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1 **Prevalence of canine enteric coronavirus in a cross-sectional survey of dogs**
2 **presenting at veterinary practices**

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5

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12

13

14 **Abstract**

15 In order to determine the prevalence of canine enteric coronavirus (CECoV) in the general
16 dog population, faecal samples were obtained in a cross-sectional study of 249 dogs
17 presenting for any reason at veterinary practices randomly selected from across the UK.
18 Demographic and clinical data was obtained for each of the samples, including signalment,
19 number of dogs in the household, reason for visiting the practice, and any recent history of
20 diarrhoea. The samples were tested by RT-PCR for the presence of both type I and type II
21 CECOV. Seven samples were positive (three from dogs in the same household), a prevalence
22 of 2.8% (95% confidence intervals 1.1-5.7). Phylogenetic analysis of partial M gene
23 sequences revealed that all seven positive samples grouped with type I CECOV, the first
24 report of this virus in the UK. None of the positive dogs presented for gastrointestinal
25 disease. Interestingly five of the positive dogs from three separate households were aged over

1 six years, suggesting that older dogs may play an important role in the persistence of CECoV
2 in such populations.

3

4 **Key Words**

5 Canine coronavirus; prevalence; type I; RT-PCR; cross-sectional.

6

7 **Introduction**

8 Canine enteric coronavirus (CECoV) is a common pathogen of dogs. Disease is typified by
9 mild enteritis; however sporadic outbreaks, usually in puppies, of haemorrhagic and fatal
10 enteritis have been attributed to CECoV (Buonavoglia et al., 2006; Evermann et al., 2005;
11 Pratelli, 2005). Severe disease outbreaks appear to be associated with the evolution of novel
12 highly pathogenic strains of CECoV (Decaro et al., 2008). Coinfection with other enteric
13 pathogens such as canine parvovirus appears to have a synergistic effect (Pratelli et al.,
14 1999b).

15

16 CECoV has recently been found to exist in two closely related forms. The original strain is
17 now designated as type II CECoV, whereas the more recently identified strain, first reported
18 in 2003, is known as type I CECoV (Pratelli et al., 2003a). The strains are named in this way
19 due to their respective homologies to types II and I feline coronavirus (FCoV) (Herrewegh et
20 al., 1998). Infection may occur with a single strain, however mixed infections with both types
21 appear to be common (Decaro et al., 2005). CECoV is distinct from the newly-recognised
22 canine respiratory coronavirus (CRCoV), which is implicated in the canine infectious
23 respiratory disease complex and falls into a separate group of the coronavirus family (Erles et
24 al., 2003).

25

1 A range of methodologies have been used to assess the prevalence of both type I and II
2 CECoV in different dog populations. Seroprevalence estimates using type II-based assays
3 range between 16%-94% (Naylor et al., 2001; Yesilbag et al., 2004), with kennelled dogs
4 tending to show a higher prevalence. In diarrhoeic dogs the prevalence of CECoV by RT-
5 PCR has been reported to range from 15-42% in pet dogs (Bandai et al., 1999; Pratelli et al.,
6 2000; Yesilbag et al., 2004) and up to 73% in kennelled dogs (Sokolow et al., 2005).

7
8 There is little data on the prevalence of CECoV infection in dogs without enteric disease, and
9 what is available is based on virus isolation, which can only detect type II CECoV, or
10 electron microscopy, which is relatively insensitive (Tennant et al., 1993) (Schulz et al.,
11 2008). This leaves a significant gap in our understanding of the epidemiology of this disease
12 in the dog population as a whole. In order to address this deficiency, we have carried out a
13 cross-sectional study of CECoV carriage as determined by RT-PCR in dogs presenting for
14 any reason at randomly-selected veterinary practices across the UK.

17 **Materials and Methods**

18 One veterinary practice was recruited by random selection from each of the twenty-three
19 regions of the United Kingdom (as defined by the Royal College of Veterinary Surgeons
20 register) in order to source a wide geographical distribution of samples.

21
22 Participating veterinarians were requested to enrol a cross-section of twenty-five dogs
23 visiting for any reason to reflect the clientele and caseload of their surgery: it was not feasible
24 to select the study dogs randomly whilst maintaining compliance. From each sampled dog, a

1 faecal sample was collected and sent by post, along with a brief questionnaire to elicit
2 information such as age, breed, sex, reason for visit and any recent history of enteric disease.

3

4 At the laboratory, samples were homogenised in a 10% dilution of minimum essential
5 medium with 10% foetal calf serum, clarified by centrifugation and genetic material extracted
6 using the Qiampr Viral RNA mini kit (Qiagen) as recommended by the manufacturer. Reverse
7 transcription was carried out using random primers and Superscript III MuLV Reverse
8 Transcriptase (Invitrogen), according to the manufacturer's instructions.

9

10 A 409bp fragment of the M gene was amplified by PCR, using Thermoprime Plus DNA
11 polymerase with 10X ReddyMix PCR buffer (Abgene), and the primer pair CCOV1/ CCOV2
12 (Pratelli et al., 1999a). These primers have previously been successfully used to detect both
13 types I and II CECoV (Benetka et al., 2006). Negative controls and at least one positive
14 control were included at every stage, from RNA extraction to PCR. The positive control
15 comprised a faecal sample, either spiked with CECoV C54 (type II, available from the start
16 of the study) or naturally infected with CECoV 07-019 (type I, became available during the
17 course of the study).

18

19 The PCR products were purified (QIAquick PCR purification kit; Qiagen) and sequenced bi-
20 directionally using primers CCOV1 and CCOV2 according to standard protocols (ABI Prism
21 BigDye terminators version 3.0 cycle sequencing kits; Applied Biosystems). For each
22 amplicon, a consensus sequence was produced using ChromasPro version 1.32
23 (Technelysium Ptl Ltd). Ambiguities identified consistently in both strands were included in
24 the consensus sequence. All primer sites were removed prior to analysis, resulting in a final
25 useable sequence of 316 nucleotides, corresponding to nucleotides 379 to 694 of CCoV type I

1 259/01 (accession af502583) and nucleotides 6782 to 7094 of CCoV type II INSAVC
2 (accession D13096). Sequences have been submitted to Genbank (EU339175 - EU339181).

3
4 Sequence alignments, nucleotide distance calculations (Jukes-Cantor), and phylogenetic
5 analysis were performed using MEGA version 4.0 (Tamura et al., 2007). Support for
6 individual nodes was sought by bootstrap analysis using 1000 repetitions.

7 Statistical analysis was performed using SPSS for Windows, Rel. 14.0.0 2005. Chicago SPSS
8 Inc.. Data were analysed using Chi Squared and Fisher's Exact Tests.

9

10 **Results**

11 Two hundred and forty-nine samples were obtained from twenty practices (figure 1);
12 numbers submitted varied from 3-33, with a mean of 12 per practice.

13

14 Characteristics of the sample population

15 Of the 249 dogs sampled, 111 (44.6%) were male, 134 (53.8%) female and for four (1.6%)
16 the sex was not specified. Dogs ranging from two months to eighteen years of age were
17 sampled. The age distribution was right-skewed (ie the tail is on the right) (figure 2). This
18 contrasts with findings from a door-to-door questionnaire study in which the age distribution
19 of the pet dogs formed a normal distribution (Westgarth C, 2008). This contrast may reflect
20 the large number of routine veterinary visits which many dogs undergo early in life for puppy
21 checks, primary vaccination and neutering; however it may also reflect possible bias in the
22 selection of cases by veterinarians.

23

24 The majority of the dogs (144, 57.8%) were from single-dog households. Fifty-four (21.7%)
25 were from two-dog households, and the remaining 51 (20.5%) came from households

1 containing three or more dogs. This contrasts with previous data from a community-based
2 survey of dog-ownership in which 77% of the households had a single dog, with 20% and 3%
3 having two dogs and three or more dogs respectively (Westgarth et al., 2007). This may
4 reflect the fact that a domestic door-to-door questionnaire would be likely to miss farms,
5 commercial kennels and breeding premises. The breeds sampled are shown in figure 3. Apart
6 from cross-breeds and Great Danes, all of the breeds most commonly sampled correspond to
7 the 20 breeds most frequently registered in 2006 (KennelClub, 2007).

8
9 Numerous conditions were given as the primary reason for taking the dog to the veterinary
10 surgeon. The most common reasons in the 228 dogs for which a specific reason was given are
11 shown in figure 4. The most common reason was vaccination (18.4%, 42/228), followed by
12 ‘routine’ (which included weight check, claw clip, parasite treatment or social reasons such
13 as accompanying another dog) and ear/ skin problems, each of which were cited as the reason
14 for visit in 12.7% (29/228) of the dogs surveyed. 7% (16/228) presented for diarrhoea, with
15 or without vomiting.

16

17 Prevalence of CECoV

18 Of the 249 samples obtained, seven were positive for CECoV, a prevalence of 2.8% (95%
19 confidence intervals 1.1-5.7). Three of the positive samples (sample numbers 01-033, 01-039
20 and 01-046) were obtained from dogs from the same multi-dog breeding household (fig 1).
21 The other four were from different households with a wide geographical distribution.
22 Samples 12-020 and 12-030 were from pups of less than 6 months of age; all of the other
23 positive samples were from dogs aged 6-11 years. Three of the seven positive dogs (sample
24 numbers 06-018, 07-019 and 12-020) had a history of diarrhoea within the previous month
25 (Table 1), although none of the seven presented at the surgery for diarrhoea. There was no

1 significant association between either presenting for, or having a history of, recent diarrhoea
2 and being CECoV positive ($p=0.4$) (Table 1), although with such a small number of positive
3 samples, it would have been difficult to detect a statistically significant association.

4
5 When sequenced, all seven positive samples grouped with type I CECoV in the M gene
6 region (figure 1). No type II CECoV was obtained from any of the samples. Sequence
7 analysis and retrospective testing of the positive samples with a real time PCR assay (adapted
8 for use in our laboratory (Decaro et al., 2005) confirmed that none of the positive samples
9 contained mixed type I/type II infections.

10
11 As the prevalence of infection was lower than anticipated, further investigations were carried
12 out to confirm the validity of the methods used. In the 233/249 samples for which the data
13 was available, 93.1% of the samples were received by the laboratory within four days, with a
14 median time in transit in the post of 2 days (figure 5). A loss of titre during transportation was
15 therefore considered possible. However in pilot experiments, a <10-fold loss in sensitivity
16 was found, using the conventional PCR assay, when CECoV spiked faecal samples were left
17 at room temperature for seven days (figure 6). As 98.3% of the samples were received at the
18 laboratory within 7 days of collection, this time delay is unlikely to have been a major
19 contributory factor to the low prevalence detected, although some potential effect cannot be
20 ruled out in those few samples which were delayed in the post for longer periods.

21
22 The sensitivity of the conventional PCR assay used was also examined in relation to the real-
23 time PCR assay of Decaro et al, 2005, and was found to have a similar sensitivity for the
24 detection of type I, type II, and mixed infections. The sensitivity was equivalent to 100-1000
25 RNA copies per μl of cDNA for the conventional PCR, as compared to 100 RNA copies per

1 μ l of cDNA in the real-time PCR. In addition, both assays (real time and conventional) have
2 subsequently been used on samples from 166 kennelled dogs, where the prevalence of
3 CECoV carriage was expected to be high. Overall concordance between the two assays was
4 96% (160/166). Of these, 30 were CECoV positive by both methods (9 type I, 6 type II and
5 15 mixed), four by real-time PCR alone (2 type I, 1 type II, 1 mixed) and two by
6 conventional PCR alone, presumably due to primer or probe binding site mismatches.
7 It was therefore concluded that the sensitivity and specificity of the methods used were
8 broadly comparable to that of the real-time PCR.

9

10 **Discussion**

11 Although the importance of CECoV as a canine pathogen is being increasingly recognised, its
12 prevalence is poorly understood. Most studies have focussed on diarrhoeic or kennelled dogs,
13 and there is little data on the prevalence of CECoV in the wider dog population, including
14 healthy pet dogs. Such information is, however, important in increasing our understanding of
15 the epidemiology of the disease. In the present study, we have targeted this population of
16 both asymptomatic and diarrhoeic dogs through a cross-sectional study survey of randomly
17 selected veterinary practices from throughout the UK. In order to maximise compliance,
18 random selection was not used at the client level, and it is possible that this may have caused
19 some bias.

20

21 Despite the large number of dogs sampled in our study, the prevalence estimate obtained was
22 relatively low (2.8%). There are few studies on prevalence in comparable dog populations,
23 but in a small study in Liverpool using virus isolation, no CECoV was obtained from 26
24 healthy pet dogs in a boarding kennel, although eight of 32 dogs with acute diarrhoea were
25 positive for type II CECoV in the same study (Tennant et al., 1993). This contrasts with a

1 more recent study in which a prevalence of 17.5% was detected by electronmicroscopy in
2 200 healthy dogs in Germany (Schulz et al., 2008). The variability of prevalence estimates
3 depends on factors such as the detection methods used and the characteristics and disease
4 status of the dog population under study. In addition, it is possible that the prevalence of
5 CECoV in a population might fluctuate over time and that any prevalence estimate will be
6 dependent on the timing of sampling.

7
8 Although 28.6% of the 248 dogs for which data was available either had diarrhoea at the time
9 of sampling or a recent history of diarrhoea, no significant association was detected with
10 CECoV shedding when compared to animals with no history of diarrhoea. However, the
11 number of CECoV positive dogs in our study was small, and this makes the power to detect
12 an association low. There are a number of possible reasons why relatively few diarrhoeic
13 dogs were positive for CECoV in our study. Mild diarrhoea in dogs is very often non-
14 infectious in origin and in some cases may simply be a natural sequel to dietary indiscretion.
15 Alternatively, other pathogens may have been involved, or if the diarrhoea had been
16 coronaviral in origin, virus shedding may have stopped by the time of sampling.

17
18 Interestingly however, although overall there was no significant association between being
19 CECoV positive and having diarrhoea, three of the seven positive dogs (all three coming
20 from different households) had had diarrhoea within the previous month. It is not clear
21 whether these seven dogs were undergoing acute asymptomatic infection or more chronic
22 shedding. Dogs have been shown to shed CECoV for a variable but potentially long time
23 following infection and clinical resolution (Decaro et al., 2005). In one natural infection
24 study, one animal was reported to shed CECoV for up to 156 days even though signs of
25 clinical disease only lasted for 10 days post-infection (Pratelli et al., 2002). Such clinically

1 normal carriers are likely to be important in maintaining infection in the general dog
2 population.

3

4 This study reports the first identification of type I CECoV in the UK. No type II CECoV was
5 identified from any of the samples in this study, although the same assay has been
6 successfully used in our laboratory to detect both types I and II CECoV in other samples from
7 UK dogs. There is some suggestion that type I CECoV may be shed at higher titres and for a
8 longer period than type II, and if this is indeed the case this could lead to an enhanced
9 probability of detecting type I CECoV in a cross-sectional study of this kind (Decaro et al.,
10 2005). Type 1 CECoV virus was first described in 2003 in Italy (Pratelli et al., 2003b) and
11 has since been reported in other countries. (Yesilbag et al., 2004), (Rennhofer et al., 2005).
12 Recent evidence, based on the discovery of a new open reading frame present in type I
13 CECoV (Lorusso et al., 2008a; Stavisky et al., 2008), with residual fragments in type II
14 CECoV, and the closely related feline coronavirus and transmissible gastroenteritis virus,
15 suggests that type I CECoV may actually represent the ancestral virus of this group (Lorusso
16 et al., 2008b).

17

18 The clinical significance of the distinction between type I and II CECoV is not clear.
19 Although classic CECoV infection is considered to cause only mild enteric disease (Tennant
20 et al., 1991), there are several reports where type II CECoV has been associated with more
21 severe haemorrhagic diarrhoea and occasional death. (Binn et al., 1974; Buonavoglia et al.,
22 2006; Zappulli et al., 2007). It has also been suggested that such signs can be seen with type I
23 (Benetka et al., 2006). Since more virulent strains appear to evolve spontaneously (Decaro et
24 al., 2008; Zappulli et al., 2007), it may be that both types I and II have the ability to cause a
25 wide spectrum of clinical manifestations, and this variation in virulence may depend on both

1 viral evolution and host factors such as age and immune status. Further work is needed on
2 different populations of dogs to help clarify this.

3

4 Recognition of CECoV in practice is hampered by the fact that commercial diagnostic tests
5 suitable for the detection of types I and II CECoV are not widely available. Electron
6 microscopy is relatively insensitive, and serological assays are based on type II CECoV,
7 which limits their use as they have unknown efficacy in detecting type I. In addition, since
8 seroprevalence in the general dog population is relatively high, such tests are difficult to
9 interpret in relation to diagnosing acute disease unless paired samples are taken. The efficacy
10 of currently available CECoV vaccines, which are all based on type II CECoV, is also
11 unclear. The only data on cross protection available to date is based on *in vitro* work and
12 suggests that the level of antigenic cross reactivity between the two types may be limited
13 (Pratelli et al., 2004). Clearly, this should also be assessed in challenge experiments *in vivo*.

14

15 In conclusion, we have shown that the newly-recognised type I CECoV is circulating at a low
16 prevalence among clinically normal adult dogs in the UK. Previous studies have concentrated
17 on kennelled dogs, and dogs with disease, which are known to be at high risk of infection
18 (Naylor et al., 2001; Sokolow et al., 2005). Subclinically infected animals within the general
19 dog population, as identified by this study, may represent an important reservoir of infection,
20 playing a significant role in the epidemiology of this disease and the evolution of the virus.

21

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2

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Table 1: Numbers of dogs with and without recent diarrhoea sampled in a cross-sectional study of 249 dogs from 20 UK veterinary practices.

1 **Figure 1:** Map of the United Kingdom. Squares show approximate locations of participating
2 practices. Black squares indicate practices which submitted positive samples; grey squares
3 indicate all other participating practices.

4 Rooted neighbour-joining tree of the seven partial M gene sequences generated in this study
5 and published reference canine, feline and human coronaviruses (outgroup). All branch
6 lengths are proportional to distances established using the Jukes-Cantor method. Bootstraps
7 are expressed as percentages, and only included where greater than 75%.

8 Key: HCoV NL63 accession DQ445912, FCoV C1Je accession DQ848678; FCoV 1146
9 accession DQ010921; FCoV DF-2 accession DQ286389; CECoV BGF10 accession
10 AY342160; CECoV 259/01 accession af502583; CECoV 23/03 accession AY548235;
11 CECoV INSAVC accession D13096. Samples from this study are coded CECoV x-y, where
12 x is the internally designated practice number, and y the individual dog reference.

13 **Figure 2:** Histogram to show age distribution of dogs sampled in a cross-sectional study of
14 249 dogs from 20 UK veterinary practices. Data missing from one dog, therefore total
15 number of dogs included is 248.

16

17 **Figure 3:** Histogram to show distribution of most common breeds of dog sampled in a cross-
18 sectional study of 249 dogs from 20 UK veterinary practices. 202 dogs are included; breed
19 was not specified for 47 dogs. Breeds with less than 4 dogs sampled are grouped under 'other
20 breeds'.

1

2 **Figure 4:** Bar chart to show the reasons for visit cited by study participants. This data was
3 available for 228 dogs. *Routine includes weight check, claw clip, flea/ worm treatment and
4 social reasons for visiting the surgery. # Sick dog included a broad range of diagnoses such as
5 pyometra, false pregnancy, and convulsions. Conditions classified as ‘misc’ (miscellaneous)
6 were those where the health status of the dog was impossible to determine, eg ‘operation’
7 which could refer to an elective or emergency surgery.

8

9 **Figure 5:** Histogram to show the length of time between collection of each faecal sample and
10 receipt at the laboratory. Data was available for 233/249 samples submitted.

11

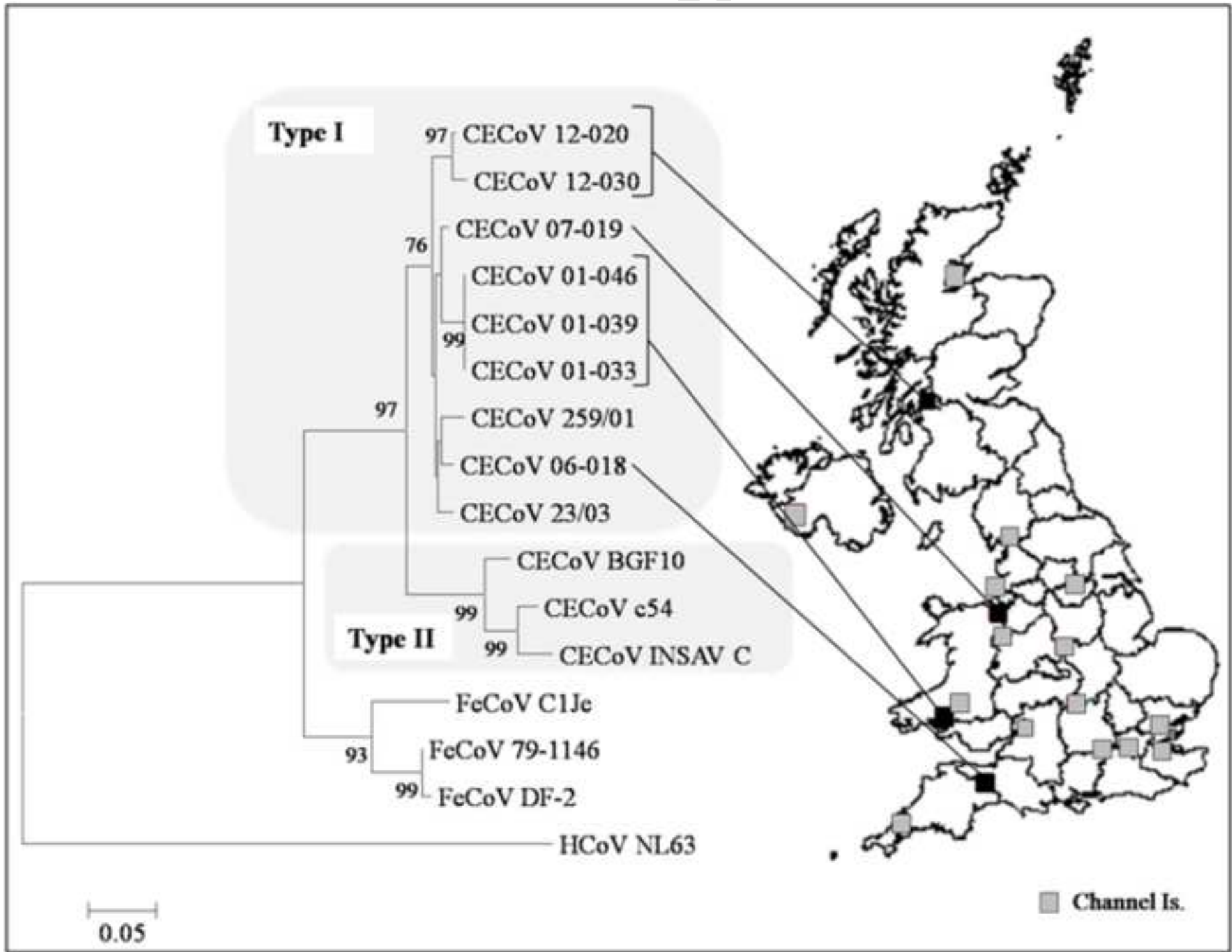
Figure 6: Faeces spiked with CECoV-C54 were kept at room temperature for a number of
days and then underwent RT-PCR using the protocol described. It can be seen that even after
7 days a faint band was visible at the -3 dilution, suggesting a loss in titre of less than tenfold.

12

	CECoV positive	CECoV negative	Total
Diarrhoea as presenting complaint	0	16	16
Owner-reported diarrhoea within previous month	3	52	55
No history of recent diarrhoea	4	173	177
Total	7	241	248*

* One negative sample had missing questionnaire data

Figure 1



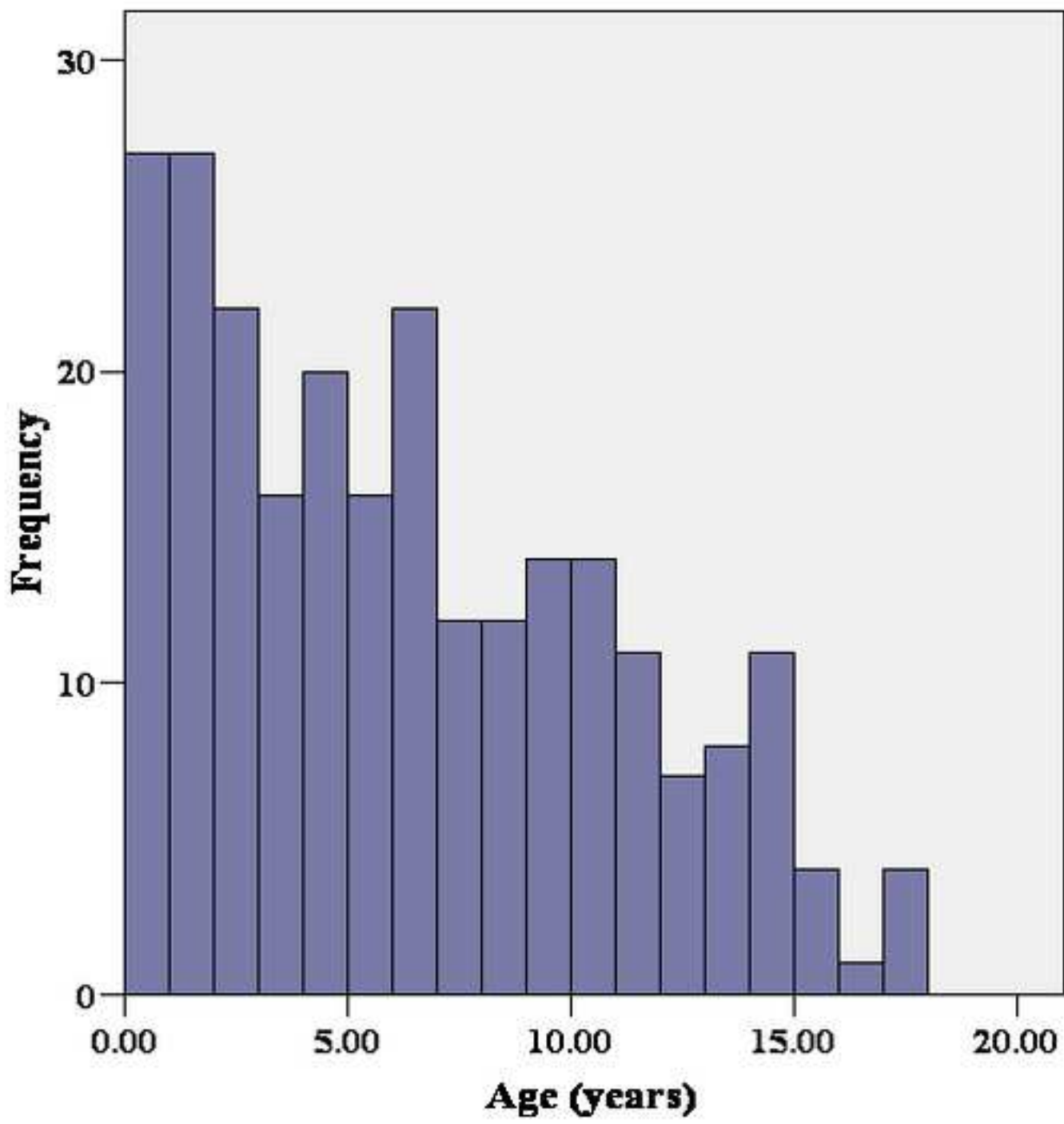


Figure 3

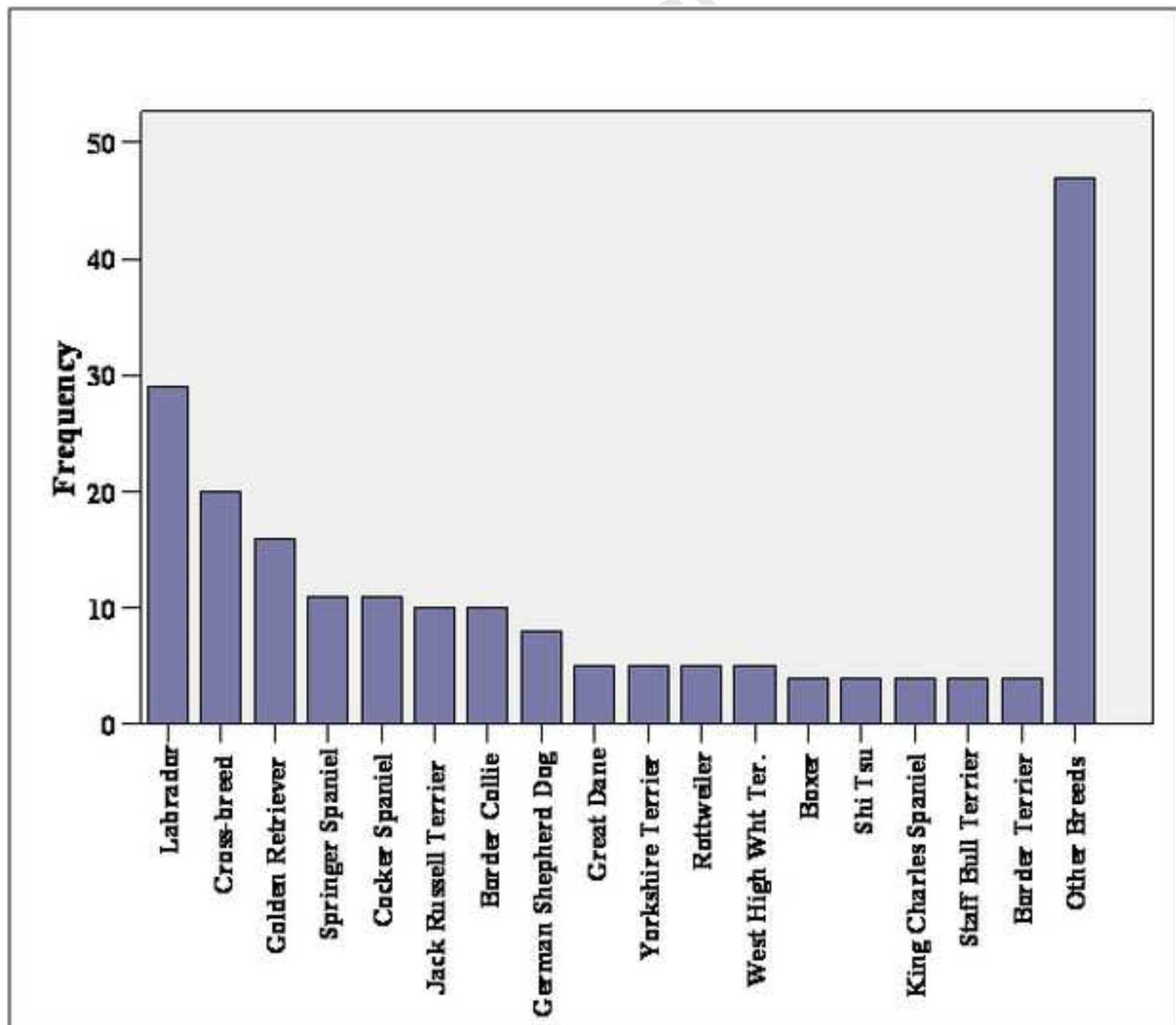


Figure 4

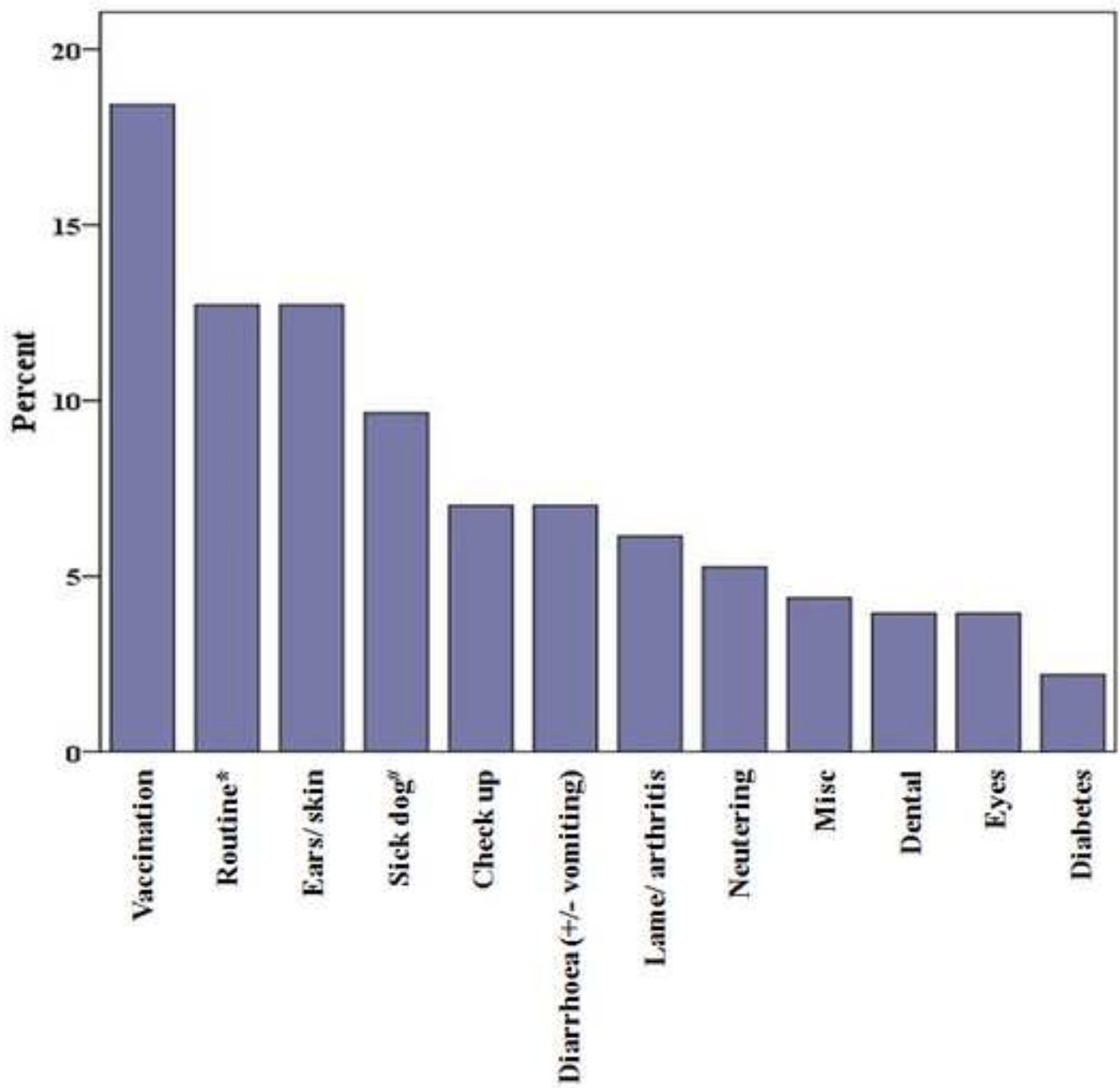


Figure 5

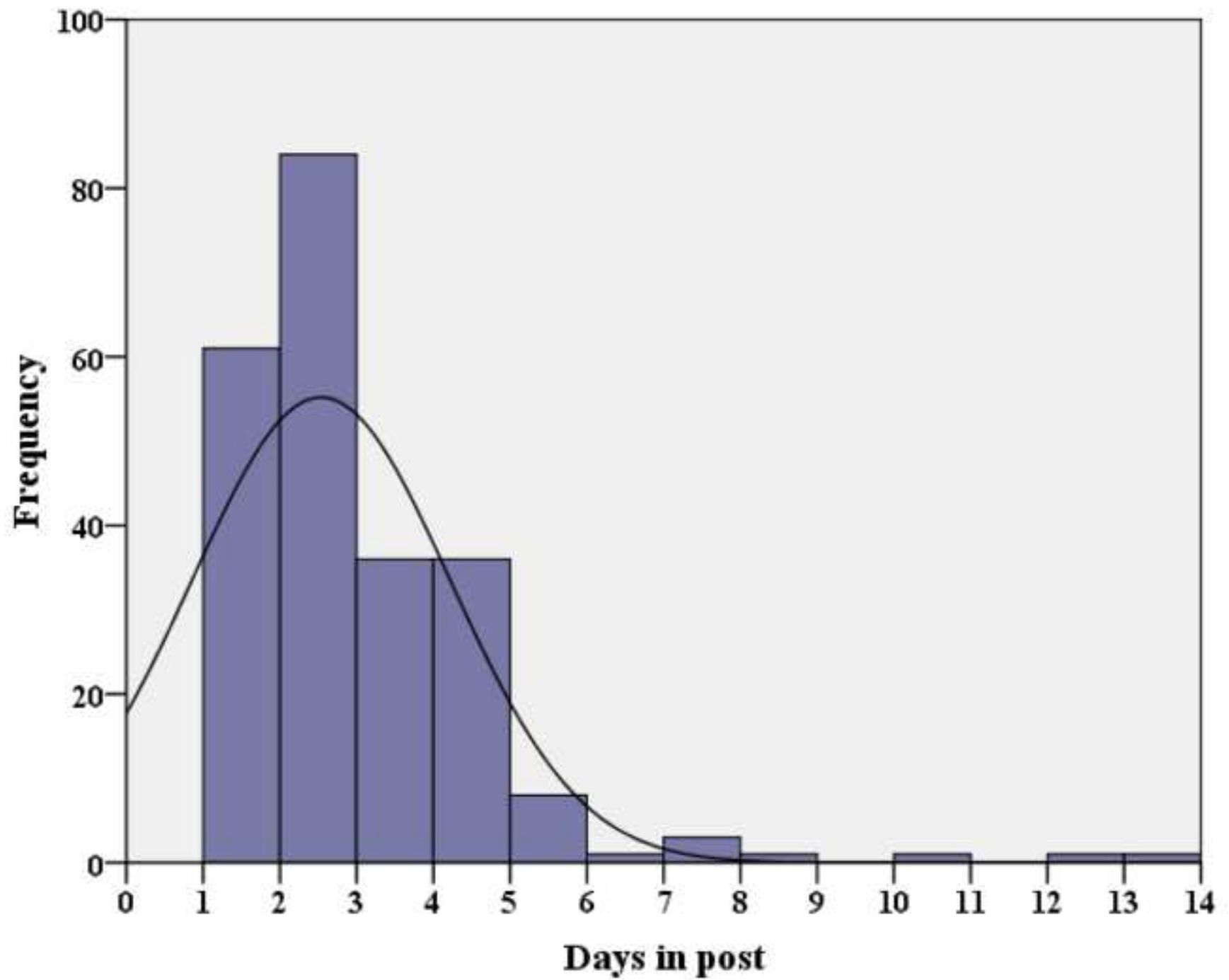


Figure 6

