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1 **TITLE PAGE:**

2 Bioassay studies support the potential for iatrogenic transmission of variant
3 Creutzfeldt Jakob disease through dental procedures

4

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18

19 ¶ These authors made an equal contribution to the study.

20

21 **KEYWORDS:** Creutzfeldt Jakob Disease, iatrogenic transmission, oral
22 tissues, dental practice, transmission.

23

24

25

26 **ABSTRACT**

27 ***Background:***

28 Evidence is required to quantify the potential risks of transmission of variant
29 Creutzfeldt Jakob (vCJD) through dental procedures. Studies, using animal
30 models relevant to vCJD, were performed to address two questions. Firstly,
31 whether oral tissues could become infectious following dietary exposure to
32 BSE? Secondly, would a vCJD-contaminated dental instrument be able to
33 transmit disease to another patient?

34

35 ***Methods:***

36 BSE-301V was used as a clinically relevant model for vCJD. VM-mice were
37 challenged by injection of infected brain homogenate into the small intestine
38 (Q1) or by five minute contact between a deliberately-contaminated dental file
39 and the gingival margin (Q2). Ten tissues were collected from groups of
40 challenged mice at three or four weekly intervals, respectively. Each tissue
41 was pooled, homogenised and bioassayed in indicator mice.

42

43 ***Findings:***

44 Challenge via the small intestine gave a transmission rate of 100% (mean
45 incubation 157 ± 17 days). Infectivity was found in both dental pulp and the
46 gingival margin within 3 weeks of challenge and was observed in all tissues
47 tested within the oral cavity before the appearance of clinical symptoms.
48 Following exposure to deliberately contaminated dental files, 97% of mice
49 developed clinical disease (mean incubation 234 ± 33 days).

50

51 ***Interpretation:***

52 Infectivity was higher than expected, in a wider range of oral tissues, than was
53 allowed for in previous risk assessments. Disease was transmitted following
54 transient exposure of the gingiva to a contaminated dental file. These
55 observations provide evidence that dental procedures could be a route of
56 cross-infection for vCJD and support the enforcement of single-use for certain
57 dental instruments.

58

59 ***Funding:*** *The study was funded by the Department of Health (England);*

60 Contract number 007/0099

61

62 **INTRODUCTION**

63 vCJD remains a challenge for public health due to uncertain prevalence in the
64 population and the possibility of cross-infection through medical procedures.
65 The disease almost certainly emerged due to the consumption of bovine
66 spongiform encephalopathy (BSE)-infected meat [1] but clinical cases have
67 not reflected the widespread exposure of the UK population. The possibility of
68 a self-sustaining and potentially amplifying “epidemic”, caused by the
69 iatrogenic transmission of vCJD from pre-symptomatic cases and
70 asymptomatic carriers to more genetically susceptible individuals, is a major
71 concern.

72

73 The prevalence of the disease in the population is estimated at between 237
74 and 109 vCJD carriers per million of the UK population (95% confidence limits
75 49-692 per million [2] and 3-608 per million [3], respectively) All clinical cases
76 of vCJD, to date, have been PRNP-129 Met homozygotes, but pre-/sub-
77 clinical carriage has been identified in 2 valine homozygotes and a
78 heterozygous patient [2][4][5]. Extended asymptomatic incubation periods in
79 these genotypes have been suggested by transgenic animal studies [6] and
80 also by studies on Kuru [7]. A recent study has identified a patient with
81 atypical sporadic CJD and valine homozygous at PRNP codon 129 [8] which
82 could represent the first case of clinical disease in this genogroup. Aside from
83 blood transfusion [5,9] there remains no evidence of iatrogenic vCJD
84 transmission to date via any surgical route.

85

86 The potential transmission of vCJD by dental practice remain poorly defined.
87 A risk assessment carried out by the Department of Health in 2004

88 (http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_4084662 ; last accessed 12th November
89 2011) , suggested a low level of risk, based on the assumption that there
90 would be insignificant levels of infectivity except within the dental pulp and
91 that only dental instruments which contacted this material posed any risk of
92 cross infection. These assumptions are tested in this study. This risk
93 assessment was revised in 2007
94 (http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_081170; last accessed 12th November 2011), based
95 on data which includes the preliminary outputs of this study.
96
97

98

99 Studies have described the presence of infectivity in hamsters following
100 intraperitoneal challenge with 263K scrapie [10], with 7.2 (gingiva) and 5.6
101 (dental pulp) log LD₅₀ i.c. units (the dose capable of causing the death of 50%
102 of challenged animals when injected intracranially into hamsters) per gram of
103 tissue. The study also showed that scrapie could be transmitted through
104 injection into the dental pulp. A recent study has shown infectivity in the root
105 of the right caudal incisor tooth in an ME7-scrapie infected mouse following
106 intracerebral challenge [11]. Collectively these studies suggest the potential
107 for transmission of the disease via the oral cavity, but comprehensive data,
108 particularly using prion strains more directly relevant to modelling vCJD in
109 humans, remains lacking.

110

111 Bioassays using tissues from vCJD patients are underway (HPA
112 unpublished), but no disease-associated prion protein (PrP^{Sc}) staining has

113 been observed in any oral tissue from vCJD patients [12]. With very small
114 number of samples involved and the absence of direct transmission from
115 human tissue to animals in low titre vCJD tissues ($<10^3$ ID/gram tissue; [13]),
116 rodent-passaged TSE strains are essential to assess the relative levels of
117 infectivity in different tissues, following exposure by different routes, as well as
118 data on spread of disease.

119

120 The present study provides evidence on the potential risks of vCJD
121 transmission by measuring relative levels of infectivity in oral tissues and
122 assessing the potential for transmission through contact of a contaminated
123 instrument with the gingival margin.

124

125 **METHODS**

126 **Primary challenge of VM mice via the small intestine**

127 All studies were conducted under a project license approved by the UK Home
128 Office. Prior to submission for approval the license was reviewed by the
129 Microbiological Services Porton Ethical review committee and signed off by
130 the Establishment Certificate Holder. Project license 30/2700 was granted by
131 the UK Home Office under the Animals (Scientific Procedures) Act, 1986. A
132 volume of 100 μ l of a 2% (w/v) titred stock of BSE301V-infectious mouse brain
133 homogenate (estimated titre $10^{8.9}$ infectious units per gram brain) [14] was
134 injected into the lumen of the upper small intestine. Groups of 10 VM mice (8-
135 10 weeks old) were anaesthetised by intraperitoneal injection of a mixture of
136 Hypnorm (fentanyl/fluoanisone) and Hypnovel (midazolam) (Schering-Plough
137 Animal Health, Welwyn Garden City, UK). With the animal in dorsal

138 recumbency, a small incision was made in the skin of the upper abdomen, the
139 upper loop of the jejunum just posterior to the duodenum was visualised and
140 an injection made through the mesenteric membrane using a 1ml syringe with
141 a 30G needle. Groups of 10 mice were sacrificed at 3-weekly intervals (3 to
142 21 weeks) post-inoculation (p.i.) or on appearance of defined clinical
143 symptoms at around 22-24 weeks [14].

144

145 **Primary challenge of VM mice via transient exposure of the gingival**
146 **margin.**

147 Dental files were selected to perform the study, due to their relatively small
148 size and ease of handling. Size "08" (21mm) dental files were immersed in
149 10% brain homogenate and incubated for 30 minutes. Files were removed,
150 and air dried at room temperature for 1 hour.

151

152 The mice were fully anaesthetized, as above, and the infected dental file was
153 gently inserted into the mouth of the mouse in parallel with the right jawbone
154 at the height of the gingival margin. It is highly likely that the far point of the
155 file (up to a maximum of 1mm will have entered the outer layer of the gingival
156 epithelium (but not the area known as the gingival sulcular
157 epithelium adjacent to the tooth socket). Due to the parallel placement, this
158 penetration would have been at a very glancing angle to the tissue and the
159 majority of the file was thus left lying in parallel contact with the gingiva along
160 the length of the jaw (jaw about 6 to 7mm length; contact region with file
161 estimated at around 5mm) for the designated 5 minute period, after which it
162 was gently withdrawn. Due to the serrated nature of these files damage to the

163 epithelium cannot be ruled out, but on no occasion was there any trauma or
164 bleeding observed during or after this procedure so any damage to the
165 epithelium will have been minimal.

166

167 The maximum load of infectivity on coated dental files was estimated. The
168 dental files are manufactured as a morse taper with an end diameter of
169 0.08mm. Assuming a 5mm section was inserted into the mouth, the maximum
170 diameter would be around 0.18mm. Surface area of a plain wire would be
171 approximately $2\text{mm}^2 (2\pi(r_{\text{av}})h + \pi r_{\text{end}}^2)$. The fluting is assumed to increase the
172 area by no more than 5 fold (maximum surface area 10mm^2). Previous
173 studies using a similar coating strategy have suggested retention of
174 approximately $0.2\mu\text{g}$ brain tissue per mm^2 [15]. Based on a titre of $10^{8.9} \text{ID}_{50}$
175 per gram brain [14] the maximum load of infectivity on the dental file is
176 estimated at $4 \times 10^{2.9} \text{ID}_{50}$ per challenge).

177

178 Groups of 10 mice were sacrificed at 4-weekly intervals (1-6 months) post-
179 inoculation (p.i.) or on appearance of defined clinical symptoms [14].

180

181 **Analysis of time-course samples**

182 The whole brain (including the medulla oblongata), spleen, salivary gland,
183 trigeminal ganglia, dental pulp, gingival margin, lingual muscle (front 2/3rds of
184 the tongue), lingual tonsil (back 1/3 of tongue including tonsular tissue),
185 salivary gland (submandibular) and saliva (following pilocarpine stimulation)
186 were collected from mice at the different time points. The individual tissues
187 from each time point were pooled and stored at -80°C prior to re-inoculation.

188 Tissue homogenates were prepared at 20% (w/v) tissue in phosphate
189 buffered saline using a Ribolyser (Fast prep 120A; Q-Biogene). As the weight
190 of tissue from the dental pulp could not be measured this tissue was diluted to
191 the minimum volume of homogenate required for re-inoculation. Ribolyser
192 beads were washed with 100µl PBS, which was used to dilute the
193 homogenates to 10% (w/v) prior to inoculation.

194

195 *In vivo analysis.*

196 The infectivity of the tissues was assessed by i.c. inoculation into the brains of
197 VM mice. Groups of 6 VM mice (6-8 weeks old) were anaesthetised by intra-
198 peritoneal injection with alfaxalone/alfadolone (Saffan, Schering-Plough
199 Animal Health, Welwyn Garden City, UK) and inoculated intra-cranially with
200 20µl of the 10% homogenate. Non-specific toxicity was observed in some
201 groups and samples were diluted (to 1% or 0.1%) as required.

202

203 Mice were monitored for clinical symptoms and sacrificed by injection of
204 barbiturate (pentobarbitone sodium) at a defined clinical end-point. Brains
205 from indicator mice were removed and stored in formalin prior to histological
206 assessment by Animal Health and Veterinary Laboratories Agency,
207 Weybridge, UK.

208

209 *In vitro analysis*

210 Homogenates were analysed by Western blot essentially as described
211 previously [14]. In brief, homogenates were digested with Proteinase-K at a
212 final concentration of 5.37 µg/ml for 30 minutes at 60°C. The enzyme was

213 inactivated by incubation with 5mM APMSF (Sigma, Gillingham, UK) in Nu-
214 Page™ gel loading buffer (Invitrogen, Paisley, UK) at 99°C for 10 minutes.
215 Samples, together with the relevant controls, were run on 4-12% Bis Tris
216 NuPage gels (Invitrogen, Paisley, UK) and transferred to nitrocellulose. The
217 membrane was blocked in 5% skimmed milk powder in phosphate buffered
218 saline containing 0.1% Tween 20 (PBS-T) for 30 minutes, washed in PBS-T
219 and incubated with primary antibody 6H4 (Prionics, Schlieren, Switzerland) (at
220 1:10,000 dilution) for 18 hrs at 4°C. The membrane was washed four times in
221 PBS-T and bound antibody was detected with anti-mouse horse radish
222 peroxidase (HRP)-conjugate (Sigma, Gillingham, UK); diluted 1:1000). Signal
223 was generated using West Dura reagent (Pierce, Cramlington, UK) and
224 imaged using a Chemidoc image analyser (Pharmacia, Sandwich, UK). The
225 Western blot method could not detect signal below a gel loading equivalent to
226 a 0.1% brain homogenate (results not shown).

227

228

229 ***Role of the funding source:***

230 The study was funded by The Department of Health (England) under contract
231 number 007/0099. The funders had no role in study design, data collection
232 and analysis, decision to publish or preparation of the manuscript. The
233 funding body were invited to comment on the manuscript and this resulted in
234 minor changes to the text of the document.

235

236 The authors were not paid to write the manuscript other than through salary
237 support as part of the grant awarded.

238

239 The contributing author had full access to all the data generated as part of the

240 study and made the final decision to submit the manuscript for publication.

241 **RESULTS**

242 **Primary transmission of infectivity from the small intestine to simulate**
243 **oral exposure to BSE.**

244 Mice were challenged via direct inoculation into the small intestine to avoid
245 any chance of contamination of the oral tissues during the primary challenge.
246 Disease transmission was observed in all animals, with a mean incubation
247 period to a defined clinical endpoint of 157 ± 17 days (Table 1A). Previous
248 studies have shown that direct i.c. challenge with the same titre of infectious
249 BSE-301V stock (estimated titre $10^{8.9}$ infectious units per gram brain [14])
250 reaches a clinical end-point in 120 ± 8.5 days.

251

252 **Analysis of relative levels of infectivity in oral tissues following**
253 **simulated oral exposure.**

254 The levels of infectivity in different oral and control tissues were assessed by
255 re-inoculation of 10% (w/v) tissue homogenate, intracranially into VM mice.
256 The mean incubation period was compared to a titration series generated
257 from BSE-301V terminal brain material as reported previously [14]. It is
258 assumed in this study that serial dilution of infectivity would be unaffected by
259 the tissue type and as such the incubation period can be used as an
260 indication of the relative titre in the different tissues. In all cases shorter
261 incubation to clinical symptoms is indicative of higher titre.

262

263 The study aimed to demonstrate the relative maximum levels of infectivity in
264 different oral tissues following simulated food-borne exposure to BSE
265 contamination. All tissues/fluids at the terminal stage of disease showed the

266 presence of infectivity (Table 2). In all tissues except for the lingual tonsil,
267 terminal tissues showed the maximal levels of infectivity recorded for that
268 tissue. Incubation periods ranged from 118 days (± 0 days, 2/2 animals
269 infected) for brain tissue through to 213 days (± 33 days, 4/5 animals infected)
270 for lingual muscle tissue. In the case of lingual tonsil, the shortest incubation
271 period (197 ± 26 days) and highest attack rate (5/5) was reached by the 15
272 week time point. The lingual tonsil material from terminal animals showed
273 lower levels of infectivity with only a single animal (1/6) succumbing to
274 disease with an incubation of 222 days.

275

276 The oral tissues most likely to be contacted during routine dental surgery,
277 (gingival margin and dental pulp), gave mean incubation periods of 152 days
278 (± 0 days, 6/6 animals challenged) and 160 days (± 55 days, 6/6 animals
279 challenged), respectively. To provide a comparison of the relative levels of
280 infectivity, titrated brain samples gave mean incubation periods of 141 ± 11
281 day (~ 1000 ID₅₀/milligram), 157 ± 18.5 (~ 100 ID₅₀/milligram) and 226 ± 94 days
282 (~ 10 ID₅₀/milligram) ([14]). This suggests that gingival margin has between
283 100 and 1000 ID₅₀/milligram, whilst dental pulp is at least 10 to 100
284 ID₅₀/milligram given that the homogenate was less than 10% (w/v).

285

286 Maximal levels of infectivity were observed in all time course tissues, other
287 than saliva, ahead of the appearance of any clinical symptoms. Maximal
288 levels were reached by week 3 (spleen), 9, (salivary gland), 12 (brain, dental
289 pulp, lingual tonsil), 15 (trigeminal ganglia, lingual muscle, alveolar bone), 18
290 (gingival margin), respectively. Clinical symptoms appeared around week 22,

291 with these animals collected as the terminally diseased group. Saliva from
292 terminal animals was the only time point which showed infectivity for this
293 sample (mean incubation 207 ± 44 days; 4/6 animal diseased).

294

295 By the first time-point at 3 weeks post-challenge, infectivity was already
296 detected in the brain, spleen, trigeminal ganglia, gingival margin, dental pulp,
297 salivary gland, alveolar bone, and lingual tonsil (with synulox), but not in
298 lingual muscle or saliva. Incubation periods ranged from 129 days (± 2 days;
299 attack rate 4/4 animals) for spleen to 273 days (1/5 animals) for gingival
300 margin (Table 2). The incubation period in the spleen sample was already at
301 the minimum level, corresponding to a maximum level of infectivity for this
302 tissue. By contrast brain tissue showed a mean incubation period of 233 days
303 with only 1 of 3 mice that survived challenge developing disease.

304

305 Brain samples were also analysed by Western blot using antibody 6H4
306 following proteinase K digestion of the 10% homogenates (Figure 1). In
307 contrast to the bioassay results, levels of detectable PrP^{res} varied significantly
308 with the conventional triple glycoform banding pattern being observable in the
309 12 week brain samples only with extended exposure (results not shown) and
310 increasing in the 15, 18 and 21 week samples to reach maximal levels only in
311 the terminal group.

312

313

314 **Transmission of infectivity from the gingival margin following transient**
315 **exposure.**

316 Dental files were used to assess whether short term contact was able to
317 transmit infectivity via the gingival margin. The exposure was designed to
318 mimic relatively atraumatic contact between a contaminated dental instrument
319 and gingival epithelium (although limited abrasion of the gingival epithelium
320 cannot be excluded – see materials and methods). The dental files were
321 coated in 10% (w/v) brain homogenate to provide a worse case challenge via
322 this route and in the absence of prior data on levels of infectivity in oral
323 tissues.

324

325 Transmission via this challenge route was shown to be efficient with 97.1%
326 (68/70) of challenged animals succumbing to disease. When the incubation
327 period of individual animals was plotted (Figure 2A and B), two distinct
328 incubation-period groups were identified (Paired T-test; $p < 0.001$). The mean
329 incubations for these two populations are shown separately in table 1B. The
330 “early” terminal group had a mean incubation period of 166 ± 18 days ($n=11$;
331 range 140-188) whilst the “standard” terminal group had a mean incubation
332 period of 247 ± 14 days ($n=57$; range 211-275).

333

334

335 **Relative levels of infectivity in early and standard terminal groups,**
336 **resulting from challenge via the gingival margin.**

337

338 The tissues from early and standard terminal groups were collected and
339 processed as separate groups for re-inoculation into indicator mice (Table 3).

340 The groups showed similar incubation periods in most tissues. Only alveolar

341 bone (174 ±6 days, 6/6 animals challenged vs 160 ±5 days, 6/6 animals
342 challenged) did not show overlapping standard deviations for early vs
343 standard terminal groups, respectively. Comparisons were not made where
344 there were less than 3 surviving animals in each challenged group (lingual
345 muscle, saliva and gingival margin).

346

347 **Analysis of relative levels of infectivity in oral tissues following transient**
348 **challenge via the gingival margin.**

349 Levels of infectivity were assessed as described above. Again, all tissues at
350 the terminal stage of disease showed the presence of infectivity (Table 3).
351 Incubation periods ranged from 126 days (+/- 5 days, 6/6 animals challenged)
352 for brain material to 198 days (+/- 43 days, 3/5 animals challenged) for lingual
353 tonsil, with all tissues showing maximal levels of infectivity in terminal animals.
354 Saliva again showed infectivity only in terminally diseased animals (160 days,
355 1/2 animals challenged) and in a single animal at the earliest time point at
356 extended incubation (353 days, 1/5 animals challenged) possibly due to
357 persistence of the original inoculum.

358

359 Maximal levels of infectivity were again reached for all tissues (except for
360 saliva) well ahead of the presentation of clinical symptoms by 4 months (brain
361 and dental pulp) and 5 months (for all remaining tissues).

362

363 By the first time point in the time course, infectivity was detected in spleen,
364 gingival margin, lingual muscle, dental pulp, salivary gland, lingual tonsil and
365 alveolar bone, but not in brain, saliva or trigeminal ganglia. Incubation periods

366 ranged from 181 days (+/- 13 days, 6/6 animals challenged) for salivary gland
367 to 314 days (+/- 116 days, 3/6 animals challenged) for gingival margin. In
368 several cases, notably in alveolar bone, lingual tonsil and gingival margin,
369 infectivity was not observed in the 2 month time-point, nor in the 3 month
370 time-point for gingival margin and lingual tonsil. This again may suggest
371 localised persistence of the inoculum followed by clearance and later
372 infiltration.

373

374 **Comparison of relative levels of infectivity between terminal groups**
375 **challenged by the small intestine or gingival margin.**

376 The relative levels of infectivity were compared between terminally diseased
377 animals from the two different challenge routes. Only the trigeminal ganglia
378 (mean incubation 136 +/- 17 days, 4/4 for small intestine route vs 160 +/- 4
379 days, 6/6 for the gingival challenge route (standard terminal group) and 159
380 +/- 6 6/6 (early terminal group) did not show overlapping standard deviations.
381 The lingual muscle samples were statistically different in the early terminal
382 group from the gingival challenge route when compared to the small intestine
383 challenge route (too few animals survived in the standard terminal group for
384 valid comparisons to be made). Comparisons were not made where there
385 were less than 3 surviving animals in each group (saliva and gingival margin
386 in addition to the lingual muscle standard group).

387

388 At earlier time points accumulation of infectivity was proportionally slower in
389 spleen and trigeminal ganglia than in the gingival challenge route. Spleen in
390 particular showed much slower accumulation of maximal levels of infectivity,

vCJD and dental practice

391 reached by week 3 in the small intestine challenge but not until month 5 in the
392 gingival challenge group.

393

394

395 **Discussion**

396 The principle aim of the study was to provide underpinning information
397 regarding the potential risks of vCJD transmission by dental procedures,
398 which would contribute to a revised dental risk assessment. The data provide
399 an important insight into potential risks, albeit in a small animal model and
400 using a worse-case approach.

401

402 The data presented here adds considerable information to the previous
403 studies related to dental transmission [10-12]. The levels of infectivity
404 observed in this study are lower than those seen in the Ingrosso study [10].
405 There are a number of potential reasons for this difference including; the
406 challenge route used (intraperitoneal vs direct introduction to the small
407 intestine), the higher end titre of scrapie vs the BSE agent (typically 10^{11} ID₅₀
408 per gram brain for 263K Scrapie compared to $\sim 10^9$ ID₅₀ for BSE-301V) and
409 the different nature of the two prion agents themselves. As BSE-301V is
410 derived from the same prion agent that caused vCJD in humans, it could be
411 argued that the lower values are more representative of the levels of infectivity
412 that might be encountered in dental patients. The absence of detectable
413 disease-associated prion protein (PrP^{Sc}) in human vCJD dental tissues [12] is
414 not incompatible with the levels of infectivity observed in this study, given that
415 the bioassay model is considered to be 100-1000 fold more sensitive than
416 even the high sensitivity Western Blot model used in the Head study. The re-
417 infection studies carried out here are also more representative of the routine
418 risks of disease transmission during dental procedures, than the highly

419 invasive procedure used previously [10], where infectious brain material was
420 injected directly into the pulp cavity.

421

422 The transmission of infectivity following direct inoculation into the small
423 intestine proved to be highly efficient. This novel route of challenge probably
424 accesses the same routes of infection that would be encountered after oral
425 uptake of infectious material but without the significant reduction in titre (of the
426 order of 2-3 log) expected on passage through the stomach. Whilst the
427 approach will inevitably result in localised trauma at the incision site, the
428 incubation period suggests that leakage into the peritoneum was not the
429 primary route of infection as intraperitoneal challenge has resulted in animals
430 reaching their clinical end-point at 196 days [16] with oral challenge at 245
431 days (unpublished; referenced in
432 [http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/do](http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_081219.pdf)
433 [cuments/digitalasset/dh_081219.pdf](http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_081219.pdf) (last accessed 12th November 2011)).
434 Rapid accumulation of infectivity in the spleen, reaching maximal levels by the
435 three week time-point, provides evidence of efficient infection through the
436 small intestine.

437

438 The observed levels of infectivity, as estimated from incubation period, are
439 higher than would have been expected in many tissues within the oral cavity.
440 The two tissues most likely to be relevant to understanding the risks of
441 iatrogenic dental transmission, the gingival margin and dental pulp, show
442 levels of infectivity of between 100-1000 and at least 10-100 infectious doses
443 (ID) per mg tissue, respectively (based on the titration series for brain material

444 shown in [14]). The maximal levels of infectivity were reached well ahead of
445 the presentation of clinical symptoms in the majority of tissues. This is likely to
446 be similar in the human situation.

447

448 At the outset of the study, there was no indication in the literature that the two
449 routes of infection would be as efficient as they proved to be. As such the
450 study used a high challenge dose in order to be able to draw conclusions as
451 to the spread of infection and accumulation of high levels of infectivity under
452 worst-case conditions. Despite this, we do not believe that the use of a high
453 challenge dose, distorts the key findings of the study. In the small intestine
454 challenge experiments, the levels of infectivity in oral tissues are actually
455 lower than the levels observed in the one limited but comparable study [10].
456 The accumulation of infectivity in the spleen is comparable to the rate seen in
457 other peripheral challenges (ip and oral) using the same model. The ability of
458 the spleen to amplify infectivity from low-dose oral or peripheral challenge
459 suggests that similar levels of infectivity would have been reached in the oral
460 tissues even with a lower challenge. The different levels of infectivity and the
461 different rate of accumulation of infectivity in different tissues also suggests
462 that the model is not simply saturated with infectivity, but rather that it
463 represents normal spread of infectivity from the intestine, potentially via both
464 lymphoreticular and direct neuronal transmission.

465

466 **Relative levels of infectivity in vivo and PrP^{Sc}-signal detectable in vitro.**

467

468 The presence of maximal levels of infectivity in the brain in mice at 12 weeks
469 is a reminder of the long pre-symptomatic phase of TSE infection. The earliest
470 clinical symptoms were evident at 18 weeks post-exposure with an average of
471 22 weeks. The 10-fold dilution data for the 15, 21 and terminal groups,
472 although limited in nature, suggest that the levels of infectivity reach a
473 maximal level and are maintained over this period, rather than continuing to
474 accumulate in the brain. The observed differences in the level of abnormal
475 prion protein, PrP^{Sc}, in the same time-course samples indicates a marked
476 separation between the level of infectivity and the detected level of its
477 surrogate marker as observed in previous studies [17, 18]. This may indicate
478 that an equilibrium is reached for the most infectious form of the agent (e.g.
479 the 14-28 unit prion protein oligomers [19]) and that this remains unaltered
480 despite the ongoing accumulation of Proteinase K-resistant aggregated
481 PrP^{res}. Alternatively, the overall level of infectivity may remain approximately
482 constant since the capacity to act as individual nuclei of infection diminishes
483 proportionately to the increasing extent of PrP^{res} aggregation. Neither
484 hypothesis was tested in the current study.

485

486 The transient exposure of the gingival margin to infectivity dried onto dental
487 files demonstrates the potential for iatrogenic transmission of infectivity
488 through contaminated dental instrument contact within the oral cavity. The
489 challenge was designed to be less invasive than previous oral inoculations
490 [10] and gingival scarification [21]. Given the relatively atraumatic instrument
491 contact, the efficiency of transmission was greater than expected with >97%
492 of challenged animals succumbing to disease, with a total population mean of

493 233 days. The identification of two sub-populations within the culled animals
494 on the basis of incubation period is intriguing. One of these populations could
495 represent animals infected by ingestion of material following oral exposure.
496 However, the use of a low challenge titre dried onto the file (estimated at
497 around $4 \times 10^{2.9}$ ID per file) and given the incubation period observed for much
498 higher challenges via the oral route (245 days; see above), would suggest
499 that ingestion is not the major infection route. The rapidly progressing (early)
500 disease may be a result of localised trauma to the gingiva, providing more
501 efficient spread of the disease, or may indicate that localised uptake has
502 accessed different infection routes, perhaps mediated by neuronal (early
503 terminal) and/or lymphatic (standard terminal) tissues, respectively. The
504 relatively rich neurological innervations of the oral cavity and links with the
505 trigeminal nucleus in the brain stem may contribute to this rapid route of
506 spread. Despite the significant differences in the incubation period of animals
507 identified as early or standard terminal groups, widespread differences in the
508 levels of infectivity in tissues were not observed on re-challenge.

509

510 The gingival challenge route is entirely novel and was designed to ask
511 specifically whether infectivity could be transmitted via transient contact rather
512 than direct inoculation [10]. To assess this, and given the very small amounts
513 of inocula that are carried on the contaminated dental files, a high titre
514 material was essential in order to test the feasibility of transmission. In terms
515 of the validity of the model, the absence of infectivity at the 2 month time point
516 for several tissues, including gingival margin, suggests that infection is not
517 simply being generally disseminated through the oral cavity. Again this

518 suggests that whilst the model is a worst-case the results are not incompatible
519 with a natural infection from a contaminated instrument at lower titres.

520

521 Similar levels of infectivity were observed at the end of the two bioassay
522 studies, with the exception of lingual muscle which was higher in mice
523 challenged via the gingival challenge route. Comparing the levels of infectivity
524 at the first point of each time course showed greater dissemination of
525 infectivity within the oral cavity for the gingival challenged animals. This might
526 have been expected with the tissues potentially retaining some of the initial
527 inocula. This is supported by the subsequent loss of infectivity in three of the
528 oral tissues by the second time-point, with gingival margin and lingual tonsil
529 remaining non-infectious at the third monthly time-point. In contrast, organs
530 which might be indicative of ingestion and systemic spread from the oral
531 cavity remained less infectious for a greater proportion of the time-course. For
532 example, the spleen showed only limited transmission and a longer incubation
533 period following gingival challenge at the first time-point, with levels of
534 infectivity not reaching those observed following challenge via the small
535 intestine until the final terminal group. Similarly, neither trigeminal ganglia nor
536 brain showed infectivity at the first monthly time-point and increased only
537 gradually over time. The high levels of infectivity in the salivary gland at the
538 first time-point and increasing through the incubation suggest that infectivity
539 was concentrated and amplified in this organ rather than being disseminated
540 from the descending nerves linked to the trigeminal ganglion, the latter
541 showing lower levels of infectivity throughout the time-course.

542

543

544 **Implications for public health.**

545 There is currently no evidence for the transmission of vCJD through any form
546 of surgical procedure, including dental practice. Proven transmission by blood
547 transfusion [5,9] suggests that surgical transmission is a potential risk via
548 procedures that contact either nervous or lymphoid tissue. For example,
549 infectivity has been found in the rectum from a vCJD patient (at 0.001%
550 infectivity of brain), but not for sporadic CJD, demonstrating the broader
551 distribution of infectivity [22-24]. Together with this observation, the highly
552 efficient transmission of infection through direct inoculation into the small
553 intestine in this study, perhaps raise concerns for endoscopic procedures.

554

555 This study uses a well established mouse model and BSE strain (as a
556 surrogate for vCJD) that has been used previously to investigate risks of
557 human to human transmission. Taken together, the observations in the
558 current study provide theoretical grounds for concern in relation to dental
559 procedures. The levels of infectivity observed in all the oral tissues, notably
560 gingival margin tissue with up to an estimated 1000 ID per mg of tissue, were
561 higher than estimated previously.

562

563 A separate component of the study, has assessed the residual protein
564 contamination on a range of dental instruments after routine cleaning and
565 disinfection in general dental practice in England [25]). The study showed a
566 number of instrument types and cleaning procedures where the upper
567 interquartile range for residual protein was in excess of 100µg. This could

568 equate to up to 100 ID per instrument even in the case of the gingival tissue.
569 Autoclaving has been shown to achieve up to a 3-log inactivation of various
570 TSE agents [26] although an autoclave designed for the dental market has
571 recently been investigated and shown to provide only a 2-log inactivation in
572 the TSE model used here (134°C, 18 minutes; Sutton et al unpublished).
573 Whilst the combined prion inactivation of multi-stage decontamination
574 procedures remains to be proven, a dental instrument soiled with infectious
575 gingival tissue, under this combination of cleaning and autoclaving, would not
576 leave a significant safety margin.

577

578 The gingival challenge was designed as a worse case scenario, with respect
579 to the loading of infectious material onto the surface of the dental instrument,
580 but was not a highly invasive procedure. The procedure resulted in very high
581 levels of transmission, suggesting that if the titre of the challenge material was
582 reduced, transmission would still be likely to occur. Even if this was a
583 relatively rare event, the very large number of dental interventions that take
584 place and the high number of procedures carried out on younger age groups
585 (in contrast to most medical surgical procedures) means the risks are not
586 negligible.

587

588 The study provides evidence to inform the ongoing debate about the control of
589 potential risks of vCJD transmission in dental practice. Preliminary data from
590 the study have already been provided to Department of Health as part of their
591 revision to the dental risk assessment
592 (http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/do

593 [cuments/digitalasset/dh_081217.pdf](#); accessed 12th November 2011). A
594 number of additional control measures have been put in place, including the
595 recent revision of the Health technical memorandum relating to
596 decontamination in dental settings in England
597 ([http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsP
598 olicyAndGuidance/DH_109363](http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_109363); accessed 12th November 2011). An emphasis
599 on universal decontamination methods and single use for difficult to clean
600 devices would appear to be sensible precautions given the observations
601 described in this study, which significantly broaden the possible routes of
602 infection through dental procedures, with wider dissemination of infectivity in
603 the oral cavity and transmission by transient exposure.

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606

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613 those of the Department of Health or the Health Protection Agency. There are
614 no conflicts of interest.

615

616

617 **Figures and tables:**618 **Table 1A: Summary of primary challenge data for different transmission routes**

619

Challenge Route	Attack rate (number of animals succumbing to disease / number of animal challenged (% attack rate))	Mean incubation / days post infection \pm standard deviation
Small intestine challenge	46/46 (100%)	157 \pm 17*
Gingival margin challenge	68/70 (97.1%)	233 \pm 33.4 [¶]

620 * range 131-230 days, median 153 days; 1 mouse died without clinical BSE
 621 symptoms at 422 days post-challenge, with no histological confirmation of
 622 BSE and was excluded from the calculation (otherwise 178 \pm 67 days). Outlier
 623 at 230 days; otherwise 156 \pm 14 range 131-192.

624 [¶] **Table 1B: Separate analysis of primary cull data from gingival challenge shows**
 625 **two populations**
 626

Challenge Route	Attack rate (number of animals succumbing to disease / number of animal challenged (% attack rate))	Mean incubation / days post infection \pm standard deviation (range). Data for TSE positive animals only.
Gingival margin; Early terminal only	11/11 (100)	166 \pm 18 (140-188)
Gingival margin; Late terminal only	57/59 (96.6%)	247 \pm 14 (211-275)

627

628

vCJD and dental practice

Weeks	Brain	Brain 0.1%	Brain 0.1%	Spleen	Spleen repeat	Spleen: 1%	Spleen: 0.1%	Saliva	Gingival margin	Lingual muscle	Dental pulp	Trigeminal ganglion	Salivary gland	Alveolar bone	Alveolar bone repeat	Lingual tonsil	Lingual tonsil + synulox
3	233 <i>N/A</i> <i>1/3</i>			129 <i>± 2</i> <i>4/4</i>	134 <i>± 0</i> <i>5/5</i>			<i>0/6</i>	273 <i>N/A</i> <i>1/5</i>	129 <i>± 0</i> <i>0/4</i>	269 <i>N/A</i> <i>1/6</i>	168 <i>± 0</i> <i>2/3</i>	184 <i>± 22</i> <i>5/5</i>		230 <i>± 25</i> <i>2/6</i>	<i>0/4</i>	380 <i>N/A</i> <i>1/5</i>
6	197 <i>± 26</i> <i>5/5</i>			142 <i>± 13</i> <i>6/6</i>	151 <i>± 23</i> <i>2/2</i>			<i>0/6</i>	243 <i>± 30</i> <i>4/5</i>	328 <i>± 193</i> <i>3/6</i>	192 <i>± 0</i> <i>4/6</i>	551 <i>N/A</i> <i>1/6</i>	158 <i>± 4</i> <i>5/5</i>		257 <i>± 18</i> <i>2/6</i>	<i>0/5</i>	424 <i>± 73</i> <i>2/6</i>
9	147 <i>± 4</i> <i>5/5</i>				132 <i>± 9</i> <i>6/6</i>	170 <i>± 47</i> <i>3/3</i>	199 <i>± 105</i> <i>4/5</i>	<i>0/6</i>	284 <i>± 127</i> <i>5/6</i>	<i>0/6</i>	346 <i>± 90</i> <i>3/5</i>	249 <i>± 69</i> <i>4/5</i>	141 <i>± 3</i> <i>6/6</i>		231 <i>± 33</i> <i>2/6</i>	<i>0/4</i>	238 <i>± 18</i> <i>2/6</i>
12	118 <i>+/- 0</i> <i>2/2</i>			130 <i>± 0</i> <i>4/4</i>	130 <i>± 5</i> <i>6/6</i>			<i>0/6</i>	184 <i>± 16</i> <i>5/6</i>	344 <i>± 101</i> <i>3/4</i>	148 <i>± 0</i> <i>6/6</i>	140 <i>± 5</i> <i>6/6</i>	139 <i>± 7</i> <i>6/6</i>		192 <i>± 11</i> <i>6/6</i>	215 <i>± 39</i> <i>5/5</i>	190 <i>± 24</i> <i>4/5</i>
15	118 <i>± 0</i> <i>6/6</i>	135 <i>± 4</i> <i>6/6</i>		153 <i>± 15</i> <i>4/5</i>	178 <i>± 49</i> <i>6/6</i>			<i>0/6</i>	188 <i>± 28</i> <i>3/6</i>	204 <i>± 24</i> <i>4/5</i>	237 <i>± 40</i> <i>5/6</i>	120 <i>± 0</i> <i>6/6</i>	140 <i>± 0</i> <i>6/6</i>		172 <i>± 7</i> <i>6/6</i>		197 <i>± 26</i> <i>5/5</i>
18		118 <i>± 0</i> <i>4/4</i>		130 <i>± 0</i> <i>3/3</i>				<i>0/5</i>	153 <i>± 5</i> <i>6/6</i>	182 <i>± 21</i> <i>4/5</i>	156 <i>± 0</i> <i>2/5</i>	115 <i>± 9</i> <i>6/6</i>	134 <i>± 9</i> <i>5/6</i>		167 <i>± 8</i> <i>5/5</i>		196 <i>± 12</i> <i>5/5</i>
21			185 <i>± 114</i> <i>6/6</i>	137 <i>± 6</i> <i>6/6</i>				<i>0/6</i>	157 <i>± 6</i> <i>6/6</i>	236 <i>± 72</i> <i>5/6</i>	186 <i>± 27</i> <i>6/6</i>	121 <i>± 6</i> <i>5/5</i>	150 <i>± 5</i> <i>5/5</i>		183 <i>± 6</i> <i>6/6</i>		377 <i>± 245</i> <i>2/6</i>
Term.			118 <i>± 0</i> <i>2/2</i>	134 <i>± 6</i> <i>6/6</i>				<i>4/6</i>	152 <i>± 0</i> <i>6/6</i>	213 <i>± 33</i> <i>4/5</i>	160 <i>± 55</i> <i>6/6</i>	136 <i>± 17</i> <i>4/4</i>	143 <i>± 6</i> <i>6/6</i>	158 <i>± 11</i> <i>4/4</i>	185 <i>± 15</i> <i>6/6</i>		222 <i>N/A</i> <i>1/6</i>

Table 2: Average incubation periods for VM mice challenged with tissues taken following small intestine challenge. The mean incubation period (Bold), standard deviation (italics) and attack rate (mice infected / mice challenged) are all shown.

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<i>Months</i>	<i>Brain</i>	<i>Brain: 1%</i>	<i>Brain: 1%</i>	<i>Spleen</i>	<i>Saliva</i>	<i>Gingival margin</i>	<i>Lingual muscle</i>	<i>Dental pulp</i>	<i>Trigemina l ganglion</i>	<i>Salivary gland</i>	<i>Alveolar bone</i>	<i>Lingual tonsil + synulox</i>	
1	0/1	0/6		252 ± 11 2/6	353 N/A 1/5	314 ± 116 3/6	311 ± 36 4/5	255 ± 47 5/5		181 ± 13 6/6	262 ± 69 3/5	296 N/A 1/6	
2	157 ± 12 3/4			163 ± 8 3/3	0/6	0/6	243 ± 63 5/6	244 ± 39 4/6	292 N/A 1/6	146 ± 8 4/4	0/6	0/6	
3	147 ± 5 5/5			163 ± 11 5/6	0/6	0/3	249 ± 46 5/6	206 ± 30 5/6	317 ± 60 2/5	143 ± 5 5/5	281 ± 77 3/6	0/4	
4	131 ± 2 4/4			162 ± 45 6/6	0/6	3/5	211 ± 24 5/6	226 ± 18 6/6	184 ± 7 6/6	214 ± 15 5/6	141 ± 0 6/6	200 ± 23 6/6	268 ± 88 3/6
5	124 ± 19 6/6			140 ± 4 6/6	0/5	4/5	189 ± 11 6/6	192 ± 10 6/6	228 ± 124 6/6	166 ± 17 6/6	136 ± 4 6/6	198 ± 34 3/6	205 ± 24 5/5
6	136 ± 7 3/3			141 ± 6 5/5	0/6	4/4	241 ± 32 6/6	206 ± 22 6/6	182 ± 13 6/6	289 ± 123 3/6	136 ± 3 6/6	297 ± 167 4/6	200 ± 5 2/6
Early terminal	120 ± 7 3/3		128 ± 4 6/6	150 ± 16 6/6	N/A 1/3	2/2	153 ± 0 5/5	155 ± 2 6/6	179 ± 19 6/6	159 ± 6 6/6	134 ± 3 5/5	174 ± 6 6/6	217 ± 64 5/5
Standard terminal	126 ± 5 6/6			134 ± 2 4/4	N/A 1/2	2/2	181 ± 0 2/2	175 ± 1 2/2	161 ± 10 4/5	160 ± 4 6/6	134 ± 3 5/5	160 ± 5 6/6	198 ± 43 3/5

636 **Table 3: Average incubation periods for indicator animals challenged with tissues taken following gingival margin challenge of VM mice.** The
637 mean incubation period (Bold), standard deviation (italics) and attack rate (mice infected / mice challenged) are shown for each tissue type taken
638 through the time course. Based on the frequency distribution, two separate groups of terminal samples were taken and treated separately, termed early
639 and standard terminal groups.

640 **Figure legends:**

641 **Figure 1:** Detectable levels of PrP^{Sc} on Western blots do not correlate with
642 the levels of infectivity. 10% brain homogenates from an uninfected brain
643 (lane 2) time-course samples week 3, 6, 9, 12, 15, 18, 21 (lane 3-9), and the
644 terminal sample (lane 10) were digested with proteinase K at 60°C for 10
645 minutes and assessed by Western blot. The observed signal does not
646 correspond with the levels of infectivity found in corresponding bioassays for
647 the week 12-21 post-exposure time-points.

648

649 **Figure 2:** Comparison of the cull dates for the mice challenged via the
650 gingival margin. Panel A; Frequency distribution plots show the presence of a
651 normally distributed population with a mean incubation period of around 250
652 days plus a small number of animals with significantly shorter incubations
653 ranging from 140-188 days. Panel B; when these two groups are compared
654 they show distinct means and distribution and are considered as distinct
655 populations ($p < 0.001$).

656

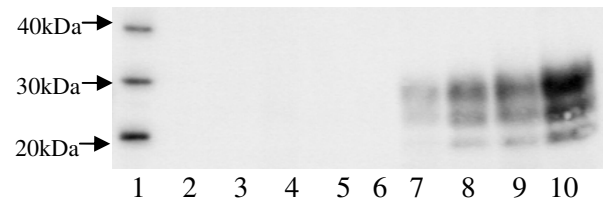
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660 **Figures:**

661 **Figure 1.**

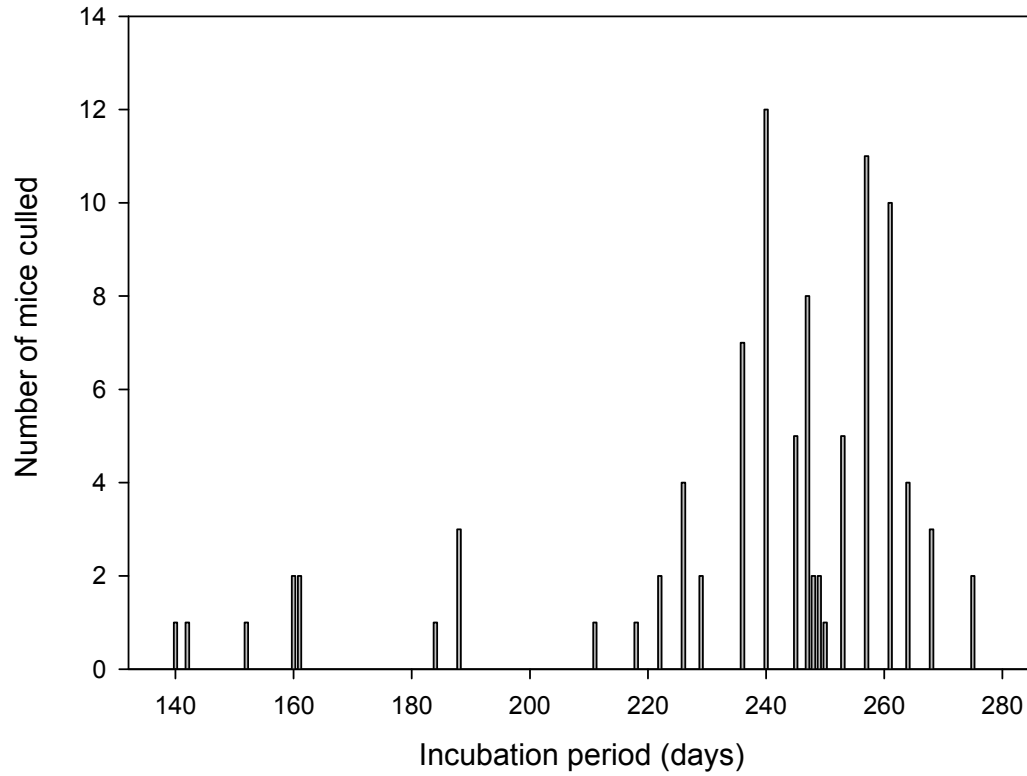


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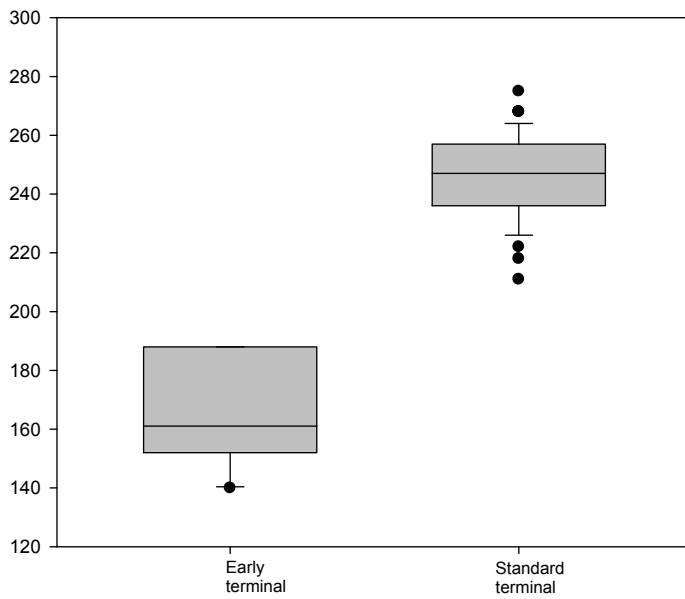
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665 **Figure 2A**



666

667 **Figure 2B.**



668

669

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