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Outbreak of encephalitic listeriosis in red-legged partridges (*Alectoris rufa*)

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Abstract

An outbreak of neurological disease was investigated in red-legged partridges between 8 and 28 days of age. Clinical signs included torticollis, head tilt and incoordination and over an initial 8 day period approximately 30-40 fatalities occurred per day. No significant gross post mortem findings were detected. Histopathological examination of the brain and bacterial cultures followed by partial sequencing confirmed a diagnosis of encephalitis due to *Listeria monocytogenes*. Further isolates were obtained from follow-up carcasses, environmental samples and pooled tissue samples of newly imported day-old chicks prior to placement on farm. These isolates had the same antibiotic resistance pattern as the isolate of the initial post mortem submission and belonged to the same fluorescent amplified fragment length polymorphism (fAFLP) subtype. This suggested that the isolates were very closely related or identical and that the pathogen had entered the farm with the imported day-old chicks, resulting in disease manifestation in partridges between 8 and 28 days of age. Reports of outbreaks of encephalitic listeriosis in avian species are rare and this is to the best of our knowledge the first reported outbreak in red-legged partridges.

Key words: *Listeria monocytogenes*, listeriosis, partridge, encephalitis, outbreak, avian listeriosis, game birds, encephalitic listeriosis

Introduction

The genus *Listeria* consists of 10 species, two of which have been associated with disease in humans and animals (Orsi et al., 2011; Bertsch et al., 2013; Lang Halter et al., 2013). Disease due to *Listeria ivanovii* appears to be rare and restricted to

46   ruminants (Orsi et al., 2011; Low & Donachie, 1997). In contrast *Listeria*

47   *monocytogenes* is associated with disease in a variety of animal species and man

48   (Gray, 1958; Low & Donachie, 1997). *L. monocytogenes* has a worldwide distribution

49   and is widespread within the environment (Gray & Killinger, 1966). There are 16

50   known serotypes but only three have been commonly described in context with

51   disease which include 1/2a, 1/2b and 4b (Low & Donachie, 1997; Seeliger & Jones,

52   1986). Phylogenetically *Listeria* spp are divided into four different lineages with 1/2b

53   and 4b predominantly belonging to lineage I, and 1/2a belonging to lineage II (Orsi et

54   al., 2011).

55   Interest in *L. monocytogenes* increased in the 1980s when it was recognised as a

56   significant food-borne pathogen causing disease in humans (Low & Donachie, 1997).

57   In animals, clinically significant infections with *L. monocytogenes* are mostly

58   recognised in ruminants (Low & Donachie, 1997). They have also been described in

59   over 17 avian species including chickens, turkeys, geese, ducks, canaries, parrots and

60   others (Gray & Killinger, 1966). However, listeriosis in birds appears to be rare and

61   outbreaks are sporadic with varying morbidity and mortality (Gray, 1958). Birds are

62   considered difficult to infect with *L. monocytogenes* and young birds are considered

63   most susceptible to disease (Bolin, 1960; Basher et al., 1984). Listeriosis in birds mostly

64   presents as septicaemia with splenomegaly, necrosis of liver and myocardium as well

65   as pericarditis (Gray, 1958; Barnes & Nolan, 2008). Reports of outbreaks of avian

66   encephalitic listeriosis are rare and originate mostly from chickens in the USA, Japan

67   and India (Cooper, 1989; Cooper et al., 1992; Kurazono et al., 2003; Vijayakrishna et

68   al., 2000). Wet weather (flooding), de-beaking or injections into the neck were

69   reported to be likely predisposing factors. Torticollis, depression and incoordination

70 were a consistent clinical sign in affected birds. These neurological signs cannot be

71 distinguished from those of the notifiable Newcastle Disease, which should be

72 considered a potential differential diagnosis to encephalitic listeriosis (Irvine et al.,

73 2009).

74 In the UK there have been occasional publications on the isolation of *L.*

75 *monocytogenes* from avian species including chickens (*Gallus gallus domesticus*)

76 (Paterson, 1937; Paterson, 1939), wild grey partridges (*Perdix perdix*) in the vicinity of

77 an infected sheep flock (McDiarmid, 1961) and a merlin (*Falco columbarius*) that

78 showed neurological signs after having eaten a sparrow presenting with neurological

79 signs (Baker, 1967).

80 The only report found on listeriosis in red-legged partridges refers to the isolation of

81 *L. monocytogenes* from two partridges in France in 1957 without providing any further

82 information (Lucas & Seeliger, 1957).

83 This report describes the first case of encephalitic listeriosis affecting young red-

84 legged partridges.

85

86 **Materials and Methods**

87 **Background:** In May 2011 four 16-day-old red-legged partridges (*Alectoris rufa*) with

88 neurological clinical signs were submitted to the Royal Veterinary College – Animal

89 Health and Veterinary Laboratory Agency (RVC-AHVLA) Surveillance Centre London

90 for post mortem examination. The partridges originated from a game bird rearing farm

91 that imported red-legged partridges as day-old chicks every two weeks during the

92 season. A total of four batches (A-D) varying in size of between 15,000 and 30,000

93 were imported in 2011. The diseased 16-day-old partridge chicks were from batch A's

94   25,000 birds which had lost approximately 30-40 chicks daily over the last eight days.

95   Some birds were found dead but the majority exhibited neurological signs resulting in

96   an inability to use the nipple drinkers. They subsequently either died or were culled.

97   Three sheds out of a total of 20 appeared to be particularly affected, whereas only

98   occasional affected birds were detected in the remaining sheds. There was no

99   improvement following treatment with various antibiotics. However losses started

100   dropping from approximately four weeks of age onwards. Total losses in the first and

101   worst affected batch A reached 5.2% at eight weeks of age (in previous years total

102   losses in partridges on this farm had been approximately 2.5%- 3% at 13 weeks). Three

103   subsequent batches of red-legged partridges were also affected in the same age range

104   but with a much lower morbidity. Their total mortality rate was within expectations

105   for age and production type.

106   Further carcasses of affected birds from the original batch A and the second affected

107   batch B were also submitted for post mortem examination at a later date as were day-

108   old partridges of batch D that had died during transit and had never entered the sheds

109   on the farm. A summary of submitted birds can be seen in table 1.

110   Sheep had been grazing the ground over the winter. However, partridges had no

111   access to outside runs until four-week of age and were kept indoors on cardboard

112   squares on top of a continuous polythene sheet. There was a good level of biosecurity

113   and cleanliness on the farm. Drinking water was supplied from the mains via holding

114   tanks together with electrolytes and multivitamin solution. The weather had been

115   unusually dry and warm during the outbreak and there had been no flooding. Data

116   from the trade control and expert system (TRACES) (a management system of the

117   veterinary authorities to track animal imports within the EU and from outside) showed

118 that the investigated farm was the only farm in the UK that had received day-old

119 partridge chicks from a specific supplier when the first and worst affected batch (A)

120 arrived. Other farms in the UK received day-old partridges from the same supplier only

121 more than a week later.

122

123

124 **Gross pathology and histopathology:** Routine post mortem examinations were

125 performed on the submitted partridges as detailed in table 1. Live partridges were

126 euthanased by cervical disarticulation following clinical examination. Swabs of brain

127 and/ or liver were taken from a total of nine birds and stored in charcoal transport

128 medium until microbiological culturing. Small tissue samples of liver, lung, yolk sac and

129 brain were removed from six one-day-old partridges of batch D that had died during

130 import transit and were pooled in buffered peptone water prior to microbiological

131 culture. Various tissue samples including brains were collected in 10% formol saline

132 and processed for routine histopathology.

133 Sections were prepared, embedded in paraffin wax, serially cut(5μm) and mounted

134 on glass microscope slides. They were stained with haematoxylin and eosin, special

135 Gram stains were also undertaken for some of the sections.

136 **Environmental sampling:** Bacteriological swabs were taken from the lining of the

137 delivery boxes of the day-old imported partridge chicks of batch C during the follow

138 up submission. Subsequently, a farm visit was carried out to collect epidemiological

139 data and environmental samples. Sterile gauze swabs were moistened with buffered

140 peptone water and swabs were taken from the floor of sheds affected by disease of

141 two different batches (A and C), a shed with birds of batch A that had not been

142   affected by disease, straw of an affected shed and clean straw from the storage stack.

143   Aliquots were taken from drinking water, multivitamin solution and two types of

144   feeding pellets. A summary of all collected environmental samples can be seen in table

145   2.

146

147   **Microbiology:** Routine cultures of samples took place on Columbia Blood agar (BA,

148   Oxoid, Basingstoke, UK) and MacConkey agar (MAC, Oxoid) at 37°C. Bacteria were

149   identified using standard laboratory techniques. *L. monocytogenes* was identified by

150   colonial morphology as beta-haemolytic colonies on blood agar and small lactose

151   fermenting colonies on MacConkey agar. They were Gram-positive short rods and

152   catalase test positive. The colonies were confirmed as *L. monocytogenes* either by API

153   Listeria (bioMérieux, Marcy-l'Etoile, France) or the CAMP test. (*L. monocytogenes* is

154   CAMP test positive, *L. innocua* is CAMP test negative (McKellar, 1994)).

155   Many samples were additionally or only cultured in selective media: they were

156   cultured directly onto *Listeria* Selective Agar (LSA, Oxoid) using a swab. Additionally

157   1g or 1ml of samples (depending on sample type) was inoculated into *Listeria* Selective

158   Enrichment Broth (LSB, Oxoid). The LSA plates were incubated overnight at 30°C and

159   daily examined for colonies typical of *Listeria*. The LSB was incubated at 30°C and

160   subcultured after 24 hours onto fresh LSA and again after 48 hours. The subculture

161   plates were examined after 24 and 48 hours for *Listeria*-like colonies as described

162   above. Where *Listeria*-like colonies were isolated on LSA they were subcultured on to

163   BA and MAC for further confirmation as described above.

164   Box liners and environmental samples were pre-enriched in buffered peptone water

165   at 37°C overnight and then cultured directly onto LSA and transferred into LSB.

166

Antibiotic Sensitivity: Two isolates of *L. monocytogenes* isolates were tested against a panel of eight antibiotics, namely Ampicillin (10µg), Doxycycline (30µg), Enrofloxacin (5µg), Lincomycin (10µg), Penicillin (1µg), Tetracycline (10µg), Tylosin (30µg) and Trimethoprim/ Sulphamethoxazole (25µg), supplied as 6mm impregnated discs by Oxoid. They were placed on an Oxoid lysed blood sensitivity agar plate together with emulsified colonies and the plate was incubated for 18-20 hours at 37°C. The zones of inhibition around each antibiotic disc were measured using an automated ProtoZone sensitivity reader (Don Whitley Scientific, Shipley, UK). Resistance was denoted by a total zone diameter of 13mm or less. Resistance gene testing was performed by using an antimicrobial resistance gene microarray for Gram positive organisms that is available through Alere Technologies (Jena, Germany). Genomic DNA was isolated as described by Perreten et al. (2005). Approximately 100 ng of DNA was labelled by a randomly primed polymerization reaction using Sequenase, version 2.0 (USB Corporation, Cleveland, Ohio) and consisted of three cycles of enzymatic reactions (Bohlander et al., 1992). This was followed by hybridisation of the labelled fragments to the array following the protocol of Perreten et al (2005). The arrays were subsequently scanned and the IconoClust software (Alere™) used to identify positive signals.

Partial sequencing: In order to provide preliminary molecular confirmation of isolate identity the virtually complete 16S rRNA gene was amplified from the initial *L. monocytogenes* isolate of the brain of a red-legged partridge (bird 1Ab) by PCR using primer pair 5' AGTTTGATCCTGGCTCAG 3' and 5' ACCTTGTTACGACTT 3' and sequenced

190   using a series of internal primers as described previously (Hunt et al., 2013).

191   Phylogenetic analysis previously described by Graves et al. (2010) was reproduced to

192   confirm placement of the isolate relative to reference strains of *Listeria*. Phylogenetic

193   analysis was performed using the MEGA5 program (Tamura et al., 2011) as described

194   in figure legends. In order to further characterise phylogenetic position, sequencing of

195   fragments of additional housekeeping genes (*sigB*, *gap* and *prs*) was also performed

196   as described by Graves et al. (2010) and phylogenetic analysis was performed as above

197   with reference to the sequences included in Graves et al. (2010).

198

199   **Serogrouping and fAFLP:** On receipt at the National reference Laboratory (NRL) for

200   *Listeria* (Public Health England, London, UK), *Listeria* isolates were cultured on blood

201   nutrient agar (BN) and incubated at 37°C for 12-24h. Bacterial nucleic acid was

202   extracted from an isolated colony grown on BN plate, using MicroLYSIS™ reagent

203   (Microzone Ltd, Haywards Heath, UK). Briefly, a 1µl loop full of bacterial growth was

204   mixed with 19µl of MicroLYSIS solution. The mixture was then subjected to 65°C for 5

205   minutes, 96°C for 2 minutes, 65°C for 4 minutes, 96°C for 1 minute, 65°C for 1 minute

206   and 96°C for 30 seconds and used directly for PCR.

207   *L. monocytogenes* was identified using an in-house duplex real time PCR exonuclease-

208   assay (LM-PCR) amplifying simultaneously a specific 112 bp fragment of the *L.*

209   *monocytogenes* haemolysin gene (*hly*A) and a specific 80bp fragment of the *L.*

210   *monocytogenes* phospholipase A (*plc*A) gene (Nogva et al., 2000). Each reaction

211   contained 25µl of 1x TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems,

212   Life Technologies, California, US), 0.3µM of each of the forward and reverse primers

213   (Eurogentec, Seraing Belgium), 0.1µM of each fluorescently labelled probes

214   (Eurogentec) and 5µl of crude or purified DNA extract. The reaction was performed

215   under 'FAST' default conditions on an Applied Biosystems 7500 FAST Sequence

216   Detection System ('TaqMan') according to the manufacturer's protocol.

217   Molecular serogrouping of *L. monocytogenes* isolates was performed using the

218   multiplex gel-based PCR assay (LSER-PCR) described by Doumith et al. (2004) using 5µl

219   of DNA extract. Pre-cast ethidium bromide-stained 1.5% agarose E-gel© (Invitrogen,

220   Life Technologies) were used to separate the multiplex PCR products by

221   electrophoresis. Results of the electrophoresis were visualised under UV light and

222   photographed using a BioRad™ UV Gel-doc© system (Hemel Hempstead, UK).

223   According to the pattern obtained (Doumith et al., 2004; Huang et al., 2011) isolates

224   were classified as being one of the four *L. monocytogenes* serogroups, as *Listeria* spp.

225   or as being non *Listeria* species.

226   Fluorescent amplified fragment length polymorphism (fAFLP) technique was used to

227   sub-type *L. monocytogenes* isolates and was performed using a modification of a

228   protocol previously described for *Campylobacter* (Desai et al., 2001). Briefly, genomic

229   DNA (15-50ng) was digested with 5U of each of two restriction enzymes, *Hind*III and

230   *Hha*I, (New England Biolabs, Hitchin UK) in the presence of RNase A and bovine serum

231   albumin. Digested fragments were ligated to two sets of specifically designed double

232   stranded adapters. These adapters served as targets for a FAM-labelled Hind-A and a

233   non-labelled Hha-A selective primers (Eurogentec, Seraing Belgium) for fragment

234   amplification by PCR. PCR products were separated on an ABi 3730XL 96capillary DNA

235   Analyzer (Applied Biosystems) alongside a GeneScan™- 600 LIZ™ Size standard, and

236   chromatographs showing FAM-fluorescing fragments were saved as .fsa files. The .fsa

237   files were exported, visualised and analysed on PEAK SCANNER™ v1.0 (Applied

238   Biosystems, Life Technologies, Paisley UK). As well as the chromatographs, PEAK

239   SCANNER™ also recorded the fragment data in a binary format in Excel files. These

240   files were then exported into Bionumerics v6.1 where they were visualised as virtual

241   electrophoresis gels and analysed. The patterns determining the fAFLP types were

242   identified using in-house built libraries.

243

244   **Results**

245   **Clinical signs:** All four live affected red-legged partridge chicks in the first submission

246   exhibited marked neurological signs including torticollis, head tilt and incoordination

247   (Fig. 1).

248

249   **Gross post mortem findings:** Other than septicaemia with perihepatitis and

250   pericarditis in one dead carcass (bird 2Be) and pneumonia concurrent with

251   neurological signs in one live bird (bird 1Aa), there were no significant gross post

252   mortem findings in the remaining carcasses. The majority of the birds had empty crops

253   and gizzards and slightly prominent ureters. Given the clinical history and the absence

254   of clear gross post mortem findings, the case was notified to Animal Health as suspect

255   Newcastle Disease as required under EU council directive 92/66/EEC. Newcastle

256   disease was ruled out following official laboratory tests carried out by government

257   laboratories.

258   **Histopathological findings:** Examination of brain tissues revealed focal sub-acute

259   encephalitis of the brainstem and cerebellum with gliosis and perivascular cuffing. In

260   addition, a microabscess was detected in one of those brains. In two of the brains

261   Gram positive bacilli were associated with the lesions (Fig. 2). Other examined tissues

262 including heart, lung, liver, intestine and sections of spinal cord were mostly

263 unremarkable other than concurrent focal fibrinogranulocytic pneumonia and

264 bronchitis with bacterial infection and possibly inhaled foreign plant material in one

265 of the birds (1Aa).

266

267 **Microbiological findings:** The bacteriological results are illustrated in table 1 and 2. In

268 summary, *L. monocytogenes* was isolated on routine bacteriological culture in mixed

269 growth from one of two brain swabs in submission 1 and on direct culture in selective

270 media from three out of four brain swabs in submission 2. This also included a swab

271 from the brain of a partridge belonging to the second (younger) Batch B. Following

272 enrichment and selective culture *L. monocytogenes* was also isolated from multiple

273 environmental samples including from the sheds of different batches (A and C) of

274 partridges regardless of whether there was a high level of neurological disease in the

275 shed or not. However, no *Listeria* spp were isolated from samples of the drinking

276 water, feed or multivitamin solution. *L. innocua* was isolated from the clean straw

277 from the storage area that served as source for the straw used in the corners of the

278 sheds. No *Listeria* spp were isolated from the transit boxes. However, *L.*

279 *monocytogenes* was isolated from an organ pool (liver, lung, yolk sac and brain) of six

280 one-day-old partridges of the fourth batch (D) that had died during import transit and

281 never entered the farm sheds.

282

283 **Antibiotic resistance:** The original *L. moncytogenes* isolate from the brain of partridge

284 b of the first submission and the *L. monocytogenes* isolate from the organ pool of the

285 day-old partridges both underwent testing for antibiotic sensitivity, showing

286 resistance to Doxycycline and Tetracycline and sensitivity to Trimethoprim/

287 Sulphamethoxazole, Lincomycin, Tylosin, Ampicillin, Penicillin and Enrofloxacin.

288 Gene array analysis revealed that both isolates were positive for both probes for the

289 *tet*M gene (both also single positive for *tet*Z; the 1Ab isolate had a single positive probe

290 for *mup*R and *van*D; the day-old isolate had a single positive probe for *tet*L).

291

292 **Partial sequencing results:** Molecular sequence analysis was carried out on the

293 original isolate from partridge 1Ab to confirm identity of the strain and to establish its

294 relationship with other *Listeria* isolates. Analysis was carried out in comparison with

295 a recent detailed analysis that described a novel species *Listeria marthii* (Graves et al.,

296 2010). Sequencing and phylogenetic analysis of the partial 16S rRNA gene, as shown

297 in Figure 3, demonstrated that the isolate is most closely related to *L. monocytogenes*

298 strains including the type strain NCTC 10357. In order to obtain more phylogenetic

299 information a more comprehensive analysis was carried out using concatenated

300 sequences of three housekeeping genes (*sigB*, *prs* and *gap*) and undertaking direct

301 comparison with previous analyses (Graves et al., 2010). As illustrated in Figure 4 these

302 analyses provided more precise description of the isolate placing it clearly within

303 lineage II of *L. monocytogenes*.

304

305

306

307 **Fluorescent Amplified Fragment Length Polymorphism (fAFLP) results:** Five isolates

308 of *L. monocytogenes* from this outbreak in partridges including samples from brain

309 swabs and environment underwent serogrouping and fAFLP testing at the NRL.

310 Additionally, a few *L. monocytogenes* isolates of animal origin kept in the AHVLA

311 bacterial strain collection underwent similar analysis. These additional isolates

312 included one from a partridge heart collected in 1992, two from pheasant livers of

313 1998 and 2002, two from chicken organs, as well as isolates from the brain of cattle,

314 sheep and goat. The results as illustrated in Fig. 5 revealed that the majority of animal

315 samples including all partridge samples belonged to serogroup 1/2a with fewer

316 belonging to 1/2b or 4. Interestingly, all partridge isolates from the current outbreak

317 were in the same fAFLP group VIIa.84. This group has not previously been detected in

318 the UK. The other isolate from a partridge in 1992 was related but not identical with a

319 similarity of 83.8% (fAFLP group: VIIa.85). In contrast all other samples of animal or

320 human origin were not closely related.

321

322 **Discussion**

323 To the best of our knowledge this is the first report of an outbreak of encephalitic

324 avian listeriosis in Great Britain and the first one in red-legged partridges worldwide.

325 Previous outbreaks of avian encephalitic listeriosis have so far been reported in

326 chickens and had morbidities <0.5% (Cooper, 1989; Cooper et al., 1992; Kurazono et

327 al., 2003). The estimated morbidity of encephalitic listeriosis in the first batch of red-

328 legged partridges in this case was higher and approximated 3%. In the subsequent

329 three batches however, estimated morbidity of neurological disease dropped

330 continuously and could not be clearly separated from the expected background

331 mortality for partridges of this age.

332 Clinical, microbiological and histopathological findings as well as the absence of gross

333 post mortem findings were consistent with encephalitic listeriosis. In line with

previous publications, isolation of *L. monocytogenes* was restricted to swabs from the brain and there was mixed growth on routine cultures in the initial submission, from which *L. monocytogenes* was isolated (Gray, 1958; Cooper, 1989). The success rate for isolating *L. monocytogenes* improved when media selective for *Listeria* spp and enrichment steps were used and swabs were taken from the brainstem area of the birds.

The original isolate was confirmed as *L. monocytogenes* by 16S RNA sequencing. Additional sequencing placed it into Lineage II, one of at least four evolutionary lineages of *L. monocytogenes* that is common in foods, widespread in natural and farm isolates and commonly associated with animal listeriosis cases as well as sporadic human cases (Orsi et al., 2011). In contrast most human listeriosis outbreaks are associated with lineage I isolates. Lineage II isolates are most commonly associated with serotypes 1/2a, 1/2c and 3a and are notable for extensive plasmid carriage and associated resistance to environmental compounds as well as bacteriocin resistance (Orsi et al, 2011). In this context, it was interesting to note that the *L. monocytogenes* isolate from the partridge was resistant to tetracyclines in spite of antibiotic resistance to tetracyclines being considered uncommon amongst *Listeria* spp. High levels of tetracyclines are even recommended for treatment of the disease by some authors (Barnes & Nolan, 2008). Morvan et al. (2010) showed that 0.77% of *Listeria monocytogenes* isolates from humans in France were resistant to tetracyclines.

The main questions that remained following the initial diagnosis in this outbreak included: how did the pathogen enter the farm and the partridges; how did it spread; why did it cause disease in so many birds; and why was disease restricted to the nervous system in spite of most earlier publications referring to listeriosis causing septicaemic disease in avian species (Gray, 1958).

L. monocytogenes has been shown to be widespread in the environment including in the soil and silage and many species including sheep, cattle and avian species have

361   been shown to harbour it in and excrete it from the intestine without any clinical signs

362   (Low& Donachie 1997). Oral infection with the pathogen is considered most common

363   (Gray & Killinger, 1966). It was therefore surprising that the diseased partridges had

364   not had any soil contact prior to disease development. There was also no indication of

365   the involvement of water or feed. However, there was widespread presence of the

366   pathogen in the indoor environment of the sheds, identifying this as potential mode

367   of spread for disease from infected birds. No *L. monocytogenes* was isolated from the

368   clean straw prior to being used in the sheds and this was therefore unlikely to have

369   introduced the pathogen into the sheds.

370   Interestingly, *L. monocytogenes* was isolated from an organ pool of day-old imported

371   partridges that had never entered the farm buildings suggesting the possibility that

372   the pathogen was introduced into the farm by imported newly hatched birds that

373   might have been infected in the hatchery or during transit. This theory would also

374   potentially provide an explanation for the differences in morbidity in the various sheds

375   depending on where the infected birds had been placed. Wet weather, flooding, de-

376   beaking and injections have been considered predisposing factors for disease in

377   previous avian outbreaks of encephalitic listeriosis with flooding having been

378   suspected as the potential mode of pathogen distribution (Cooper et al., 1992;

379   Kurazono et al., 2003). However, neither weather conditions nor management factors

380   are believed to have played a significant role in this case given a very dry and warm

381   spring and good management and biosecurity levels on the farm. Horizontal spread of

382   infection from in-contacts is therefore being hypothesised. Birds are considered

383   difficult to infect with *L. monocytogenes,* however, Basher et al. (1984) found that

384 newly hatched chicks could be infected by natural contact to experimentally infected

385 young chicks and subsequently develop disease and die.

386 Additional testing supported the theory that the pathogen had been introduced into

387 the farm by imported day-old chicks by identifying a similar antibiotic resistance

388 pattern between the different *L. monocytogenes* isolates with resistance to

389 tetracyclines likely due to the *tet*(M) gene..

390 The bacterial isolates from this outbreak also all belonged to serogroup 1/2a, which is

391 commonly associated with clinical cases of listeriosis in animals in contrast to

392 outbreaks in humans that are mostly associated with serogroups 1/2b or 4b from

393 lineage I (Orsi et al., 2011). Interestingly, the isolates from the previously reported

394 outbreaks of avian encephalitic listeriosis in chickens in the USA and Japan all

395 belonged to serogroup 4b (Cooper, 1989; Cooper et al., 1992; Kurazono et al., 2003),

396 as did one of the isolates from red-legged partridges in France in 1957; the other

397 isolate was no further classified than serogroup 1 (Lucas & Seeliger 1957). These had

398 been to the best of our knowledge the only reported *L. monocytogenes* isolates from

399 red-legged partridges worldwide.

400 Further subtyping by fAFLP revealed that all isolates from this outbreak also belonged

401 to the same fAFLP type: VIIa.84. This fAFLP type was different to any previously

402 identified by Public Health England (Corinne Amar, personal communication). It was

403 also different to other animal isolates analysed at the same time. Most closely related

404 was an isolate collected from the heart of a partridge from a different farm in 1992

405 and analysed now at the same time that had 83.8% similarity in its fAFLP profile. This

406 raises the question as to whether a partridge specific strain of *L. monocytogenes* might

407 exist. Unfortunately, this was the only *L. monocytogenes* isolate of a partridge

408 available from the AHVLA bead collection due to the rarity of diagnosed listeriosis in

409 avian species in the UK. Isolates from pheasants, chickens and brains of ruminants

410 could be classified into different fAFLP groups and some even different serogroups

411 e.g. 1/2b or 4.

412 This very interesting and unusual case left several questions unanswered. It remains

413 unclear what underlying factors allowed disease to develop to such an extent and why

414 it manifested itself as neurological disease. There was no indication for visceral disease

415 caused by *L. monocytogenes*. It also remained unclear why there had been no similar

416 reports from other farms though the absence of additional imports from the same

417 supplier source at the same time as the first batch was acquired might account for

418 this. The morbidity in subsequent batches was much lower and cases might have been

419 missed amongst "normal" mortality rates.

420

421 *L. monocytogenes* is a well-recognised zoonotic pathogen but the zoonotic risk in this

422 case was considered low as the majority (99%) of human infections are foodborne

423 (Mead et al., 1999; Swaminathan & Gerner-Smidt, 2007). There have however been

424 reports of cutaneous or ocular infection due to contact with infected animals

425 (McLauchlin et al., 2004) and the farmer and his workers were made aware of

426 potential risks. No human health issues were observed during this outbreak.

427 A further important aspect of this outbreak with marked neurological signs and a rise

428 in mortality was that avian notifiable disease, specifically Newcastle Disease, could not

429 be ruled out on clinical grounds, notification of suspect disease took place and

430 subsequent official laboratory tests were required. There can be a large variation in

431 the nature and severity of clinical signs seen in cases of avian notifiable disease

432 (Newcastle disease or Avian Influenza). Therefore any progressive neurological signs

433 in poultry and game birds coupled with an unexplained rise in mortality or drop in

434 production should prompt the consideration of avian notifiable disease as an

435 alternative differential diagnosis (Irvine et al., 2009).

436 On the basis of the findings in this report, listeriosis should also be considered an

437 unusual but potential cause for neurological signs in avian species – not only in

438 individual birds but also in an outbreak-like situation.

439

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448

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584

Table legends:

Table 1: Summary of bacteriological findings, brain histopathology and other findings

in the submitted red-legged partridges. Live submitted birds were showing

neurological signs.

Submission (date)	Batch	Individual identifier	Age (days)	Bacteriology	Histopathology of brain	Other
1 (06/05/11)	A	Bird 1Aa (live)	16	ND	Focal subacute encephalitis of the brainstem and cerebellum with associated Gram positive bacilli	Acute pneumonia and bronchitis
1 (06/05/11)	A	Bird 1Ab (live)	16	Brain: mixed growth including Lm on routine culture	As above	No growth on fungal culture; liver lead: 3.2µmol/kg DM
1 (06/05/11)	A	Bird 1Ac (live)	16	Brain: NSO Liver: NSO	As above but no Gram positive bacilli visible	No growth on fungal culture
1 (06/05/11)	A	Bird 1Ad (live)	16	Liver: NG	As above with no Gram positive bacilli visible but microabscess in one section	
2 (18/05/11)	A	Bird 2Aa (live)	28	Brain: mixed incl Lm on LSA	ND	
2 (18/05/11)	A	Bird 2Ab (dead)	28	Brain: mixed incl Lm on LSA	ND	
2 (18/05/11)	B	Bird 2Bc (dead)	14	Brain: mixed incl Lm on LSA	ND	
2 (18/05/11)	B	Bird 2Bd (dead)	14	Brain: NSO	ND	
2 (18/05/11)	B	Bird 2Be (dead)	14	Liver: NSO	ND	Marked perihepatitis, pericarditis, very poor body condition
2 (18/05/11)	C	Bird 2Cf (live)	1	Brain: NSO	ND	Holding head back following transit during import
Death in transit (02/06/11)	D	Birds x 6 (dead)	1	Organ pool- incl Lm on LSB	ND	

589 ND, not done; Lm, Listeria monocytogenes; NSO, no significant organism; NG, no
590 growth; LSA, Listeria Selective Agar; LSB, Listeria Selective enrichment Broth;
591

592   Table 2: Summary of the bacteriological results from environmental samples

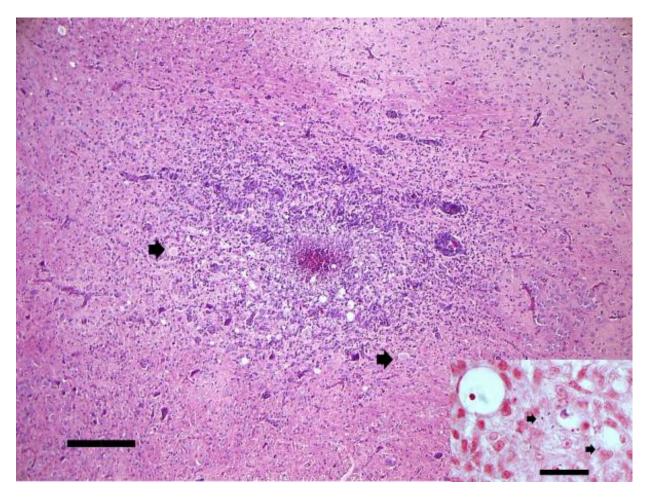| Submission	Batch	Sample	Bacteriology - selective Listeria culture
2	C	Transit box 1 – box liner	NSO
2	C	Transit box 2 – box liner	NSO
2		Water – drinking water	NG
2		Pellets	NG
2		Super fine crumbs	NG
Farm- floor swab	A	Shed 8 - affected	mixed incl **Lm** on LSB
Farm- floor swab	A	Shed 10 - affected	NSO
Farm- floor swab	A	Shed 4 - not affected	mixed incl **Lm** on LSB
Farm- floor swab	C	Shed 19 - affected	mixed incl **Lm** on LSB
Farm- floor swab	A	Straw – shed 10 affected	mixed incl **Lm** on LSB
Farm		Clean straw	mixed incl *Listeria innocua* on LSA/ LSB
Farm		Multivitamin solution	NG
Farm		Water	NG

593   NSO, no significant organism; NG, no growth; MG, mixed growth; Lm, *Listeria*
594   *monocytogenes*; LSB, Listeria Selective enrichment Broth; LSA, Listeria Selective Agar

595

596

597   **Figure legends:**

598   Fig. 1: Torticollis in a 16-day-old live partridge chick.

599

600

601   Fig. 2: Brain, parasagittal section, border of brainstem and deep cerebellar white

602   matter of a red-legged partridge: Microabscess with mixed mainly mononuclear

603   leucocyte infiltration with perivascular lymphocytic cuffing, gliosis and central

604   necrosis. Note swollen degenerate axons within vacuoles (arrows) (HE, scale bar =

605   200μm). *Inset*: Gram positive bacilli (arrows) within the microabscess (Gram stain,

606   scale bar = 20 μm).

607

608

609   Fig. 3: Phylogenetic analysis of representatives of the genus *Listeria* (Graves et al.,

610   2010) inferred from 16S rRNA gene comparison. The tree was constructed in

611   MEGA5 using the neighbor-joining approach following CLUSTAL alignment of

612   sequences trimmed to a 1397 bp consensus contig and applying the Jukes-Cantor

613   substitution model. Numbers at nodes correspond to proportions of 500 resamplings

614   that support the topology shown with values >50% indicated. Bar = 0.005

615   substitutions per nucleotide position. The isolate from the case referred to in this

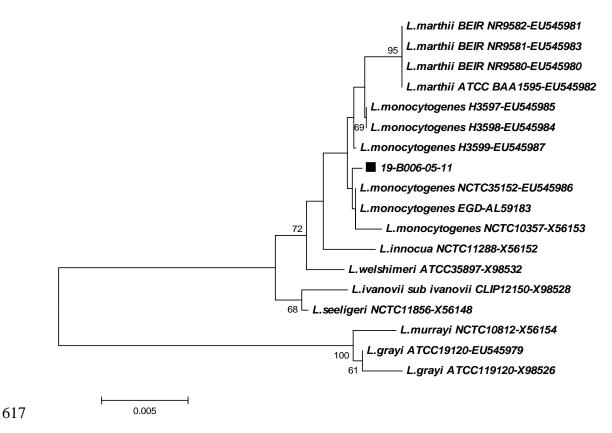616   report (bird 1Aa) is identified as 19-B006-05-11.

617 |———| 0.005

618

619 Fig. 4: Phylogenetic analysis of representatives of the genus *Listeria* based on

620 concatenated *sigB*, *gap* and *prs* sequences showing the position of 19-B006-05-11

621 relative to sequences described by Graves et al. (2010). The tree was constructed in

622 MEGA5 using the neighbor-joining approach following CLUSTAL alignment of

623 sequences and applying the Jukes-Cantor substitution model. Numbers at nodes

624 correspond to proportions of 500 resamplings that support the topology shown with

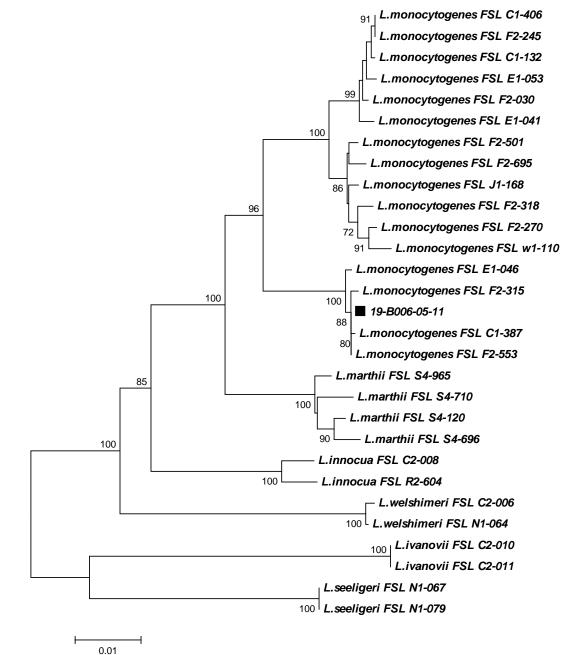625 values >70% indicated. Bar = 0.01 substitutions per nucleotide position.

626

627 Fig. 5: Dendogram of fAFLP similarity. *L. monocytogenes* isolates of various human

628 and animal sources including isolates from partridges and environment of this

629 outbreak (first five isolates) and one isolate from a partridge from a different case in

630 1992 (Partridge- heart) underwent serogrouping and fAFLP testing for similarity. The

631 first isolate 'Partridge – brain' refers to the initial isolate (19-B006-05-11) in this

632 outbreak from the brain of the submitted 16-day-old partridge. The second isolate

633 'Partridge – organ pool' refers to the isolate from the organ pool of one-day-old

634 partridges that had never entered the farm.

Percentage of similarity	Serogroup	Source of isolates	fAFLP type	Source information
	1/2a	Animal	VIIa.84	Partridge - brain
	1/2a	Animal	VIIa.84	Partridge - organ pool
	1/2a	Environmental	VIIa.84	Partridge's shed
	1/2a	Animal	VIIa.84	Partridge - brain
	1/2a	Environmental	VIIa.84	Partridge's shed
	1/2a	Animal	VIIa.85	Partridge - heart
	12a	Food	III.3	Food: meat
	1/2a	Food	III.3	Food: fish
	1/2a	Animal	III.3	Cattle - brain
	1/2a	Human	III.3	Human - blood
	1/2a	Animal	III.32a	Pheasant - liver
	1/2a	Animal	III.5a	Pheasant - liver
	1/2a	Environmental	XIV.3a	Food processing environment
	1/2a	Food	XIV.3a	Food: butter
	1/2a	Food	XIV.3a	Food: poultry
	1/2a	Food	XIV.3a	Food: cheese
	1/2a	Food	XIV.3a	Food: meat
	1/2a	Human	XIV.3a	Human - blood
	1/2a	Food	XIV.3a	Food: cream
	1/2a	Human	XIV.3a	Human - ascitic fluid
	1/2a	Animal	XIV.3a	Chicken - heart
	1/2a	Human	XIV.3a	Human - placenta
	1/2a	Human	XIV.3a	Human - CSF
	1/2a	Human	XIV.23	Human - blood
	1/2a	Animal	XIV.23	Sheep - brain
	4	Food	V.3	Food: meat
	4	Food	V.3	Food: cheese
	4	Animal	V.3	Sheep - brain
	4	Human	V.3	Human - blood
	4	Human	V.3	Human - aneurysm sac
	4	Human	V.3	Human - CSF
	4	Animal	IIA4.1	Goat - brain
	4	Human	IIA4.1	Human - blood
	1/2b	Human	IVb.55	Human - blood
	1/2b	Animal	IVb.55	Cattle - brain
	1/2b	Animal	IVb.56	Chicken - liver

635