Epidemiological survey of equine influenza in Andalusia, Spain.

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Abstract

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

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

Introduction

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

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

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

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

Material and Methods

Study design

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

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

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

Sample collection and serological analyses

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

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

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

Spatio-temporal cluster analysis

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

Statistical analysis

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

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

Results

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

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

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

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

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

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

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

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

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

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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.

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

Variable	Categories	% Positive	Number/overall*	*P* 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.