

1 **Epidemiological survey of equine influenza in Andalusia, Spain.**

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22 **Abstract**

23 Equine influenza is a highly contagious respiratory disease considered the most important
24 respiratory disease in equids. Although influenza A virus (IAV) has caused outbreaks in
25 equids worldwide, surveillance on these species in Spain has not been conducted. A cross-
26 sectional study was carried out to determine the individual and herd prevalence of
27 antibodies against H3N8 and H7N7 IAV in equids in Andalusia (southern Spain).
28 Antibody against IAV was measured by the single radial haemolysis assay. A spatial scan
29 statistical analysis was carried out using a Bernoulli model. Risk factors associated with
30 IAV infection were assessed by multivariate analysis. Antibodies to H3N8 IAV were
31 detected in 241 out of 464 unvaccinated equids (51.9%; 95% CI: 47.4–56.5).
32 Seropositivity against the H7N7 subtype IAV was not found in any of the analysed
33 animals. Significantly higher seropositivity was found in geriatric (OR= 6.1, $P = 0.008$,
34 95% CI= 1.6 – 23.1) and adult (OR= 4.8, $P < 0.001$, 95% CI= 2.5 – 9.0) equids compared
35 to young. Specific antibodies against A/equine/Shropshire/2010 (H3N8) or
36 A/equine/Newmarket/5/2003 (H3N8) only were confirmed in 11 and 45 of the animals,
37 respectively. The spatial analysis showed a statistically significant cluster centred in the
38 west part of Andalusia. The results confirmed widespread H3N8 subtype IAV exposure
39 in equine species in Andalusia. Conversely, the absence of seropositivity against H7N7
40 IAV obtained in the present study suggests that this subtype has not circulated in southern
41 Spain in recent years. Because of the animal health and economic consequences of IAV
42 in equids, further surveillance and molecular studies are required to monitor and
43 characterize the most prevalent IAV circulating in these species in Spain.

44

45 **Keywords:** *influenza virus; horses; mules; donkeys; risk factors; spatial analysis.*

46 **Introduction**

47 Influenza A virus (IAV) is an enveloped single-stranded negative-sense RNA virus
48 belonging to the *Influenzavirus A* genus (family *Orthomyxoviridae*). Influenza viruses are
49 classified according to their two major surface glycoproteins: haemagglutinin (H1–H18)
50 and neuraminidase (N1–11) (Tong et al. 2013). Although highly pathogenic avian
51 influenza virus (H5N1) has been isolated from donkeys (Abdel-Moneim et al. 2010), only
52 viruses of the H7N7 and H3N8 subtypes have been shown to circulate endemically among
53 equine species. The H7N7 subtype, which was the first IAV isolated in horses in 1956
54 (Sovinova et al. 1958), is considered extinct as this virus has not been detected from
55 equids for over three decades (Webster, 1993). The H3N8 subtype was initially isolated
56 in 1963 in Florida (Waddell et al. 1963) and subsequently spread globally. H3N8 IAV
57 diverged into European and American lineages in the late 1980s (Daly et al. 1996). The
58 American lineage was further divided into the Kentucky, South American, and Florida
59 sub-lineages, with a more recent divergence between Florida clade 1 and clade 2 (OIE
60 2009). Although viruses from both clades have caused outbreaks in equine species
61 worldwide (OIE-WAHIS), Florida clade 1 have predominated in the USA but have been
62 the cause of outbreaks in Africa, South America, Asia and Europe. Florida clade 2 strains
63 are endemic in Europe and have also been implicated in outbreaks in Asia (Elton and
64 Bryant, 2011).

65 Equine influenza (EI) is a highly contagious and widespread infectious disease of horses
66 and other equine species (OIE 2016). This disease is considered the most important
67 respiratory disease in equids, as outbreaks rapidly spread through susceptible populations.
68 Transmission occurs by direct contact, or indirectly through fomites and in aerosols. IAV
69 infection in equids is a typically self-limiting respiratory disease characterized by fever,
70 lethargy, coughing, dyspnoea and nasal discharge, particularly in naïve or unvaccinated

71 individuals (Firestone et al. 2011). Even though the mortality rate associated with IAV
72 infection is low in horses, infected animals are prone to secondary bacterial infections
73 that can lead to pneumonia and death (Back et al. 2011). Equine influenza causes severe
74 economic losses for the horse industry by causing disruption of equestrian events,
75 restrictions of movements and preventive and control measures (Cullinane et al. 2010).

76 Although the H3N8 IAV is endemic in equids in Europe, epidemiological information on
77 this subtype is still very limited in some regions. In Spain, EI outbreaks have not been
78 reported and survey studies on IAV in equids have not been carried out. The aim of the
79 present study was to determine the seroprevalence, risk factors and spatial distribution of
80 IAV in equids in Andalusia (southern Spain).

81 **Material and Methods**

82 *Study design*

83 A cross-sectional study was carried out to determine the individual and herd prevalence
84 of antibodies against IAV in equids in Andalusia (36° N–38° 60′ N, 1° 75′ W–7° 25′ W).
85 Andalusia is the region with the largest number of equids in Spain with a total of 223 696
86 equids recorded during a census conducted in 2015, including 189 790 horses, 19 926
87 mules and 13 980 donkeys (CAP 2015). Initially, a total of 441 equine herds were
88 surveyed by official veterinarians as part of a regional surveillance health programme
89 carried out in Andalusia between 2011 and 2015. The sampling was stratified by
90 provinces according to the proportion of horses in each province. The herds in each
91 province were selected by simple random sampling from the official records of herds
92 obtained from the Regional Government of Andalusia (CAP 2015). Based on the
93 epidemiological questionnaire, 270 herds, in which vaccination programmes against IAV
94 have been previously implemented, were excluded for this study. Finally, blood samples

95 from 234 unvaccinated horses were collected in the remaining 171 herds. Based on the
96 size of the selected herds (ranging from 1 to 260; median= 7), an estimated within-herd
97 prevalence of 50% and a confidence level of 95% (95% CI), between one and five horses
98 were randomly sampled to detect seropositivity within each herd. Sampled horses were
99 selected using systematic sampling. Additionally, blood was collected from 169 donkeys
100 (from 41 herds) and 61 mules (from 39 herds) using convenience sampling. The
101 geographical distribution of the analysed equine herds is represented in Figure 1. None
102 of the sampled equids showed clinical signs compatible with equine influenza at the time
103 of sample collection.

104 An epidemiological questionnaire was carried out during the sampling through an on-
105 farm interview with the owners, in order to obtain data related to the herd and animals.
106 The explanatory variables collected from the questionnaire were grouped as (a) individual
107 data: age range (young: <5 years, adult: 5–14 years or geriatric: >14 years), gender (male
108 or female), breed (purebred or crossbred), vaccination history (type of vaccine used and
109 date of vaccination); (b) herd data: province, municipality, herd size (small: 1–5, medium:
110 6–12 or big >21 animals), activity (farming, leisure or work), type of housing (outdoors
111 (kept outdoors during the day), indoors (kept in shelter during the day) or mixed (free
112 access to both types of housing)), direct contact with other horses, transport of horses
113 within the last 6 months (< 6 months or > 6 months), presence of other equine species,
114 presence of other animal species (birds, cats, dogs and pigs); (c) biosecurity measures:
115 cleaning and disinfection at least one time per week.

116 *Sample collection and serological analyses*

117 Blood samples were collected by jugular venipuncture using sterile collection system
118 tubes without anticoagulant (Vacutainer[®], Becton-Dickinson, USA) and transported to

119 the laboratory under refrigeration within 24 h of sampling. Samples were centrifuged at
120 400 g for 15 min, and sera separated and stored at -20°C until analysis.

121 Antibody levels against IAV were measured using the single radial haemolysis (SRH)
122 assay performed as described in the OIE Terrestrial Manual (OIE 2016). Samples were
123 tested in parallel using A/equine/Shropshire/2010 (H3N8) (Florida clade 1),
124 A/equine/Newmarket/5/2003 (H3N8) (Florida clade 2) and A/equine/Prague/1956
125 (H7N7) strains as antigens. Serum from a hyper-immunized experimental pony (Scott et
126 al. 2012) and the relevant European Pharmacopoeia reference antiserum (Eq Influenza
127 Subtype 1 Strain A/equine/Newmarket/1977 (H3N8) Horse Antiserum) for the
128 A/equine/Prague/1956 (H7N7) strain were included as positive controls on each plate as
129 appropriate. Serum samples with a clear zone of haemolysis were considered to be
130 positive.

131 *Spatio-temporal cluster analysis*

132 A spatial scan statistical analysis was carried out at municipality level using a Bernoulli
133 model to detect significant clusters of IAV presence in equine herds (Kulldorff et al.
134 2006). The number of Monte Carlo simulations was set to 999 for the cluster scan statistic.
135 Analyses were run using SaTScanTM v9.4.4. Clusters were considered to be significant
136 at $P < 0.05$.

137 *Statistical analysis*

138 The prevalence of antibodies by SRH was estimated from the ratio of positives to the total
139 number of samples, with the exact binomial confidence intervals of 95% (95% CI)
140 (Thrushfield, 2007). Associations between serological results and explanatory variables
141 were analysed using a Pearson's chi-square test. All statistically significant variables
142 (likelihood ratio and Wald test, P -value < 0.15) in the bivariate analysis were selected as

143 potential risk factors. Cramer's V coefficient between pairs of variables was computed to
144 prevent collinearity. Finally, a generalized estimating equation (GEE) was carried out to
145 study the effect of the variables selected on the basis of bivariate analysis. The number of
146 seropositive animals was assumed to follow a binomial distribution and both the "herd"
147 and "province" were included as random effects. A forward introduction of variables was
148 used, starting with the variable with the lowest *P*-value in bivariate analysis. At each step,
149 the confounding effect of the included variable was assessed by computing the change in
150 the odd ratios (OR). Confounding variables were those that, when added to the model,
151 changed the OR by more than 30%. The model was re-run until all remaining variables
152 presented statistically significant values (likelihood-ratio Wald's test, $P < 0.05$) and a
153 potential relationship with the response variable existed. The fit of the models was
154 assessed using a goodness-of-fit test (Hanley et al. 2003). All the statistical analyses were
155 performed using SPSS 20.0 (Statistical Package for Social Sciences, Inc., Chicago, IL,
156 USA).

157

158 **Results**

159 Antibodies against H3N8 subtype IAV were detected in 241 of the 464 (51.9%; 95% CI:
160 47.4–56.5) equids tested (Table 1). Seropositivity against H7N7 subtype IAV was not
161 found in any of the 464 analysed sera. The distribution of individual and herd prevalence
162 to A/equine/Shropshire/2010 (H3N8) and A/equine/Newmarket/5/2003 (H3N8) strains
163 among species is shown in Table 1. The Bernoulli model identified one statistically
164 significant cluster (radius: 30.1 Km; $P < 0.027$) centred in the west part of Andalusia
165 (Figure 1).

166 A total of nine explanatory variables were selected from the univariate analysis (Table 2).

167 The "breed" was excluded from the multivariate analysis due to collinearity with the

168 variable presence of “shelter”, while “cleaning and disinfection” and “presence of other
169 equids” had collinearity with “species”. The final GEE model showed that the main risk
170 factor associated with IAV seroprevalence in equine species was age. Significantly higher
171 seropositivity was found in geriatric (OR= 6.1, $P = 0.008$, 95% CI= 1.6 – 23.1) and adult
172 (OR= 4.8, $P < 0.001$, 95% CI= 2.5 – 9.0) equids compared to young. Similar results were
173 found when only horses were selected (geriatric: 87.5%, 42/48; OR= 32.8, $P = 0.001$,
174 95%CI= 3.6 – 89.5; adults: 70.3%, 97/138; OR= 28.6, $P = 0.001$, 95%CI= 4.1 – 62.5 vs
175 young animals: 25.0%, 12/48).

176 **Discussion**

177 The overall seroprevalence obtained in the present study (51.9%) indicates high H3N8
178 subtype IAV exposure in equids in southern Spain. Reported rates of seropositive equids
179 from different countries range from 26.4% in Israel to 67.6% in the UK (Aharonson-Raz
180 et al. 2014; Barquero et al. 2007). However, the comparison of seroprevalence among
181 studies should be interpreted carefully due to differences in methods used to measure
182 antibodies, number of samples tested, equine species analysed and epidemiological
183 context.

184 The seroprevalence detected in mules (62.3%) and donkeys (30.8%) in the present study
185 was higher than that found in these species in Turkey (mules: 12.8% and donkeys: 9.4%)
186 (Ataseven and Daly, 2007) and in mules in Brazil (37.5%) (Gaiva e Silva et al. 2014).
187 Most of the seropositive animals had positive results to both H3N8 IAV strains, which
188 could be due to co-infections or, more probably, cross-reactivity between the two IAV
189 Florida clades. However, specific seropositivity against A/equine/Newmarket/5/2003
190 (H3N8) and A/equine/Shropshire/2010 (H3N8) strains suggest circulation of these two
191 H3N8 IAV strains among the equine population tested. Nonetheless, the circulation of
192 other strains detected in Europe should be considered. Seronegative results to H7N7 IAV

193 indicate absence of circulation of this subtype in southern Spain, which is consistent with
194 reports that the virus no longer circulates in equids (Loroño-Pino et al. 2010; OIE 2017).

195 Vaccination is considered the main tool to prevent IAV in equids (Elton and Bryant,
196 2011). It was previously suggested that at least 70% of a given equine population needs
197 to be fully vaccinated to prevent EI epidemics (Baker, 1986). However, mathematical
198 modelling has suggested that this figure can be as low as 40% if the vaccine strain
199 perfectly matches circulating viruses, but may need to be as high as 90% if there is
200 substantial antigenic drift between the vaccine strain and circulating viruses (Park et al.
201 2009). Based on the epidemiological questionnaire carried out to select the non-
202 vaccinated horse herds for this study, the 61.2% of the initially surveyed herds were
203 vaccinated against IAV in the last 5 years (data not shown). Therefore, continuous
204 monitoring of field IAV strains circulating in equids in Spain as well as vaccination
205 programmes against these strains should be implemented in the studied area in order to
206 verify the effectiveness of vaccines and avoid outbreaks due to lack of immunological
207 protection.

208 The herd seroprevalence levels obtained in this study indicated a widespread circulation
209 of IAV in equids in southern Spain. However, the spatial distribution was not uniform
210 across the municipalities. The spatial analysis showed a significantly higher IAV
211 circulation in the western region of Andalusia (Huelva province). Interestingly, the cluster
212 was located surrounding the Doñana National Park (DNP), the main wetland for water
213 birds in Spain. The strategic location within important wild bird migratory flyways
214 between Europe and Africa, the environmental conditions and the high density of horses,
215 including unvaccinated feral and semi-wild horse populations (Retuerta and Marismeño
216 breeds) in DNP, are possible factors implicated in the higher spread of IAVs in this area.
217 The equine H3N8 IAV lineage is thought to have first emerged in horses in South

218 America as a result of direct transmission from the wild bird reservoir (Murcia et al. 2011)
219 and a further ‘spill-over’ of the H3N8 subtype from wild birds to horses was detected in
220 China in 1989 (Guo et al. 1992). Furthermore, antibodies against the avian IAV H3N8
221 subtype have been previously detected in wild birds in Spain (Busquets et al. 2010; Pérez-
222 Ramírez et al. 2012). Although the equine-specific H3N8 IAV strains used in this study
223 have circulated in horses for at least 40 years, some cross-reactivity with currently
224 circulating avian H3N8 IAV strains may remain. Further studies could include testing of
225 equine sera against currently-circulating strains of equine and avian H3N8 IAV. Our
226 results constitute an important step for understanding IAV spread in the studied area and
227 will provide valuable information for the development of more cost-effective surveillance
228 and control programmes in Spain.

229 The multivariate analysis showed that the prevalence of IAV antibodies in equids was
230 age-related, in accordance with several previous studies (Aharonson-Raz et al. 2014;
231 Guildea et al. 2011; Laabassi et al. 2012). The higher seroprevalence in older animals
232 reflects a cumulative likelihood for exposure to IAV and persistence of antibodies.

233 In conclusion, this study confirms a widespread H3N8 subtype IAV exposure in equine
234 species in Andalusia. Conversely, the absence of seropositivity against H7N7 IAV
235 obtained in the present study suggests that this subtype has not circulated in southern
236 Spain in recent years. The identification of spatial clusters can serve to inform risk-based,
237 more cost-effective strategies towards better prevention and control of EI in Spain.
238 Because of the animal health and economic consequences of IAV in equine populations,
239 further surveillance and molecular studies are required to monitor and characterize the
240 most prevalent IAV strains circulating in these species in Spain. In order to better prevent
241 IAV outbreaks in naïve populations, specific measures, including quarantine of imported

242 equids and vaccination programmes should be implemented in the studied area,
243 particularly surrounding the DNP, where the spatial cluster was detected.

244

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248

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370

371 **Table 1.** Individual and herd-level seroprevalence against influenza
 372 A/equine/Shropshire/2010 (H3N8) and A/equine/Newmarket/5/2003 (H3N8) in different
 373 equine species in southern Spain.

Species		Positive/ total (%; 95% Confidence interval)			
		H3N8 subtype	A/equine/Shropshire/ 2010 (H3N8) strain *	A/equine/Newmarket/ 5/2003 (H3N8) strain	Co-occurrence
Horse	Individual	151/234 (64.5; 58.4-70.7)	139/227 (61.2; 54.9-67.6)	139/234 (59.4; 53.1- 65.7)	127/234 (54.3; 47.9- 60.7)
	Herd	123/171 (71.9; 65.2-78.7)	113/167 (67.7; 60.6-74.7)	112/171 (65.5; 58.4-72.6)	103/171 (60.2; 52.9- 67.6)
Donkey	Individual	52/169 (30.8; 23.8-37.7)	47/169 (27.8; 21.1-34.6)	37/169 (21.9; 15.7-28.1)	32/169 (18.9; 13.0- 24.8)
	Herd	13/41 (31.7; 17.5-45.9)	10/41 (24.4; 11.3-37.5)	12/41 (29.3; 15.3-43.2)	8/41 (19.5; 7.4- 31.6)
Mule	Individual	38/61 (62.3; 50.1-74.5)	37/61 (60.7; 48.4-72.9)	20/61 (32.8; 21.0-44.6)	19/61 (31.15; 19.5- 42.8)
	Herd	29/39 (74.3; 60.7-88.1)	28/39 (71.8; 57.7-85.9)	16/39 (41.0; 25.6-56.5)	15/39 (38.5; 23.2%, 53.7)
Total	Individual	241/464 (51.9; 47.4-56.5)	11/457 (2.4; 1.0-3.8)	45/464 (9.7; 7.0-12.4)	178/464 (38.4; 33.9- 42.8)
	Herd [†]	162/241 (67.3; 61.3-73.2)	150/237 (63.3; 57.2-69.4)	140/241 (58.1; 51.9-64.3)	127/241 (52.7; 46.4-59.0)

374 *Sera from seven horses from four herds could not be tested against
 375 A/equine/Shropshire/2010 (H3N8) due to low volume. [†]Two or more equine species
 376 coincided in ten herds.

377

378 **Table 2.** Distribution of variables identified as significant ($P < 0.15$ in the univariate
379 analysis) and included in the multivariate analysis to determine the risk factors associated
380 with AIV seroprevalence in equids in Andalusia (southern Spain).

Variable	Categories	% Positive	Number/overall*	<i>P</i> value
Equine species [†]	Horses	64.5	151/234	<0.0001
	Mules	62.3	38/61	
	Donkeys	30.7	52/169	
Age [†]	Geriatric	75.6	65/86	<0.0001
	Adults	58.6	143/244	
	Young	24.6	33/134	
Sex	Male	51.8	142/274	0.99
	Female	51.9	98/189	
Breed [†]	Pure	46.4	170/366	<0.0001
	Crossbred	71.4	65/91	
Herd size [†]	Big	47.2	50/106	0.07
	Medium	42.2	27/64	
	Small	55.8	164/294	
Shelter [†]	Indoors	69.2	71/107	<0.0001
	Outdoors	44.1	120/272	
	Mixed	53.4	39/73	
Cleaning and disinfection [†]	Yes	52.4	118/225	0.06
	No	44.2	77/174	
Activity	Farming	58.5	38/65	0.41
	Leisure	49.7	143/288	
	Work	53.2	58/109	
Transport of equids [†]	< 6 months	61.8	63/102	0.01
	> 6 months	47.2	93/197	
Entrance of other equids	< 1 months	60.4	64/106	0.86
	> 6 months	58.8	10/17	
	1-6 months	54.8	17/31	
Presence of other equids [†]	Yes	56.7	89/157	0.03
	No	47.0	117/249	
Presence of pigs	Yes	49.8	23/46	0.98
	No	50.0	100/201	
Presence of cats [†]	Yes	56.7	101/178	0.02
	No	45.8	108/236	
Presence of birds	Yes	45.8	172/332	0.33
	No	51.8	38/83	
Presence of dogs	Yes	51.8	176/340	0.26
	No	44.6	33/74	

381 *Missing values were omitted. †Variable included in the multivariate analysis.

382

383

384 **Figure legend**

385 **Figure 1.** Map of Andalusia (southern Spain) showing the gradient of seroprevalence at
386 municipal level. The circle indicates the significant spatial cluster of herds with equids
387 seropositive for AIV.