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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 in 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). Antibodies againsts IAV were 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 animals. 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.

1. 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, 2017), Florida clade 1 has 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

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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., 2016). 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).

2. Material and methods

2.1. Study design

A cross-sectional study was carried out to determine the individual and herd prevalence of antibodies against IAV in equids in Andalusia (36°N–38°60′N, 1°75′W–7°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 Fig. 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.

2.2. 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 −20°C 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.

2.3. 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 $P < 0.05$.

2.4. 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 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).

3. 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 (Fig. 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 variable presence of "shelter", while "cleaning and disinfection" and "presence of other equids" had collinearity with "species". The final GEE model showed that the main risk factor associated with IAV seroprevalence in equine species was age. Significantly higher seropositivity was found in geriatric (OR = 6.1, $P = 0.008$, 95% CI = 1.6–23.1) and adult (OR = 4.8,