE typified by syncitia and multinucleate cell formation was observed from day three post infection until harvesting on day six. Surprisingly, s~~Syncytial CPE was ~~eventually~~ observed on the VeroE6 cell monolayers only after 12 days post infection ~~on~~in flasks that had been checked daily.

Electron microscopy. Electron micrograph pictures show … [More info here please] (Figure 1C).

Genomic organisation.

Commented [AAC19]: I didn't see reference to these primer sets in the Methods. Did I miss them, or are they still to be added?

Commented [BK20]: Jenn, can you please add something about how you were able to eventually get this growing on Vero cells or whatever passage? Maybe it was just a higher MOI or something? And obviously update the methods too – thanks!

Commented [BK21]: Jenn, can you please add something about how you were able to eventually get this growing on Vero cells or whatever passage? Maybe it was just a higher MOI or something? And obviously update the methods too – thanks!

Commented [BK22]: Sandy? Clare?

AchPV3 ~~had~~ has similar genomic organization to existing pararubulaviruses. The genome ~~was~~ is 15,600 bp in length, obeying the rule-of-six, and hasd the coding capacity to encode eight proteins; the nucleocapsid (513 aa in length), matrix (376 aa), fusion (527 aa), attachment (587 aa), polymerase (2273 aa) proteins as well as the overlapping V protein (238 aa), W protein (168 aa) and phosphorprotein (395 aa). These overlapping reading frames ~~were~~ are facilitated by the existence of an RNA editing site at position 2410 of the genome. Although viral RNA was extracted from cell culture supernatant containing predominantly intact virons, presence of low levels of virus mRNA contamination allowed for the determination of the P gene RNA editing site.

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Relationship with other paramyxoviruses. AchPV3 is related to, but distinct from, previously described pararubulaviruses. Phylogenetic analysis of the full-length N protein of AchPV3 demonstrated that the virus clustered phylogenetically with AchPV2, and further expanded the pararubulavirus genus comprised of AchPVs, ThkPVs, TioPV, TevPV and the ~~human~~ known zoonotic pathogens MenPV and SosPV (Figure 2). Phylogenetic comparison of available partial polymerase gene sequences also showed AchPV3 to be distinct from viral sequence fragments previously detected using consensus PCRs in the same bat population [24] and from other bat populations elsewhere [8,9,15] (Figure 2, inset). Amino acid sequence identities of AchPV3 proteins were highest with other pararubulaviruses ~~and SosPV~~, followed by other rubulaviruses when compared with members from other genera within the *Paramyxovirinae* (Table 1).

Commented [AAC25]: This is a pararubulavirus.

Discussion

Here, we ~~have found~~report ~~another~~ novel pararubulavirus, named AchPV3, from African fruit bats. Genomic analysis of ~~the virus,~~ AchPV3, reveals it to be a typical representative of this species phylogenetically clustering with other members of the pararubulavirus genus, and using a conserved mRNA editing site. This further increases the evidence of the role ~~for~~ of bats as reservoirs for this group of viruses.

~~The~~ A meta-analysis of paramyxovirus polymerase gene fragments from this and other studies [5,8,9,15] demonstrates the occurrence of these viruses throughout the Old World. ~~These~~ Two of these bat associated pararubulaviruses (MenPV and SosPV) ~~are linked with~~can cause disease in humans, while others (AchPV2 and TioPV) ~~disease, including the known human pathogens MenPV and SosPV, as well as those that~~ are ~~suggested~~ considered to be capable of infecting humans on the basis of serological evidence, ~~AchPV2 and TioPV~~ [11,13,14,17]. Owing to their distribution across a wide geographical area and their association with human disease, pararubulaviruses warrant further study. Specifically, in order to separate potential zoonoses from harmless viruses, more work must be done to understand the host restriction of paramyxoviral tropism and the mechanisms and ecology of viral infection in the natural reservoir host.

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With respect to determining mechanisms of host restriction, AchPV3 did not appear to grow in Vero cells. This is distinct from AchPV1 and AchPV2, which were isolated in ~~parallel~~ Vero cells from the same sample set [14], from the other bat-derived rubulaviruses specific to this sub-clade (i.e. MenPV, TioPV both grow readily ~~on~~ in Vero cells [13,25]), as well as other rubulaviruses (e.g. MprPV, PorPV [26,27]). Notably, however, attempts to isolate the human pathogen SosPV and its nearest relative ThkPVs ~~on~~ in Vero cells failed [16,17], with SosPV having to be sub-cultured in neonatal mice prior to being capable of ~~non-syncitial~~producing CPE ~~on~~ in Vero E6

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cells [17]. Further study of this restriction of *in vitro* host range ~~will~~ might aid understanding of the barriers to zoonotic transmission for bat associated rubulaviruses.

MAYBE CITE THE ANIMAL STUDIES OF ACHPVs IN HERE AND RELATIONSHIP WITH ACHPV2.

Furthermore, AchPV3 has significant potential to provide insight into the behavior of paramyxoviruses in their bat hosts. AchPV3 was isolated, and subsequently had to be passaged, by freeze thawing entire cell culture flasks, possibly indicating a cell-associated infection and/or control and sequestration of the virus. This possibility was supported by the low amounts of AchPV3 ~~that was~~ released into the media (data not shown), complicating genome sequencing. There was also only subtle CPE observed in the cell culture flasks, but this may have resulted from the primary nature of the cell culture line being used rather than any inherent pathogen difference.

Commented [AC28]: Can we really say for sure that it will?

Commented [BK29]: Jenn can you please have a read of this and see if any of it is still relevant or should it just be deleted? I'm not sure how hard you had to work to get it growing in Vero cells?

Commented [BJ(GA30R29]: So, it was unable to be isolated in Vero cells but the PaKi x2 stock was eventually able to grow in VeroE6 cells...there may be some discussion possible around the difference between Vero and VeroE6 cells? Also, the entire stock that grew in veroE6 was used for sequencing and that is lit[illegible] all we have done with this virus. So I'm wondering if I sho[illegible] a further experiment to inoculate veroE6 cells again, see if I get the same result, harvest the stock and pass again onto veroE6 and see if CPE appears more quickly...what do you think? Could provide some more discussion about adaption to veroE6 or something...

Commented [AC31]: Can we show these data – perhaps in supplementary information?

Commented [TM(GA32]: KThe NGS sequencing wet work workflow we have developed isn't impaired by low amounts of virus unlike previous methods used in the past. We can obtain full genome sequence from total RNA amounts of <100pg. Consequently, this comment should be removed IMO.

Commented [AC33]: This is not a great sentence/paragraph to finish on. Needs something stronger here.

Acknowledgements

We ~~would like to~~ thank the Veterinary Services Directorate (Ghana) for logistical help in sampling wild bats in Accra. The authors acknowledge the support of the Australian Microscopy and Microanalysis Research Facility to the microscopy facilities of the Australian Animal Health Laboratory. ~~We also thank Mary Tachedjian for technical assistance.~~ This study was funded by a Wellcome Trust research training fellowship (KB) and KB is funded by a Clinical Research Career Development Fellowship (xxxxxxxxx). PRM was also funded by the Wellcome Trust. JLNW is supported by the Alborada Trust and the RAPIDD program of the Science and Technology Directorate, Department of Homeland Security.

Commented [AC34]: Correct? Also, should we thank the Wildlife Division of the Ghana Forestry Commission for granting the permit to conduct the field work?

Commented [AAC35]: Kate – weren't these urine samples collected with funding from the 3Rs grant? If so, that should be acknowledged here.

Figure legends

Figure 1. Growth of AchPV3 in culture. *Pteropus alecto* primary kidney cells uninfected (A), and infected (B) with AchPV3 showing multinucleate syncytial cells (B, arrows). Under electron microscopy (C) the characteristic *Paramyxovirus* ribonucleoprotein (RNP) was seen (indicated in C, inset)

Figure 2. Midpoint rooted maximum likelihood phylogenetic trees based on a 585 amino acid alignment of the nucleoprotein of *Paramyxovirinae* members (main) and a 176 amino acid alignment of the polymerase protein for the same taxa, and rubulavirus and pararubulavirus fragments detected in bats (inset). Samples starting with U are from *Eidolon helvum* in Ghana [5]. AchPV3 is starred, scale bars represent expected number of substitutions per site, and bootstrap values (of 100) of relevant sites are shown.

Commented [TM(GA36]: Kate, unfortunately I can't see the star next to AchPV3. Probably not shown on my version of acrobat.

Tables

Table 1. Pair-wise amino acid identities for AchPV3 nucleocapsid (N) and phosphoproteins (P) with other Paramyxovirinae.

		Achimota virus 3	
Genus		N	P
Pararubulavirus	AchPV1	66	43
	AchPV2	75	47
	ThkPV1	60	38
	ThkPV2	66	39
	ThkPV3	60	41
	MenPV	64	41
	TioPV	65	39
	SosV	58	42
	TevPV	77	29
Rubulavirus	MuV	51	24
	MapPV	47	26
	SimPV41	45	25
	hPIV2	44	25
	SimPV5	47	25
	PorPV	49	25
	hPIV4	42	24
Morbillivirus	RPV	22	9
	MeV	23	8
	CDV	23	10
Henipavirus	HeV	27	7
	NiV (M)	27	7
	NiV (B)	27	8
Avulavirus	NDV	31	20
Respirovirus	SeV	19	5
Unclassified	BeiV	25	10
	JPV	22	9
	MosV	25	8
	TPMV	23	8

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s22

From: Barr, Jenn (H&B, Geelong AAHL) s22
Sent: Thursday, 1 August 2019 9:08 AM
To: Tachedjian, Mary (H&B, Geelong AAHL); Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Smith, Ina (H&B, Black Mountain); Baker, Michelle (H&B, Geelong AAHL); linfa.wang s22 ; Todd, Shawn (H&B, Geelong AAHL); A.Cunningham s22 aferlasvet s22 suuire s22 Crameri, Sandra (AAHL, Geelong AAHL); claire.holmes s22 Pablo.Murcia s22 James Wood; Gary Crameri
Subject: RE: AchPV3 paper

Hi guys,
Yes Mary, we did purify the viral S/N through a 20% sucrose cushion prior to extracting RNA with the Zymo Direct zol RNA Miniprep kit and included the DNasel step. I did this for both the PaKi stock and the VeroE6 stock.
Cheers,

Jenn

Duplicate Email - Removed

s22

From: Tachedjian, Mary (H&B, Geelong AAHL) s22
Sent: Friday, 2 August 2019 12:51 PM
To: Barr, Jenn (H&B, Geelong AAHL); Baker, Kate; Marsh, Glenn (H&B, Geelong AAHL); Smith, Ina (H&B, Black Mountain); Baker, Michelle (H&B, Geelong AAHL); linfa.wang s22 Todd, Shawn (H&B, Geelong AAHL); A.Cunningham s22 aferlasvet s22 suuire s22 Crameri, Sandra (AAHL, Geelong AAHL); claire.holmes s22 Pablo.Murcia s22 James Wood; Gary Crameri
Subject: RE: AchPV3 paper

Thanks Jenn!

Cheers

MT

Mary Tachedjian
Senior Experimental Scientist
Health and Biosecurity
CSIRO Australian Animal Health Laboratory (AAHL)
s22

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